IN-SITU INJECTABLE THERMOSENSITIVE GEL BASED ON POLOXAMER AS A NEW CARRIER FOR TAMOXIFEN CITRATE

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ABSTRACT

Objective: To evaluate poloxamer (Pl) based *in-situ* injectable thermosensitive gel of Tamoxifen citrate (TMC) compared to orally administered TMC regarding retention in different tissues. Methods: The inclusion complexes of TMC with β-cyclodextrin (β-CD), hydroxypropyl β-cyclodextrin (HP-β-CD) and sulfobutyl-7-ether β-cyclodextrin (SBE-β-CD) were prepared by solvent evaporation method and evaluated for drug-excipient compatibility tests as well as *in-vitro* release studies. Poloxamer analogs were mixed in different ratios and evaluated for gelation temperature and rheological properties. Finally, the optimized thermosensitive hydrogel formula was evaluated for *in-vitro* drug release as well as *in-vivo* drug retention in rat tissues, plasma and liver. Results: TMC/SBE-β-CD complex showed the highest drug release rate. The optimum concentrations of poloxamer analogs for the in situ gel-forming delivery system were 20% (w/v) Poloxamer 407 (F127) and 15% (w/v) Poloxamer 188 (F68) that exhibited sol -gel transition at 36.5°C ± 0.5°C. TMC/SBE-β-CD complex incorporated in the optimized thermosensitive gel base exhibited elevated drug level in cancer tissues and low level in plasma and liver compared to oral TMC suspension. Conclusion: The optimized formula of TMC/SBE-β-CD hydrogel could contribute in elevating drug level at targeted tissues and improving drug anticancer activity.

Keywords: SBE-Cyclodextrin, Inclusion complex, Injectable hydrogel.

INTRODUCTION

Tamoxifen citrate (TMC) is a nonsteroidal anti-estrogen frequently considered the drug of choice for the treatment of breast cancer as it has a relatively low toxicity than most chemotherapeutic agents such as alkylating agents, anti-metabolites and anti-tumor antibiotics [1-4]. However, the drug is a highly lipophilic and susceptible to first pass metabolism and P-glycoprotein (P-gp) pump efflux in the liver and intestine when orally administered [5]. One of the many approaches adopted to enhance solubility and bioavailability of poorly-water soluble drugs is complexation with cyclodextrin [6-8]. This complexation involves entrapment of the drug hydrophobic moiety in the cyclodextrin (CD) cavity that has similar hydrophobic nature as the drug. The outer surface of CD is characterized by being hydrophilic; thus enhancing aqueous solubility of the drug [9].

Although solubility enhancement of TMC may improve its bioavailability from oral dosage forms, the drug side effects cannot be reduced. Therefore, there was an urgent need for a new dosage form that can reduce TMC side effects through localizing its presence at cancerous tissue. The use of *in-situ* injectable poloxamer-based thermosensitive gel was expected to help reaching this goal. *In situ* gel formation occurs due to one or combination of different stimuli such as physiological stimuli e.g., temperature and pH [10], physical changes in biomaterials e.g., solvent exchange, swelling, and self-assembly [11-13], and chemical reactions e.g., enzymatic, chemical and photo-initiated polymerization [14, 15] or based on UV-irradiation [16]. Temperature modulation system has been studied as an approach to reduce TMC side effects. It relies on the use of systems containing thermosensitive materials such as, polysaccharides (e.g., cellulose derivatives, xyloglycan and Chitosan) and poloxamers [16]. Aqueous solutions of poloxamer 407 exhibited a thermo reversible property; a perfectly reversible thermogelling phenomenon characterized by a sol-gel transition temperature ($T_{sol \to gel}$). Below the $T_{sol \to gel}$, the sample behaves as a fluid (sol) whereas above that temperature it changes to semi-solid gel [17]. Poloxamer is a copolymer of hydrophobic and hydrophilic blocks of propylene oxide (PO) and ethylene oxide (EO) blocks [18]. As temperature increases above $T_{sol \to gel}$, the PO blocks dehydrate causing the copolymer molecules to aggregate into spherical micelles whereas the PO blocks oriented towards the core and the EO chains arranged in the outer shell [19, 20].

The main objective of this study was to minimize the toxicity of TMC by localizing its presence at cancerous tissue through the use of *in-situ* injectable poloxamer-based thermosensitive gel. In order to achieve our goal, the solubility and release rate of the lipophilic drug were enhanced through SBE-β-CD complexation. The inclusion complex was incorporated in thermosensitive gel carrier and evaluated for drug retention in tissues, plasma and liver.

MATERIALS AND METHODS

Materials

Tamoxifen citrate was kindly supplied by Amriya Company for Pharmaceuticals Industries, Egypt. β-cyclodextrin (βCD) and hydroxy propyl β-cyclodextrin (HP-βCD) were purchased from Sigma Aldrich, USA; whereas sulfobutyl ether-7-β-cyclodextrin sodium salt (SBE-βCD) commercially known as Captisol®, was kindly supplied from Cydex Inc, USA. Poloxamer 407 and poloxamer 188 were purchased from Sigma Aldrich, Germany under the commercial names Pluronic F127® (F127) Pluronic F68® (F68), respectively. Methanol and acetonitrile of HPLC grade were also purchased from Sigma-Aldrich, Inc., Germany. Water for HPLC was obtained from ADWIC, Egypt whereas disodium hydrogen phosphate, sodium dihydrogen phosphate and sodium chloride were purchased from EL-Gomhoria Company, Egypt.

Preparation of TMC-CD inclusion complexes and physical mixtures

Binary physical blends in the molar ratio of 1:2 TMC: CDs were prepared by mixing 3.54 mM TMC with 7.1 mM of CDs (equivalent to 402 mg, 489 mg and 767 mg of β-CD, HP- β-CD SBE- β-CD, respectively) in a ceramic mortar with pestle for 30 minutes. The produced mixtures were then passed through sieve no 8 to ensure complete homogeneity. The 1:2 drug/carrier ratio was obtained from phase solubility diagrams (data not shown).

The solvent evaporation method was employed for preparing drug-complexes where an equivalent weight of 7.1 mM of the CDs where dissolved in glass beaker containing 50 ml distilled water with the aid of magnetic stirrer[21, 22]. A weight of 100 mg TMC (equivalent to 3.548 mM) was added to the CD solutions. Solutions were left on the stirrer (24-48 hr) till clear solutions were obtained. The solutions were kept at 40°C in an incubator till complete evaporation of solvent and dry complexes were obtained.

Preparation of TMC-CD complexes loaded thermosensitive sol-gel systems

Incorporation of the selected TMC-CD complex system (TMC/SBE-β-CD) in a thermosensitive gel base was performed according to the cold method described in literature [23]. In brief, a series of solutions of F127 (20 w/v %) with increasing concentration of F68 (5, 10 and 15 w/v %) were prepared by slow addition of poloxamers to distilled water which was then stirred with magnetic stirrer and allowed to dissolve overnight at 4°C. Tamoxifen citrate (10 mg) was initially dissolved in lowest amount of methanol before being added to the cold F127/ F68 solutions with gentle mixing. Thermosensitive sol-gels containing TMC/SBE-β-CD complex were prepared in the same way but with the complex added directly to the poloxamers mixture without being initially dissolved in methanol.

Characterization of TMC-CD complexes

Fourier Transform Infrared (FTIR) spectroscopy

Spectra of samples of TMC, CDs, their physical mixtures and complexes were characterized using Shimadzu infrared spectrophotometer (FTIR Shimadzu 82400S, Lab Wrench). Discs of mixtures of the samples with IR grade KBr in the ratio of (9:1, KBr: samples) were prepared by hydraulic press under 5.5 metric ton of pressure. The spectra were collected within the scanning range of 400 to 4000 cm^{-1} and with a resolution of 4 cm^{-1} using a mercury cadmium telluride (MCT) detector.

Differential scanning calorimetry (DSC):

Accurately weighed samples (7-8 mg) of TMC, CDs, their physical mixtures and complexes were analyzed using Shimadzu DSC (DSC-50; Shimadzu Corporation, Japan). Temperature of samples was elevated from room temperature (20-22°C) to 200°C, at rate of 10°C/minute under flow of nitrogen at rate of 30 ml /minutes to avoid sample oxidation.

Characterization of thermosensitive sol-gel systems

Measurement of gelation temperature:

A small transparent beaker containing a few milliliters of thermosensitive system and a magnetic bar was placed in low-temperature (4°C) adjusted thermostat controlled water bath. An Omron digital thermometer (Model: ECO Temp II, Omron Healthcare, INC., Lake Forest, IL) was immersed in the solution which was heated gradually with continuous stirring of 30 rpm. When the magnetic bar stopped moving due to gelation, the temperature displayed on the digital thermometer was determined as gelation temperature [23, 24].

Rheological behavior measurements:

A Piezoelectric axial vibrator (PAV) was used to determine the frequency dependent viscoelasticity of the thermosensitive sol-gel formulations below and above the gelation point at 18°C and 37°C, respectively. Determination of viscoelastic behavior of preparations was carried out over a wide range of frequency (1-4000 HZ) [25]. Samples were carefully applied between the stainless steel plates with a spatula to ensure no formulation shearing did occur [26]. The gap between the plates was varied between 9 μm to 89 μm by spacers. The vibration actuated by Piezoelements had amplitude of only several nanometers; thus, all measurements were maintained in the linear viscoelastic region.

In-vitro TMC release studies from inclusion complexes and physical mixtures

Release of drug from samples (equivalent to 10 mg of TMC) of physical mixtures or complexes-freshly screened through sieve No. 8 was conducted using USP apparatus 2 dissolution tester (Hanson SRII 6 flasks, California, USA). Distilled water (900 ml) at temperature of 37±0.5C° was used as the dissolution medium with the paddle speed maintained at 75 rpm. At fixed time intervals (15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165 and 180 minutes) 3 ml samples withdrawn with a syringe then filtered with a filter-syringe (micro-filter pore size 0.45 mm) before being assayed spectrophotometerically (Perkin Elmer Lambda Ez 201, U.K) for

drug content at a wave length of 237 nm. The withdrawn samples were replaced with equal volume of fresh dissolution medium. All the release experiments were run in triplicates.

A comparison among the release profiles of different complexes was done with dissolution efficiency parameter (DE) which is defined as the area under a dissolution curve between defined time points t_1 and t_2 expressed as a percentage of the curve at maximum dissolution, Y_{100}, over the same period of time [27, 28].

$$Dissolution\ Efficiency(DE) = \left[\frac{\int_{t2}^{t1} Y \cdot dt}{Y_{100}(t_2 - t_1)}\right] \cdot 100 \qquad Eq.\ 1$$

Where, Y is the percentage of drug dissolved between time points' t_1 and t_2.

In-vitro TMC release studies from thermosensitive gels

In-*vitro* release of drug from TMC/SBE-β-CD complex embedded in thermo-sensitive gel was evaluated in phosphate buffer saline (PBS, PH 7.4). Appropriate amount of optimized gel base formula containing 10mg TMC or its equivalent weight of selected inclusion complex, were placed in two open ended tube (1 cm in diameter) with cellulosic membrane (Spectra/Por®, molecular porous membrane tubing No.2, Mwt cut off 12-14000 D, Spectrum Laboratories, Inc., USA) fixed on one end. The other end of tube was attached to the bottom of a dissolution tester apparatus 1 shaft. The membrane used was soaked in the release medium overnight before use. After sample addition the tube was lower enough to allow the membrane to be in touch with the surface of PBS buffer (500 ml) maintained at 37°C± 0.5°C. The dissolution tester shaft was allowed to rotate at 50 rpm and at specific time intervals 3 ml samples were withdrawn and replaced with equal volume of fresh medium. Samples were filtered using 0.22 μm sterile syringe filters and analyzed for TMC content via Perkin Elmer spectrophotometer at a wave length of 237 nm. The release experiments were run in triplicates.

In-vivo study

Study design

Female Sprague Dawley rats' 250 gm ± 20 gm in weight that were 4 or 5 weeks old were supplied by the Holding Company for Biological Products and Vaccines [VACSERA], Egypt. All rats were maintained in a light controlled room maintained at 22°C ±2°C and 55% RH ± 5% RH (Animal House of Pharmacology Department - Faculty of Pharmacy - Helwan University). Approval to carry out the *in-vivo* study was obtained from the Animal Ethics Committee of Faculty of Pharmacy, Helwan University. The rats were divided into 2 groups, 7 rats each and fasted overnight (12 h) with free access to water before the experiments. One group of animals received oral free TMC (suspension) prepared by shaking 20 mg of TMC in 16 ml distilled water, 2 ml of the prepared suspension was administered orally to each rat. The second group was directly injected in the breast tissues with thermosensitive gel (sol form). All formulae were administered at a dose of 10 mg/ Kg body weight. The site of injection was marked to facilitate tissue separation.

Blood samples (8 hours post administration) were collected by retro-orbital puncture from the rats after being mildly anaesthetized using diethyl ether, and placed into previously heparinized eppendorff tubes (approximately 1 ml). Rats were then euthanized with diethyl ether and tissues were collected to quantify the content of drug in them. Plasma was separated by centrifuging the blood samples at 2000 rpm for 5 min at 4°C. Liver and breast tissues were rinsed with isotonic buffer and homogenized according to previously reported method [29]. Tissue samples were homogenized (1:5, w/v) in 50 mM Tris-HCL at pH 7.4 and acetonitrile was used for drug extraction. The prepared homogenates contains 20% of liver tissues and 10% of breast tissues. Plasma and tissues samples were stored at -80°C till quantitative analysis [4].

Plasma levels of TMC were analyzed using a modified HPLC reported method [5]. A mobile phase of acetonitrile: methanol (85: 15 % v/v) containing 0.02% triethylamine was used for the analysis at a flow rate of 1.5 ml/ min. Column was Agilent Zorbax Octa-decyl-silyl (ODS), with dimensions 250 mm x 4.6 mm. Plasma and tissue samples were prepared for analysis by adding equal volume of acetonitrile to each sample, followed by vortexing for 30 seconds and centrifugation at 4000 rpm for 15 minutes. The upper layer was transferred to another tube, filtered through a 0.45um Millipore filter, and then 10 µl was injected into the HPLC column. Measurement was done with UV detector at wavelength of 237 nm (UV detector: Model SPD- 10A VP, Shimadzu, Japan). Samples were quantified for TMC content against a calibration curve constructed from spiking plasma samples with 0.1 ml of the standard TMC solutions followed by acetonitrile extraction.

Statistical analysis

An unpaired student t-test was used in the comparison of the amount of drug retained *in-vivo* between the liver, breast tissues and plasma after 8hrs of administration using InStat₃ program (version 3, Graph Pad software, Inc., La Jolla, CA 92037, USA).

RESULTS AND DISCUSSION

Characterization of Tamoxifen citrate cyclodextrin complexes

Fourier Transform Infrared Spectroscopy

Spectra of samples of TMC, β-CD, SBE-β-CD, HP-β-CD, drug- CD's inclusion complexes and poloxamers were characterized (charts not shown). The detection revealed that the spectrum of TMC-CDs inclusion complexes exhibited only one new highly intense narrow peak observed at 1639 cm⁻¹. The obtained peak is characteristic to the hydrogen bond that formed between the C=O group of TMC and the OH group of the CDs in the complex. The results showed no significant difference in the abundant peaks of the spectrum of free polymer and may be explained on the basis of incomplete drug inclusion within the polymer's cavity.

Differential scanning calorimetry (DSC)

The DSC thermogram of TMC powder exhibits a single sharp endothermic peak at 144.68°C [30] corresponding to its melting point and indicating its crystalline state (Figure 1-A). Whereas, β-CD had an endothermic peak at 102°C corresponding to its dehydration [31] (Figure 1-B). In the TMC/β-CD physical mixture the two endothermic peaks appeared at almost the same corresponding temperature of the pure components but with lower intensity due to dilution (Figure 1-C). In the TMC/β-CD prepared complex there was shift in β-CD endothermic peak to lower temperature of 82°C and reduction in the TMC peak enthalpy compared to the physical mixture. The peak shift in complex compared to physical mixture reflected complex formation. However, appearance of TMC peak in the complex was indicative of partial complexation where some free drug molecules still existed in the system.

The physical mixture of TMC/HP-β-CD thermogram showed two endothermic peaks at 62°C and 150°C for HP-β-CD dehydration and melting of TMC, respectively, which were at the same temperature as individual curves (Figure 1-F). However, in the TMC/HP-β-CD complex the TMC melting peak disappeared completely as a result of full complexation (Figure 1-G). Moreover, the broad HP-β-CD endothermic peak was changed to a relatively sharp one and shifted to higher temperature of 79°C. Absence of drug melting peak as a result of complete complexation was correlated to what has been previously reported in literature [32].

Thermogram of SBE-β-CD (Figure 1-H) revealed an endothermic peak at 77.43°C related to the dehydration of CD [33]. The thermogram of prepared TMC/SBE-β-CD complex showed complete disappearance of the drug melting peak similar to that observed with TMC/HP-β-CD complex (Figure 1-J). The results obtained indicated that the extent of drug complexation varied according to the type of cyclodextrin used. In case of β-CD, complexation was partial and some TMC molecules were still free in the matrix whereas in case of HP-β-CD and SBE-β-CD all drug molecules were involved in inclusion complexation.
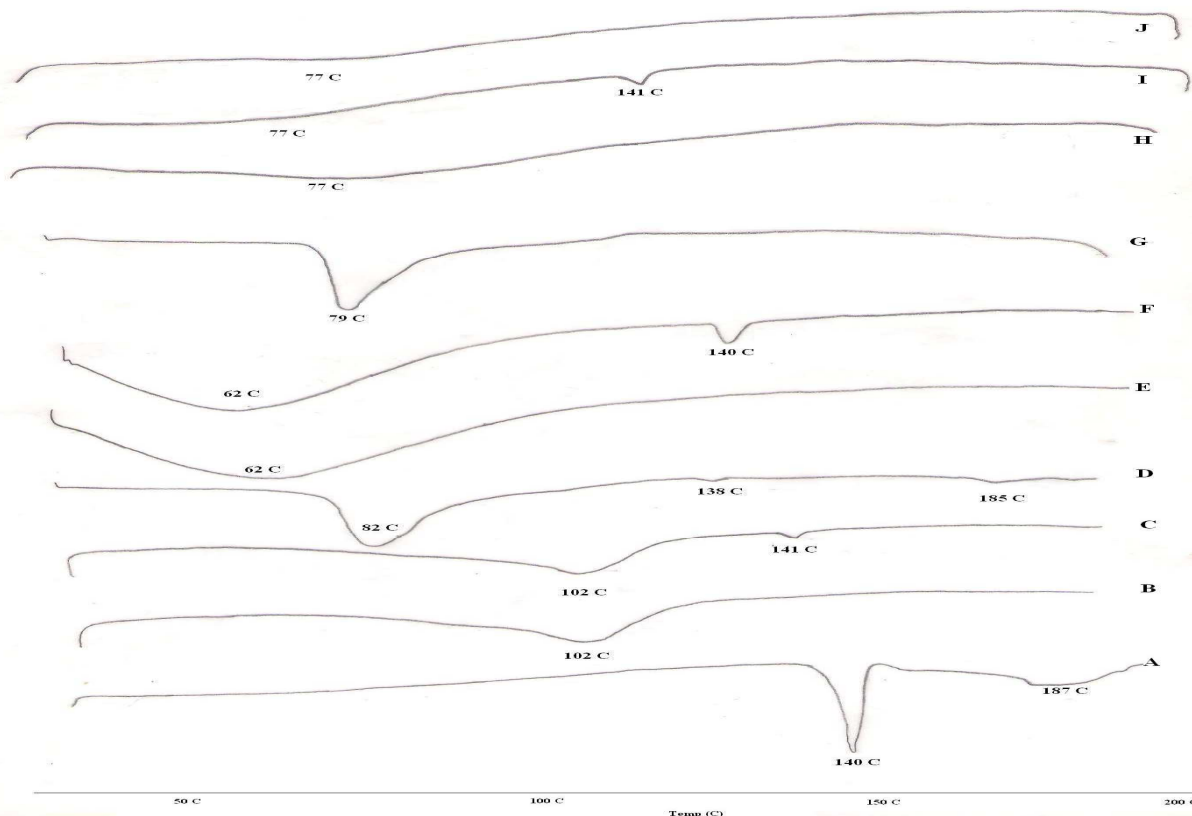

Fig. 1: DSC thermograms of (A) TMC, (B) β-CD, (C) TMC/β-CD physical mixture, (D) TMC/β-CD complex, (E) HP/β-CD, (F) TMC/HP-β-CD physical mixture, (G) TMC/HP-β-CD complex, (H) SBE-β-CD, (I) TMC/SBE-β-CD physical mixture, (J) TMC/SBE-β-CD.

***In-vitro* TMC release studies from inclusion complexes and physical mixtures**

Figure 2 represented the release profile of TMC from different inclusion complexes and their physical mixtures. The data showed that all inclusion complexes as well as physical mixtures of TMC-CD systems exhibited better release profile than pure TMC. Among the three types of cyclodextrins, SBE-β-CD complex provided the highest rate and the best DE factor (61.1%), (Figure 2, Table 1). The dissolution efficiency data can be explained on the basis of the very low pK_a of sulfonic acid groups that provided SBE-β-CD with multiple negative charges at physiologically compatible PH values [34]. The

four carbon butyl chain coupled with repulsion of the end group negative charges allows the extension of CD cavity. This often results in stronger binding to drug candidates compared to other modified CDs. The water solubility of SBE-β-CD (>500mg/ ml at 25℃) [35] is significantly higher than the parent β-CD (18.5 mg/ml at 25 ℃) [36]. Therefore, based on DE results of TMC/SBE-β-CD inclusion complex was selected for formulation of thermosensitive gel in situ [37-39].

The highest safety profile of injectable SBE-β-CD confirmed our selection. This polymer does not exhibit the nephrotoxicity of β-CD and moreover SBE-β-CD complexation had been reported to provide protection against drug-induced cytotoxicity [40].
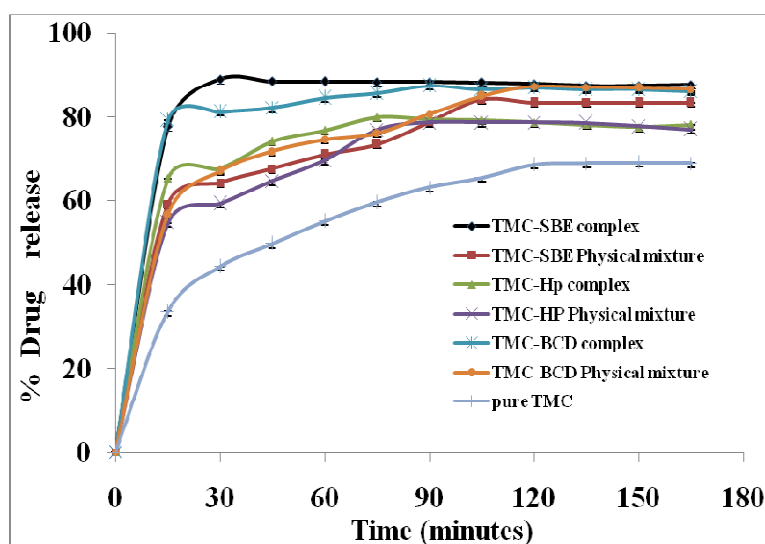

Fig. 2: Release profile of TMC from different inclusion complexes and their physical mixtures.

Table 1: Dissolution efficiency values for the different prepared systems

Formulation	Dissolution efficiency percentage
Pure drug	27.83 ± 0.59%
TMC/SBE- βCD physical mixture	45.62 ± 0.39%
TMC/SBE- βCD complex	61.1 ± 0.32%
TMC/HP-βCD physical mixture	42.13 ± 0.69%
TMC/HP-βCD complex	49.53 ± 0.45%
TMC/βCD physical mixture	45.14 ± 0.47%
TMC/βCD complex	50.14 ± 0.24%

Table 2: The composition of thermosensitive sol-gel formulations

Formula	Drug	Poloxamer407 (Pluronic 127)	Poloxamer188 (Pluronic 68)	Gelling Temperature (℃)	Comment*
F_1	____	20%	____	21℃ ± 1℃	N.
F_2	____	20%	5%	25.5℃ ± 1℃	N.
F_3	10 mg pure TMC	20%	5%	26.5℃ ± 0.5℃	N.
F_4	TMC-SBE-βCD complex equivalent to 10 mg Pure TMC	20%	5%	26.5℃ ± 0.5℃	N.
F_5	____	20%	10%	30.5℃ ±0.5℃	N.
F_6	10 mg pure TCM	20%	10%	32.5℃ ±0.5℃	N.
F_7	TMC-SBE-βCD complex equivalent to 10 mg Pure TMC	20%	10%	33℃ ± 0.5℃	N.
F_8	____	20%	15%	**36℃ ± 1℃**	**S.**
F_9	10 mg pure TMC	20%	15%	**36.5℃ ± 0.5℃**	**S.**
F_{10}	TMC-SBE-βCD complex equivalent to 10 mg Pure TMC	20%	15%	**37℃ ± 0.5℃**	**S.**
F_{11}	____	____	15%	No Gelling Characters	N.
F_{12}	10 mg pure TMC	20%	____	22℃ ± 0.5℃	N.

* N: Not exposed to further examination, S: Selected for further examination.

Poloxamers present in w/v%. Each raw shows all contents of each formulations. The volume of each formula is 5 ml.

Preparation of Thermosensitive gel

Measurement of gelation temperature

The $T_{sol-gel}$ transition temperature of the formulations developed - with and without drug - having different ratios of poloxamer F127 to poloxamer F68 was determined (Table 2). Transition temperature exhibited an increment when percent of poloxamer F68 increased in the formulation. This increment was attributed to the thickening power of poloxamers in water corresponding to the molecular weight and ethylene oxide/propylene oxide ratio [41, 42]. The optimum concentrations of poloxamer analogs for the in situ gel-forming delivery system were 20% (w/v) Poloxamer 407 (F127) and 15% (w/v) Poloxamer 188 (F68) that exhibited sol -gel transition at 36.5^{o}C ± 0.5^{o}C. Therefore, formulations F9 and F10, which have the closest gelling temperatures to the human body temperature (36.5° and 37°C, respectively) were selected for further studies.

The thermosensitive property of gels was evaluated by sol/gel transition temperatures ($T_{sol \rightarrow gel}$). At this particular temperature a drastic change occurs in the rheological behavior and the elasticity modulus which is a measure of the energy stored and recovered per cycle of deformation and reflection of the solid-like component of the system [43]. A gelation in the range of 25-37°C is optimum for development a thermosensitive formulation. With gelation temperature lower than 25°C the formulation might exist in the gel form at room temperature leading to difficultly in manufacturing, handling, and administering. On the other hand, when the gelation temperature is higher than 37°C it would result in a formulation that remains in the liquid state till administration.

Rheological behavior measurements

The sol-gel transition temperature ($T_{sol} \rightarrow _{gel}$) was chosen as the temperature at which both moduli were equal, reflecting similar elastic and viscous properties (17). Complex viscosity (η^*) obtained under dynamic conditions of oscillatory tests (frequency range of 1-

1000 Hz at 18°C and 37°C) is comprised of real part known as dynamic viscosity (η') and imaginary out-of-phase part (η'') according to the following equation [25, 26].

$$\eta^* = \eta' + i\,\eta'' \qquad \text{Eq. 2}$$

Where η^* is the complex viscosity. i is the imaginary unit, $i^2 = -1$

Viscous properties of samples are identified by η', whereas η'' represents their elastic properties. Both η' and η'' are related to the loss modulus (G'') and storage modulus (G') of the sample via the relationships:

$$\eta' = G''/\omega \qquad \text{Eq. 3}$$

$$\eta'' = G'/\omega \qquad \text{Eq. 4}$$

Where ω is the angular frequency, which is related to the frequency (f) by:

$$\omega = 2\pi f \qquad \text{Eq. 5}$$

All formulations at 18°C were in a liquid form and showed a Newtonian behaviour with no significant changes in viscosity over a broad range of shear rates. The PAV measurements in Figures 3 top part, showed that at 18°C, most of formulations exhibited viscosity η_1 near from 0.1 to 0.3 Pas and negligible η_2 (Newtonian behaviour, whereas η_1 is constant and $\eta_2 \approx$ zero). While at 37°C, all formulations showed non Newtonian behaviour and high viscosity values (Figures 3 bottom part for F2, F5, and F8 compared to Figures 5, 6 for F9, F10 respectively).

The PAV measurements of hydrogel samples presented in Figures 3-6, also demonstrated the effect of poloxamer concentration on rheology. The data in Figure 3 revealed that the viscosity η_1 increased from 130 m Pas to 190 m Pas with increasing the percentage of Pluronic 68 from 5% (F2) to 15% (F8) at 18°C. In contrast, samples F9 and F10 (similar in P68 content) had the same viscosity and moreover had higher elastic modulus and higher viscosity than the other samples at the same temperature (Figure 4).
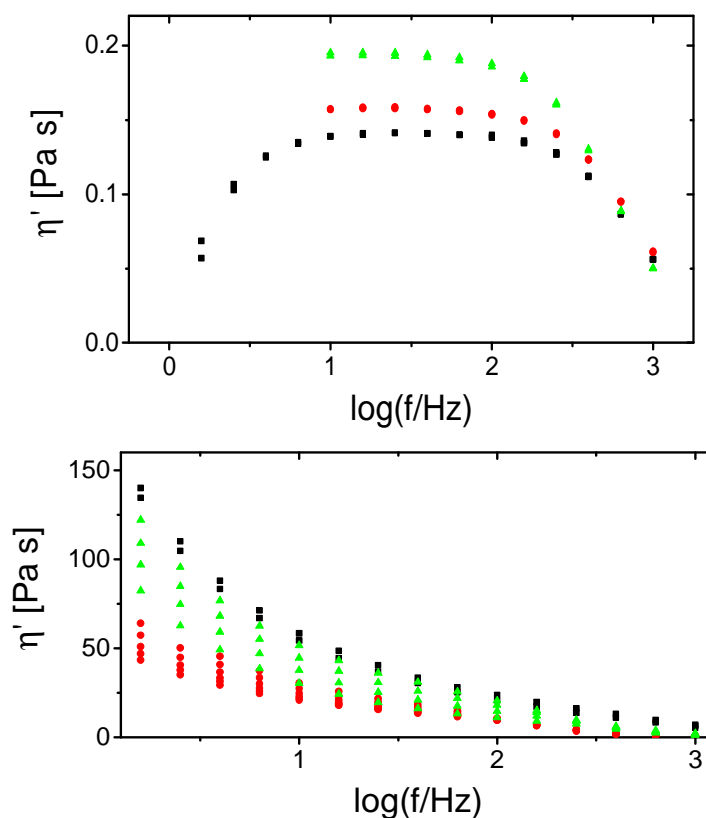

Fig. 3: PAV measurements of samples F2 (20%F127+5%F68, black square), F5 (20%F127+10%F68, red circle) and F8 (20%F127+15%F68, green triangle) at 18°C (top figure) and 37°C (bottom figure).
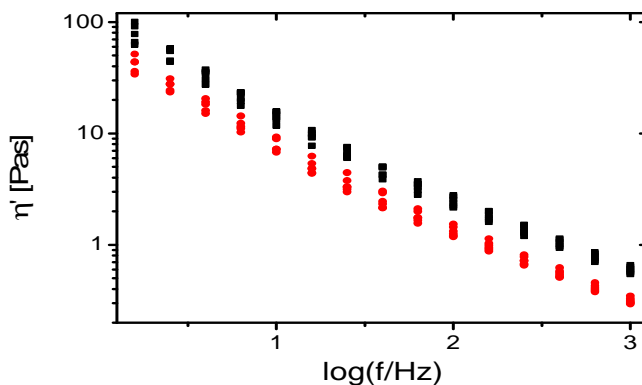
Fig. 4: PAV measurements of sample F9 (S106) (black color symbols) and F10 (S107) (red color symbols) at 18ºC.

On the other hand, at 37℃ a dramatic shear-thinning behavior was observed in the profiles of all formulations. As the angular frequency increased from 1 to 1000 (by log value from 0 to 3), the viscosity dropped 100 folds or more. The remarkable shear thinning behavior at 37℃ indicates the temperature induced gel structure formation of the poloxamers based formulation [42]. The samples had a Newtonian behavior below the gelation temperature that behaves linearly with frequency and non-Newtonian behavior above this measurement

(Figures 5, 6) [44,45]. The PAV measurements during 8 month- storage period of F9, F10 at RT and dark place revealed that he fresh samples were more elastic than viscous as $\eta_1 > \eta_2$ indicating to the gel. While, the aged samples exhibited liquid like behavior as $\eta_2 > \eta_1$. Also, the magnitude of η_1 and η_2 decreased with time (Figure 7). Consequently, F9 and F10 thermosensitive gel formulae were selected for further drug release studies based on gelation temperature values that were found to be around human body temperature (data in Table 2).
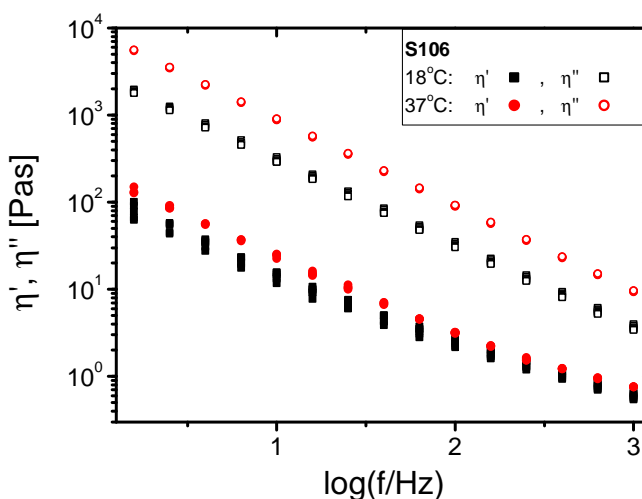

Fig. 5: PAV measurements of sample F9 (S106) at 18 (black color symbols) and 37ºC (red color symbols) (η': closed symbols, η": hollow symbols).
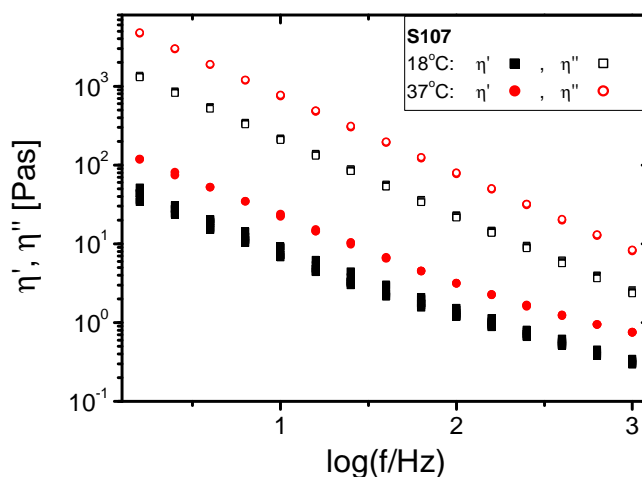

Fig. 6: PAV measurements of sample F10 (S107) at 18 (black color symbols) and 37ºC (red color symbols) (η': closed symbols, η": hollow symbols).
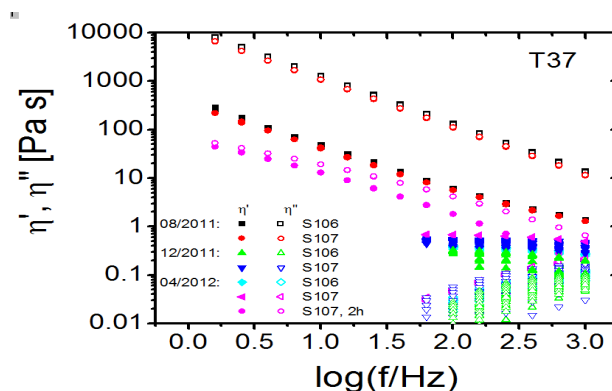
Fig. 7: PAV measurements of fresh and aged samples F9 (S106) and F10 (107) at 37ºC (η': closed symbols, η": hollow symbols).

In-vitro TMC release studies from thermosensitive gels

Figure 8 showed that the time of complete drug release for F10 was 11 hr compared to 27 hr for F9. Difference in release profiles was mainly attributed to high solubility of TMC/SBE-β-CD complex compared to pure TMC (Figure 2) that obviously appeared in the release time of drug from formulation (Figure 8). In other words, the release of drug from TMC-SBE-β-CD complex-containing thermosensitive gel (F10) was faster than from gel loaded with free drug (F9) thus, indicated that the enhancement of drug release by complexation with SBE-β-CD (F10) was still prominent after incorporation in the thermosensitive gel.
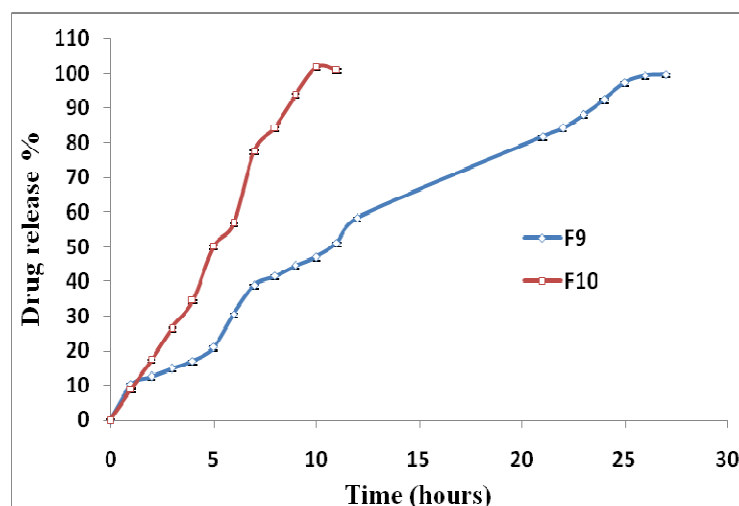

Fig. 8: Release profile of F9 (formulation consists of 20% P127 + 15% P68 + 10 mg TMC) and F10 (formulation consists of 20 % P127 + 15% P68 + TMC/SBE-β-CD complex equivalent to 10 mg TMC).

The results suggested that formulation of TMC in poloxamer thermosensitive gel might slow drug diffusion from gel if applied to cancerous tissues and consequently enhance drug- retention that can be ensured from *in-vivo* experiment. We hypothesized that the cyclodextrin would slow down the diffusion of lipophilic TMC from hydrogel and that the polymeric gel would slow down the diffusion of TMC/SBE-β-CD complex, and thus, ultimately enhancing the retention of the injected TMC-CD in breast tissues. Therefore, F10 was selected for *in-vivo* retention studies.

In-vivo studies, HPLC quantification and statistical analysis:

To confirm the ability of TMC/ SBE-β-CD -Gel to retain the TMC in tissues, drug concentration in rat breast tissues, plasma and liver were determined 8 hr after breast tissue injection F10 and compared to orally delivered suspension and TMC/ SBE-β-CD solution injection as control. The selection of this time point (8 hr) based on the reported TMC pharmacokinetics data in literature. It was reported that TMC elimination is biphasic, with an initial half-life of around 7.3 hr and a terminal half-life of 7-11 days with the reported T_{max} of 4-7 hr for free TMC [46]. As demonstrated in Figure 9, TMC/ SBE-β-CD -Gel (F10) significantly extended the retention of

TMC in breast tissues ($p < 0.05$). This is likely because the hydrogel limited the diffusion of the TMC- inclusion complex from the tissues.

We have also quantified the amount of TMC recovered from blood and liver to examine the re-distribution of TMC/ SBE-β-CD -Gel 8 hr after tissue injection. Figure 9 showed no significant difference in plasma drug level in case of direct tissue injection and oral administration. The authors attributed this result to the neglected effect of SBE-β-CD complexation on TMC plasma concentration. Previously from literature, it was found that the TMC metabolite plasma concentration was not affected by complexation of TMC with hydroxybutenyl- β -cyclodextrin after oral as well as intravenous injection [47].

However, when TMC was administered orally a significant fraction was recovered from liver 8 hr after dosing compared to direct injection of gels into breast tissues. One explanation is in vivo, F10 might have immediately gelated into a semi-solid state, and thus, prevented the significant outflow of the TMC-CD from breast tissues. Meanwhile, in the absence of the hydrogel, a significant fraction of the TMC-CD injected into might have been forced out of tissues because of the increased intracellular pressure generated by the

injection. In other words, the lowest percent of the injected thermosensitive gel was diffused out of the breast tissue and into liver for re-distribution. The results were in agreement with our previous finding and literature reporting that thermo-sensitive gels aimed to enhancing the retention of drugs when delivered locally [48, 49].
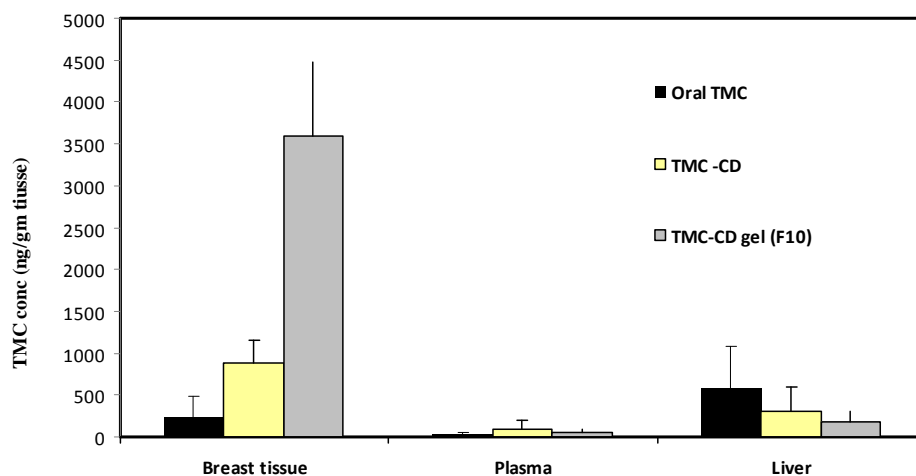

Fig. 9: Quantification of TMC in breast tissues, plasma and liver 8 hr after a single oral dose and a single breast tissue injection with TMC-SBE-β-CD solution and hydrogel F10 (*n = 7*).

CONCLUSION

In this study, we have shown that dispersing TMC- cyclodextrin complex into a thermosensitive polymeric gel is a promising candidate for TMC delivery. The hydrogel helped to enhance the retention of drug in breast tissues. Moreover, other lipophilic chemotherapeutic agents may also be locally delivered into tumors using similar cyclodextrin-in-thermosensitive gel system to increase the residence time of the chemotherapeutic agents in the tumors, and thus, to improve their anti-tumor efficacy and decrease their non-targeted toxicity.

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