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Thermosensitive *in situ* gel of Timolol Maleate for the treatment of open angle glaucoma

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ABSTRACT

The objective of this study was to develop a novel chitosan- chondroitin 6 sulphate insitu gel system for sustained drug delivery of timolol maleate. To increase the low bioavailability and short ocular residence time of timolol maleate eye drops, aqueous solutions of drug in combinations with the polymers were prepared to identify suitable compositions with regard to gel forming properties and drug release behavior. Mixtures of solutions of Pluronic (15-25% w/w), chitosan (0.05-0.3% w/w) of low molecular weight (Mw), chondroitin 6 sulphate (0.05-0.3% w/w) were prepared. Timolol maleate release was determined using a membraneless dissolution model in artificial tear solution up to 8 hours and the samples were analyzed spectrophotometrically at 294 nm. The rheological behavior of solutions in response to dilution or temperature changes and also the phase change temperature (PCT) were determined using a brookfield viscometer. The formulation consisting of 15% Pluronic, 0.05% chondroiitn 6 sulphate and 0.05% low Mw chitosan, depicted highest release efficiency coupled with an acceptable mean release time and is suggested as a suitable ophthalmic preparation for sustained release of timolol maleate. This *in situ* gel was liquid in non-physiologic conditions (pH 4 and 25ºC) and transferred to the gel form upon exposure to physiologic conditions (pH 7.4 and 37ºC). The PCT of this *in situ* gel did not change upon dilution.

Keywords: *in situ* gel, pluronics, timolol maleate, chitosan, chondritin 6 sulphate.

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INTRODUCTION

Topical delivery of drugs into the lower cul-de-sac using eye drops is a conventional approach for treatment and diagnosis of ocular diseases. The cornea is the primary location for these drugs to penetrate into eyes. Human cornea generally consists of three layers: the epithelium, stroma and endothelium. Epithelium, the most external layer, is composed of a number of well-organized and tightly packed cells and serves as a selective barrier for the penetration of ophthalmic drugs. It is noted that a high drug concentration at the cornea membrane surface is required for most of the hydrophilic drugs to ensure their essential delivery through the ocular barrier. Unfortunately, the dropped drug solution is immediately diluted by the tear fluid, followed by rapid elimination from the pre-cornea area because of the lacrimal secretion and nasolacrimal drainage. A large proportion of the topically applied drug is immediately diluted in the tear film and excess fluid spills over the lid margin and the remainder is rapidly drained into the naso-lachrymal duct. A proportion of the drug is not available for therapeutic action since it binds to the surrounding extra-orbital tissues. These processes lead to a typical corneal contact time of about 1–2 min in humans, and an ocular bioavailability that is commonly less than 10%. Due to poor ocular bioavailability, many ophthalmic drugs are applied in high concentrations. This causes both ocular and systemic side-effects. Several new preparations have been developed for ophthalmic use not only to prolong the contact time of the vehicle at ocular surface, but at the same time slow down the elimination of the drug. Successful results were obtained with inserts and collagen shields, although these preparations present some disadvantages, such as noncompliance, especially by elderly people and many patients lose the device sometimes without becoming aware of it. From the point of view of patient acceptability, a liquid dosage form is preferable. Novel delivery concepts and approaches are in high demand for improving the effectiveness, safety, and convenience of eye drops. Recently, in situ gel forming systems [1], especially the thermo-sensitive ones, have showed their potential in increasing the residential time and possible controlled release of drug molecules for eye diseases because of their capacity to improve bio-adhesiveness of ophthalmic solutions. Thus in situ-forming gel ophthalmic drug delivery systems can be used which are prepared from polymers that exhibit reversible phase transitions and pseudoplastic behavior to minimize interference with blinking. Such a system can be formulated as drug containing liquid suitable for administration by instillation into the eye, which upon exposure to physiological conditions will shift to the gel phase when instilled in the cul-de-sac [2], thus increasing the precorneal residence of the delivery system and enhancing ocular bioavailability. Thus in this study attempts have been made to design a in situ gel formulation incorporating varied concentrations of pluronics [3,4], low molecular weight chitosan and chondroitin 6 sulphate.

Poloxamer (Pluronic®), a block copolymer that consists of polyethylene oxide (PEO) and polypropylene oxide (PPO) units, is known for exhibiting the phenomenon of reverse thermal gelation under a certain concentration and temperature. At a concentration of 18% (w/w) or higher in aqueous solution, poloxamer 407 (P407), is transformed from a low viscosity solution to a gel under the ambient temperature. But this lower concentration solution will lose the

gelation ability after diluted by lacrimal fluid. Therefore, poloxamer 188 (P188), was added to P407 solution as a regulatory substance and exhibited a good perspective to increase the gelling temperature (GT) of P407. Different gel enhancing polymers has been used in combination with poloxamer: mono amine-terminated poloxamer with hyaluronic acid, the mixture of 0.3% carbopol and 14% Pluronic, linear poly(N-isopropylacrylamide-g-2- hydroxyethyl methacrylate) gel particles, and also poloxamer 407 and 188 (21 and 5%w/v respectively), with carbopol 1342P NF (0.1% - 0.2%). Pluronic F127 (PF127) consists of polyoxyethylene units (70%) and polyoxypropylene blocks (30%)[5]. It is a thermosensitive polymer solution, which behaves as a liquid below its low critical solution temperature (LCST) and forms gel when the environmental temperature reaches or is above the LCST. At a concentration of 15% or higher in aqueous solution PF127 is transformed from a low viscosity solution to a semisolid gel upon heating from 4 °C to temperature greater than 23 °C and this thermogelation is reversible upon cooling. The phenomenon of thermogelling is characterized by a sol–gel transition temperature. That is to say below this temperature, the sample is fluid allowing a comfortable and precise delivery, above this transition temperature, the solution becomes gel according to the increment of local temperature. The thermogelification results from the interaction between the different molecules of Pluronic. The increment of the temperature modifies the hydration spheres around the hydrophobic units which in turn induces higher interactions between these different units. This made PF127 attractive in formulating thermoreversible gels for ophthalmic and controlled delivery of many drugs. In recent years PF127 has attracted specific interest in designing various formulations, particularly insitu gels containing various drugs have been used for treating patients with ocular conditions. Chitosan, a polysaccharide derived from naturally abundant chitin, is composed of randomly distributed β-(1-4) linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). It is used in fields as different as food, biomedicine and agriculture among others[6]. A novel approach was developed to produce thermosensitive neutral hydrogel based on chitosan/polyol salt combinations that could undergo sol-gel transition at a temperature close to 37 °C[7]. Chondroitin 6 sulphate [8, 9, 10], is a sulfated glycosaminoglycan composed of a chain of alternating sugars. It is an unbranched biodegradable polysaccharide, which has been used in ocular delivery and exhibits mucoadhesive properties. Timolol maleate (TM) is the most commonly used drug that treats the open-angle glaucoma. Timolol maleate is a non-selective betaadrenergic receptor blocking agent. It is a white, odorless, crystalline powder, which is soluble in water, methanol, and alcohol. As systemic absorption of TM may cause respiratory and cardiovascular side effects, it is important to minimize the systemic absorption and enhance ocular bioavailability of TM.

Thus the overall objective of this study was to develop and evaluate PF127 gel forming solution to improve the ocular bioavailability and hence decrease the systemic absorption and side effects of TM. It is a challenge to enhance its bioavailability and safety through different pharmaceutical approaches. To achieve this objective varied insitu gels containing 0.5% TM were designed using least possible excipients concentration. While designing this formulation, to find the effects of different concentrations of low molecular weight chitosan, chondroitin 6 sulphate and poloxamer, a general factorial design was used in development of the thermosensitive *in situ* gels.

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MATERIALS AND METHODS

Experimental

PF127 was obtained from Sigma, USA. TM was from Merck, USA. Low molecular weight chitosan 150000 was obtained from Fluka, Switzerland. Chondroitin 6 sulphate was obtained from Sichuan Biosyn Pharmaceutical Co, China. All other chemicals and solvents were reagent grade and from Merck chemical company, Germany.

Preparation of In Situ Gel Formulations

The chitosan solutions (0.05, 0.1 and 0.3% w/w), were prepared by dispersing the required amount in acetic acid solution (2% w/v) with continuous stirring until completely dissolved. For preparation of Pluronic solutions (15-25% w/w), the required amount of polymer was dispersed in distilled, deionized water with continuous stirring for 1 h at room temperature. The partially dissolved Pluronic solutions were stored in the refrigerator (at 4ºC) until the entire polymer was completely dissolved (approximately 24 h). The chitosan/Pluronic solutions were prepared by dispersing the required amount of Pluronic in the desired concentration of chitosan with continuous stirring for 1 h. The partially dissolved solutions were then refrigerated until solutions were thoroughly mixed (approximately 24 h). The reported composition of chitosan/Pluronic mixture was the final concentration of chitosan and Pluronic content in the mixture. Chondroitin 6 sulphate was then added in varying concentrations of 0.05, 0.1 and 0.3 % w/w to the above solutions with continuous stirring until thoroughly mixed. For preparation of TM containing polymer solutions, 0.5% of TM was added to the above solutions with continuous stirring until thoroughly mixed. Benzalkonium chloride solution was added 0.006% as preservative in all solutions. All the sample solutions were adjusted to pH 4.0 ± 0.1 or 7.4 ± 0.1 by 0.5 M sodium hydroxide solution, sterilized at 121ºC and 15 psi for 20 min and then stored in the refrigerator prior to the evaluation of their rheological properties. Three formulation variable factors i.e., chitosan, chondroitin sulphate and Pluronic concentrations each at three different levels were studied (Table **1**) by a general full factorial design. Twenty eight formulations (Table **2**) were designed including a plain 15% pluronic formulation.

Table 1. Variables and their Levels Used in Production of the *In Situ* Ophthalmic Gels of Timolol maleate

LEVELS			VARIABLES
III	II	I	
0.3%	0.1%	0.05%	Chitosan concentration
0.3%	0.1%	0.05%	Chondroitin 6 sulphate concentration
25%	20%	15%	Pluronic concentration

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Table 2. Composition of the In situ ophthalmic gels of TM containing 0.5% drug (P = Pluronic, C = Chitosan low molecular weight, CS = Chondroitin 6 sulphate)

Code	Concentration %		
	P	C	CS
P_1	15		
PC_2	15	0.05	0.05
PC_3	15	0.1	0.05
PC_4	15	0.3	0.05
PC_5	15	0.05	0.1
PC_6	15	0.1	0.1
PC_7	15	0.3	0.1
PC_8	15	0.05	0.3
PC_9	15	0.1	0.3
PC_{10}	15	0.3	0.3
PC_{11}	20	0.05	0.05
PC_{12}	20	0.1	0.05
PC_{13}	20	0.3	0.05
PC_{14}	20	0.05	0.1
PC_{15}	20	0.1	0.1
PC_{16}	20	0.3	0.1
PC_{17}	20	0.05	0.3
PC_{18}	20	0.1	0.3
PC_{19}	20	0.3	0.3
P_{20}	25	0.05	0.05
PC_{21}	25	0.1	0.05
PC_{22}	25	0.3	0.05
PC_{23}	25	0.05	0.1
PC_{24}	25	0.1	0.1
PC_{25}	25	0.3	0.1
PC_{26}	25	0.05	0.3
PC_{27}	25	0.1	0.3
PC_{28}	25	0.3	0.3

Measurement of Gelation Temperature

Ten milliliters of the sample solution and a magnetic bar were put into a transparent vial that was placed in a low temperature water bath. A thermometer with accuracy of 0.1 °C was immersed in the sample solution. The solution was heated at the rate of 2 °C/min with the continuous stirring of 500 rpm. The temperature was determined as GT, at which the magnetic bar stopped moving due to gelation [11, 12]. Each sample was measured at least in triplicate.

Effect of Dilution on Gelation

The measurements were made at 15-37°C, the temperature in the conjunctival sac of the eye. The sol-gel transition temperature of poloxamer was determined from shearing stress measurements at 500 rpm, the temperature was increased 4°C every 10 min. The GT was defined as the point where a sudden shift in shearing stress was observed. To mimic the

properties in the eye, if all applied polymer solution (40 µl) was immediately mixed with the available tear fluid (7 µl), which would be the worst case scenario; the polymer solution was mixed with simulated tear fluid in a ratio of 40:7.

Rheological Studies

The rheological studies were carried out in a Brookfield cap+ viscometer (Brookfield engg lab inc, Model CAP 2000 +L, USA). The viscosity and shear stress of the sample solutions were measured at various shear rates at 25°C and 37°C, respectively. The behavior of the gels was studied in two conditions: in the physiologic (37°C and pH 7.4) and non-physiologic (25°C and pH 4) conditions. An ideal gel should show a Newtonian flow in nonphysiological condition while, pseudoplastic properties at physiological conditions. In order to simulate the physiological disposition of gels more literally, the polymer solutions were diluted by simulated tear fluid (STF) in a ratio of 40:7 and then adjusted to physiological pH value (7.4 ± 0.1) by adding the required amount of sodium hydroxide before the rheological studies were conducted at 37 ± 0.1 °C.

***In Vitro* Release from PF127 Gel Formulations**

A membraneless dissolution model was used for in vitro studies. The PF127 formulations (2 gm) were transferred into tared vials and mounted vertically in a water bath at 34 ± 0.1 °C and shaken at 20 rpm. Care was taken that the gel contained no air bubbles and that the surface was smooth. Artificial isotonic tear solution (10 ml) pre-equilibrated at the experimental temperature (34 °C), was used as the release medium. Sample (1 ml) was drawn at various time intervals and replaced with 1 ml fresh artificial isotonic tear solution.

The content of TM was determined by UV at 294 nm after suitable dilution. The release profile of TM was obtained by plotting the cumulative amount of drug released from each PF127 formulation against time. The dilution of the release medium due to replenishment following each aliquot withdrawal was taken into account in the calculation of the cumulative amount of TM released from the gel. Each experiment was performed in triplicate.

STATISTICAL ANALYSIS

Differences in drug release parameters from *in situ* gels were statistically analyzed by two-way analysis of variance (ANOVA). Statistically significant differences between *in vitro* drug release of formulations were defined as $p<0.05$.

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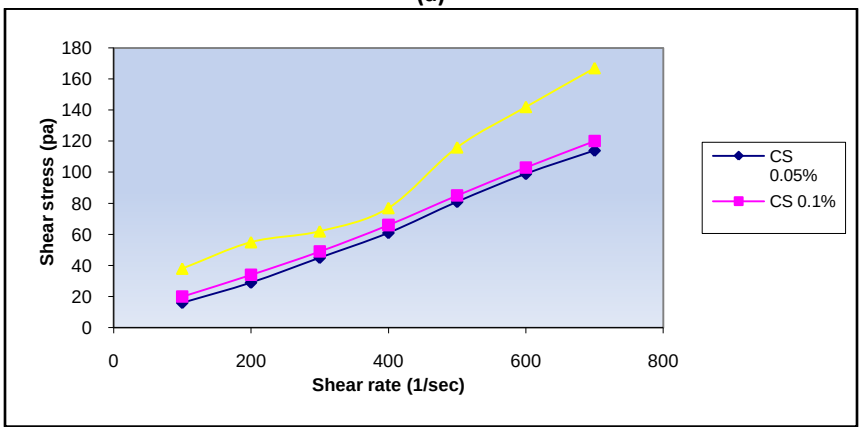
RESULTS

(a)



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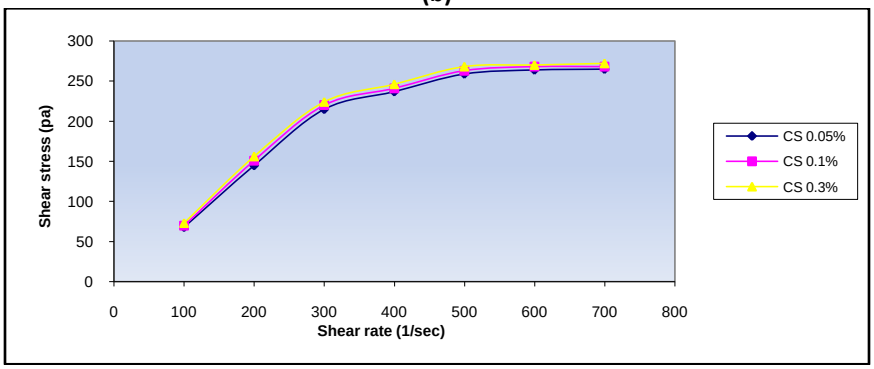
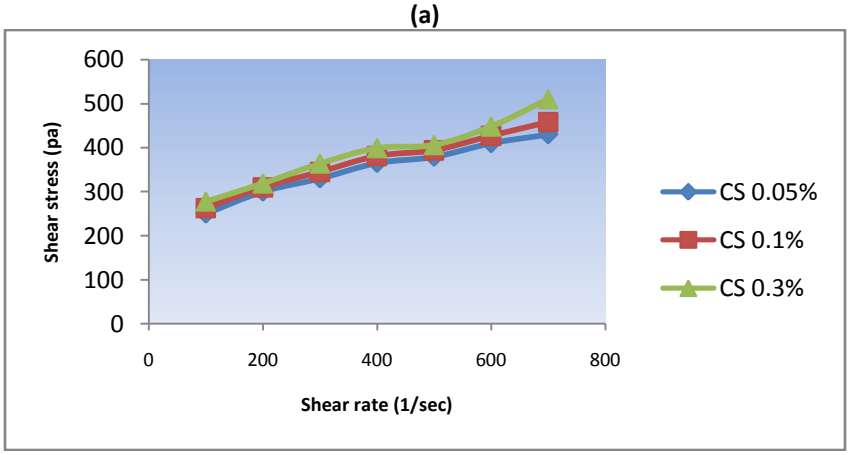


Fig. (1). Rhegogram of *in situ* gels containing 15% Pluronic F127 and 0.05% chitosan with different chondroitin 6 sulphate concentrations in (a) nonphysiologic conditions (pH 4 and 25ºC) and (b) physiologic conditions (pH 7.4 and 37ºC).

(a)



(b)

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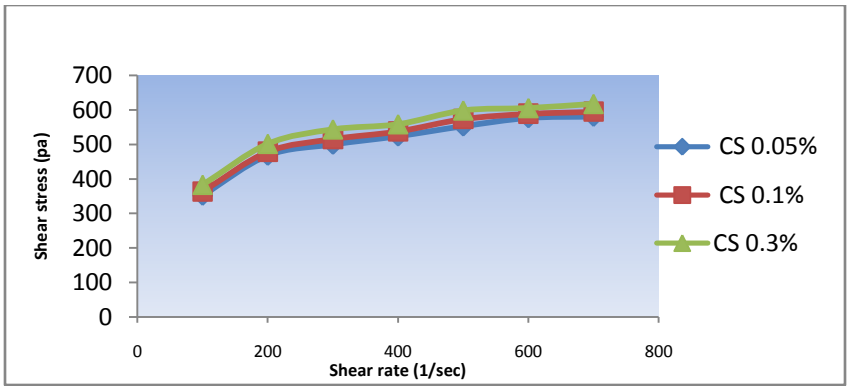
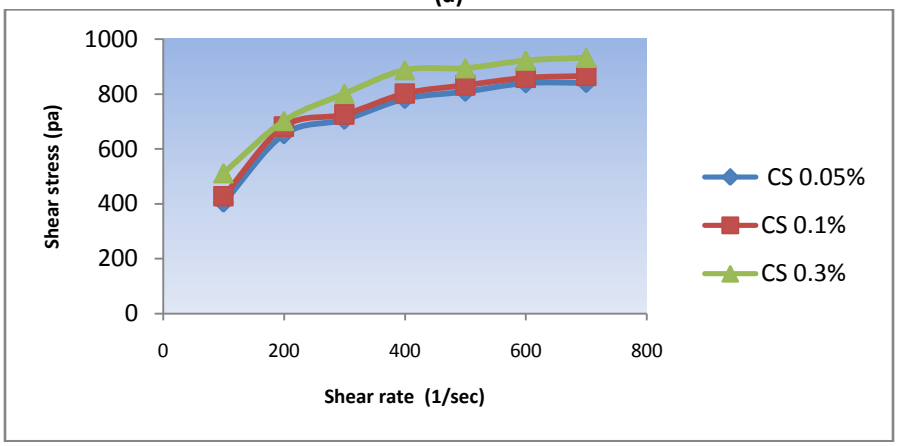


Fig. (2). Rhegogram of *in situ* gels containing 20% Pluronic F127 and 0.05% chitosan with different chondroitin 6 sulphate concentrations in (a) nonphysiologic conditions (pH 4 and 25ºC) and (b) physiologic conditions (pH 7.4 and 37ºC).

(a)



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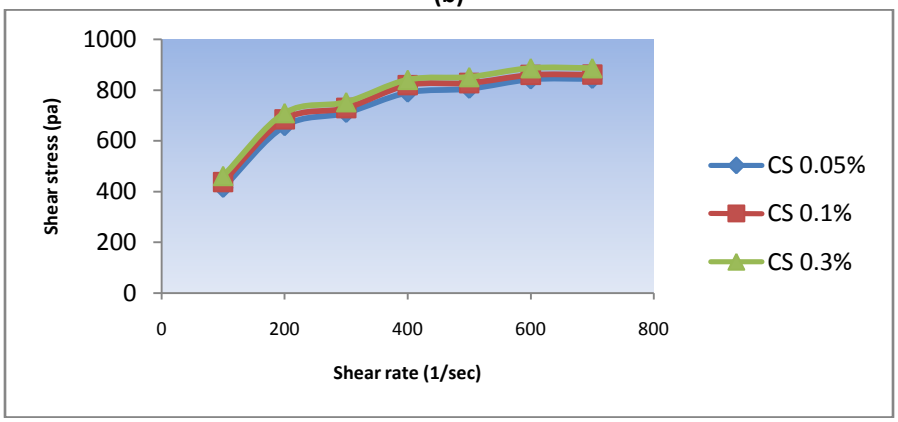

Fig. (3). Rhegogram of *in situ* gels containing 25% Pluronic F127 and 0.05% chitosan with different chondroitin 6 sulphate concentrations in (a) nonphysiologic conditions (pH 4 and 25ºC) and (b) physiologic conditions (pH 7.4 and 37ºC).

(a)

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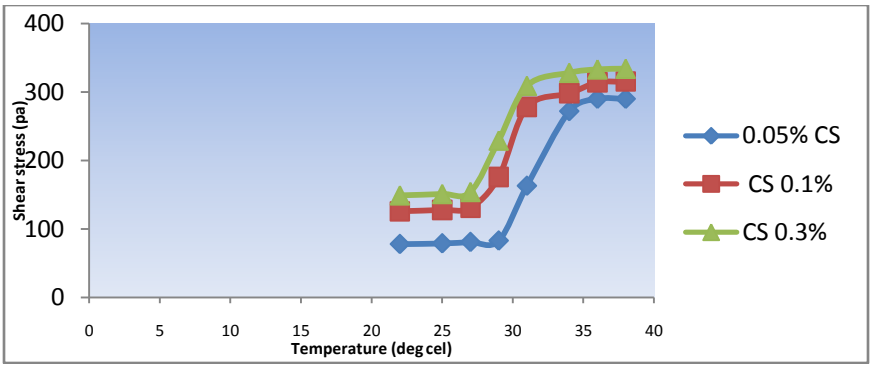

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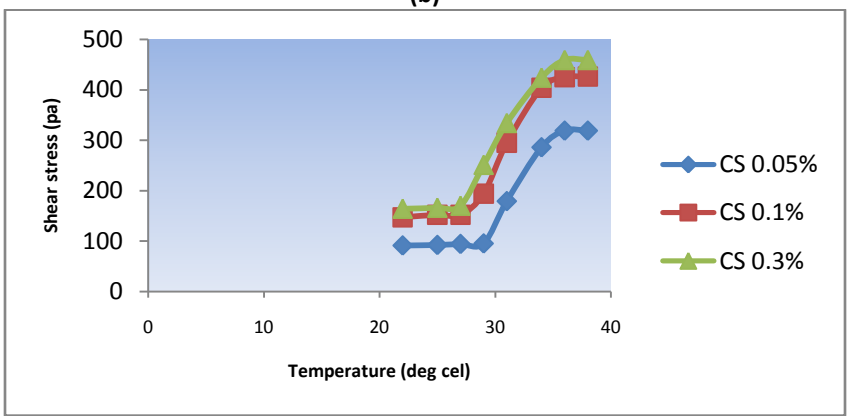

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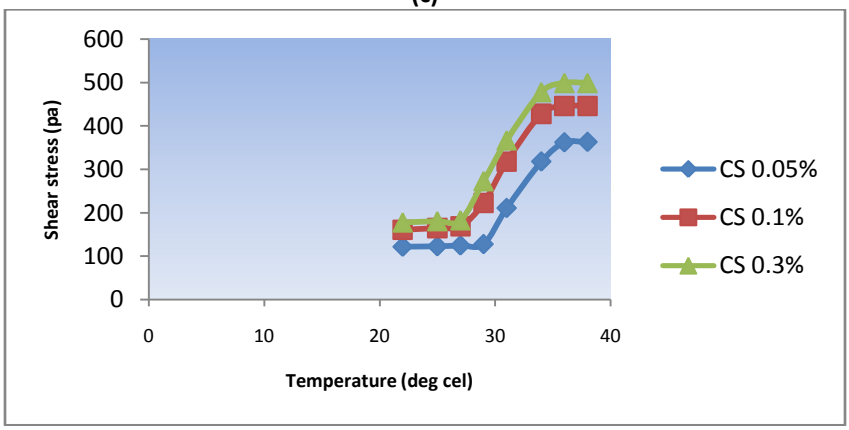

Fig. (4). Phase Change Temperature (PCT) of *in situ* gels containing 15% Pluronic F127 and varying concentrations of chitosan: a) 0.1%, b) 0.2% and c) 0.3% of different concentrations of chondroitin 6 sulphate.

(a)

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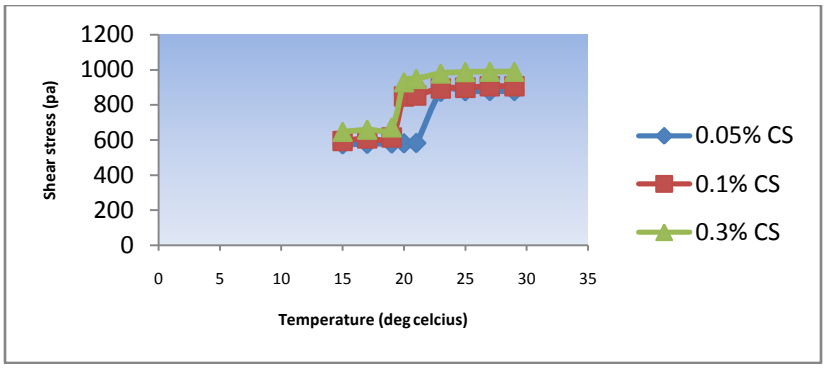

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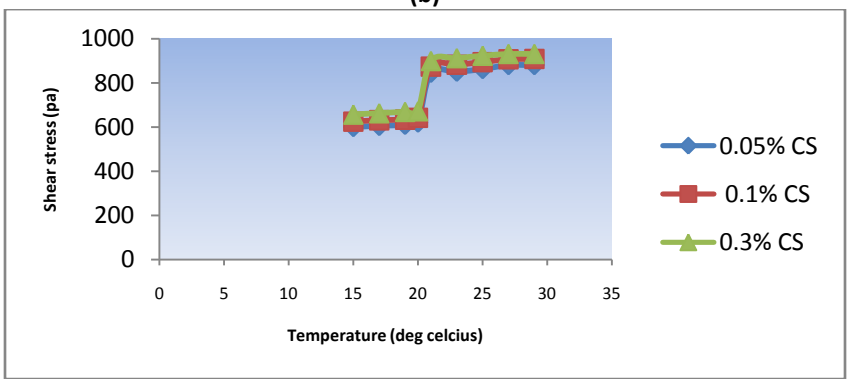

(c)

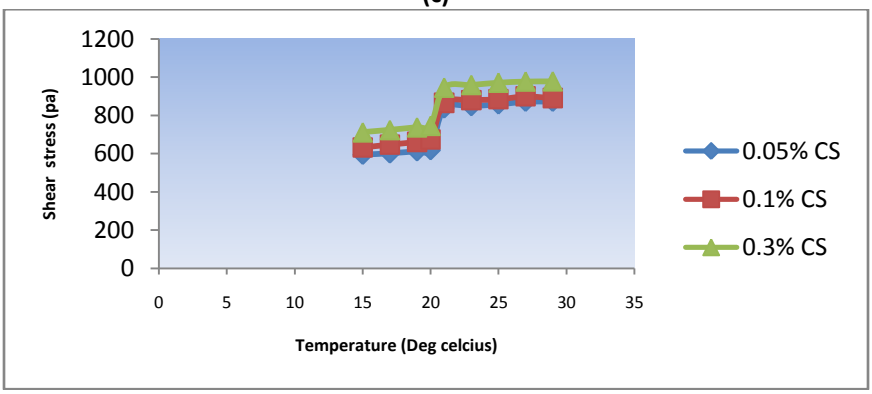

Fig. (5). Phase Change Temperature (PCT) of *in situ* gels containing 25% Pluronic F127 and varying concentrations of chitosan: (a) 0.1%, (b) 0.2% and (c) 0.3% of different concentrations of chondroitin 6 sulphate.

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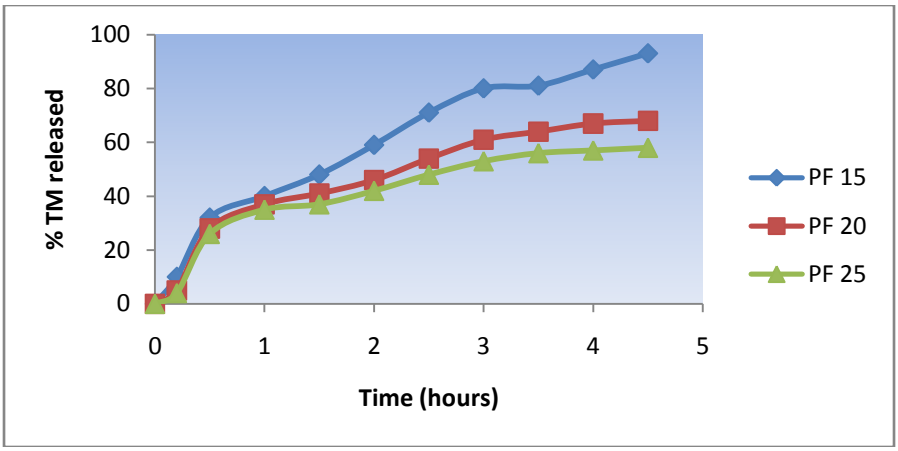

Fig. (6). Release profile of TM from gel formulations containing various concentrations of PF 127, (n=3). Results are represented as mean ± SD.

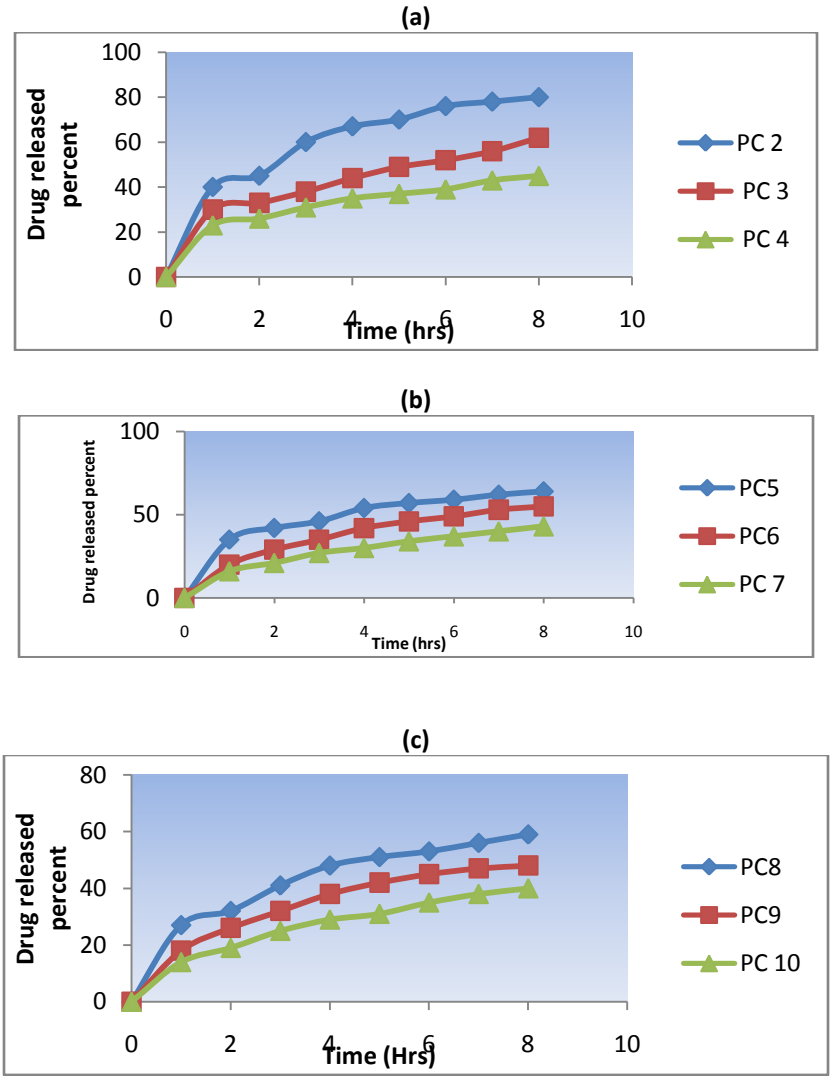

Fig. (7). Effect of different concentrations of chitosan on timolol release from *in situ* gels containing 15% Pluronic F127 and (a) 0.05%, (b) 0.1% and (c) 0.3% of (n=3). Results are represented as mean ± SD.

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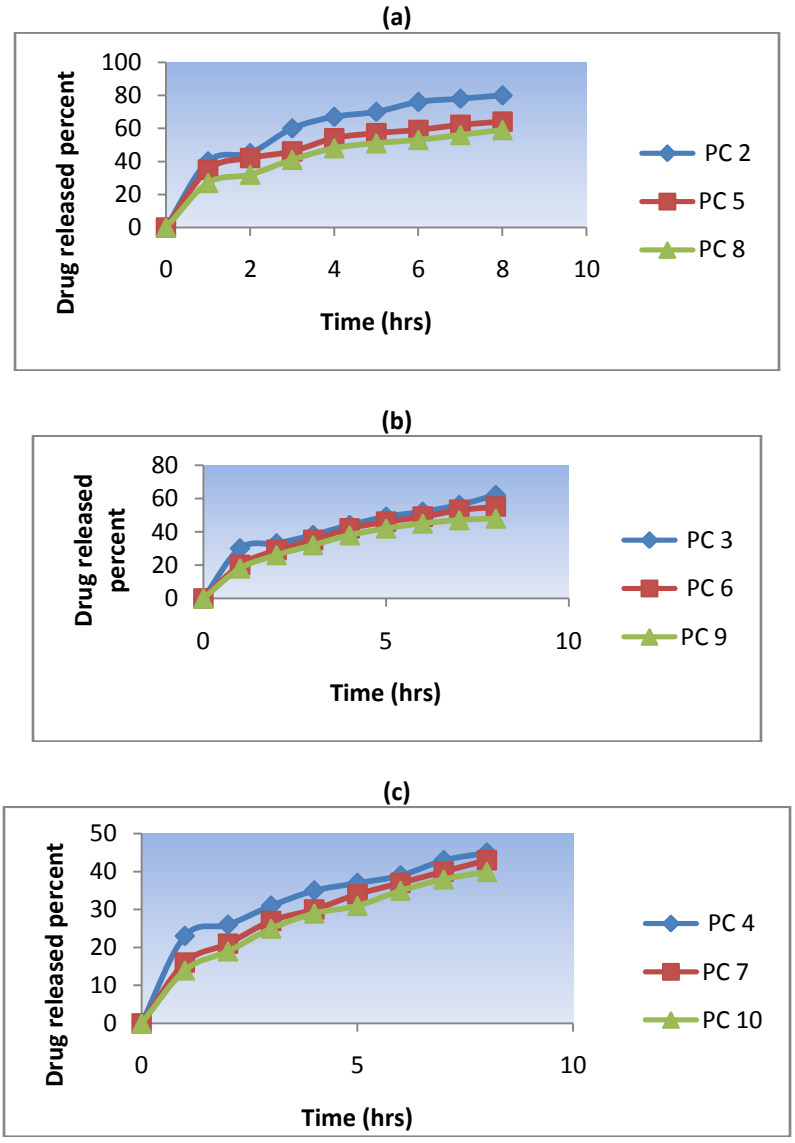

Fig. (8). Effect of different concentrations of chondroitin 6 sulphate on timolol release from *in situ* gels containing 15% Pluronic F127 and (a) 0.05%, (b) 0.1% and (c) 0.3% of chitosan (n=3). Results are represented as mean ± SD.

Figs. (1-3) shows the effect of 0.05% of chitosan with different concentrations of chondroitin 6 sulphate in combination with 15%, 20% and 25% of Pluronic in physiologic and non-physiologic conditions. As Figs. (1) and (2) indicate both 15% and 20% of Pluronic show Newtonian and pseudoplastic flow in non-physiologic and physiologic conditions respectively. However, when concentration of Pluronic is increased to 25% (Fig. 3) the gels show a non-Newtonian flow in both non-physiologic and physiologic conditions which is inappropriate for instillation in the eye. Figs. (4) and (5) show phase change temperature (PCT) of *in situ* gels containing 15% and 25% of Pluronic F127, different concentrations of chitosan and different concentrations of chondroitin 6 sulphate. As these two figures indicate the gels with 25% Pluronic show sharper PCT around 20^{o}C while those with 15% Pluronic have greater PCT at

about 32-37°C. In Table 3 the results of PCT before and after dilution of the gels with the artificial tear are compared and all the formulations show higher PCT after dilution. Fig. (7) shows the effect of different concentrations of chondroitin 6 sulphate and Fig. (8) the effect of different concentrations of chitosan on timolol release from *in situ* gels containing 15% Pluronic F127. As these figures indicate increasing the concentration of chitosan from 0.05% thru 0.1% to 0.3% and also increasing the concentration of chondroitin 6 sulphate from 0.05% thru 0.1% to 0.3%, decreases the rate of drug release.

Table 3. Comparison of the Phase change temperature (PCT) of the in situ ophthalmic gels of TM before and after dilution with simulated tear fluid. (n=3) Results are represented as mean ± SD.

Code	PCT before dilution (deg cel)	PCT before dilution (deg cel)
P_1	40.5 ± 0.3	43.5 ± 0.4
PC_2	37.8 ± 0.1	38.4 ± 0.2
PC_3	36.6 ± 0.0	37.1 ± 0.1
PC_4	35.9 ± 0.3	36.7 ± 0.1
PC_5	37.1 ± 0.2	37.0 ± 0.0
PC_6	36.4 ± 0.1	36.2 ± 0.8
PC_7	35.8 ± 0.1	35.5 ± 0.3
PC_8	36.5 ± 0.4	37.7 ± 0.5
PC_9	35.7 ± 0.5	36.2 ± 0.6
PC_{10}	35.2 ± 0.6	35.0 ± 0.5
PC_{11}	27.6 ± 0.6	28.5 ± 0.2
PC_{12}	26.8 ± 0.3	27.9 ± 0.7
PC_{13}	26.3 ± 0.2	27.1 ± 0.1
PC_{14}	26.9 ± 0.0	28.0 ± 0.1
PC_{15}	26.2 ± 0.1	27.3 ± 0.3
PC_{16}	25.7 ± 0.2	26.2 ± 0.8
PC_{17}	26.5 ± 0.3	27.4 ± 0.6
PC_{18}	26.3 ± 0.3	26.9 ± 0.2
PC_{19}	25.1 ± 0.4	25.8 ± 0.1
P_{20}	21.9 ± 0.6	22.6 ± 0.0
PC_{21}	20.4 ± 0.7	21.2 ± 0.0
PC_{22}	19.8 ± 0.7	20.7 ± 0.7
PC_{23}	21.1 ± 0.1	21.8 ± 0.6
PC_{24}	20.2 ± 0.1	21.3 ± 0.3
PC_{25}	19.0 ± 0.2	20.2 ± 0.3
PC_{26}	20.4 ± 0.3	20.7 ± 0.5
PC_{27}	19.7 ± 0.8	20.6 ± 0.2
PC_{28}	18.4 ± 0.0	19.5 ± 0.0

The release of 0.5% TM from gel formulations regressed against time using least square analysis. The slope of the regression line is a measure of the rate at which TM is released from PF127 gel formulations. The regression analysis indicated that as the concentration of PF127 increased the amount of the drug released decreased (slope: PF127 15%=934 _g min−1, PF127 20%=884 _g min−1 and PF127 25%=845 _g min−1). These results indicate that the structure of the gel functioned as an increasingly resistant barrier to drug release as the concentration of PF127 increased, which is depicted in Fig 6. The mechanism for such enhanced resistance may

be due to reduction in the number and dimension of water channels and to the increase in the number and size of micelles within the gel structure. The shorter intermicellar distance leads to greater numbers of cross-links between neighboring micelles leading to higher viscosity and lower rate of drug release. This assumption may be potentiated by the rheology study that indicates direct proportionality between gel concentration and viscosity. The drug release from 15% PF127 containing chitosan from 0.05% thru 0.1% to 0.3% and also increasing the concentration of chondroitin 6 sulphate from 0.05% thru 0.1% to 0.3%, was examined at 34 °C. The rank order of drug release was as follows: PC2 > PC3, PC5 > PC8 > PC6 > PC9 >PC4, PC7 > PC10. In general, there was a reduction in TM release obtained upon addition of the viscosity-enhancing agent chitosan to 15% PF127 gel. The increased viscosity might have contribution to the decreased rate of drug release from these formulations compared with 15% PF127 gel containing 0.5% TM. The slowest rate of drug release was obtained from the formula containing 0.3% chitosan concentration. This could be due to the formation of micelle junction zones between 0.3% chitosan and polyethyloxylene–polypropyloxylene block copolymers (Pluronic). Although the polymers differ in chemical structure, both have hydrophobic regions in their chains: of chitosan and polypropyloxylene block of Pluronic. Another feature common to the two polymers is that their gels exhibit inverted thermal reversibility, that is, they gel with heating and melt with cooling. Both the inverted temperature behavior and the presence of hydrophobic regions in the polymers provide evidence for the formation of micelle junction zone. Water molecules structured around the hydrophobic regions of polymer chains in a sol become disordered with increases in temperature. Newly exposed hydrophobic regions attract one another to form bonds, whereas hydrophilic areas rearrange to maximize their contact with the aqueous medium. The resulting structures are micelles, which continue to grow in size and number at higher temperatures, leading eventually to more rigid gel structure. Consequently, drug release is retarded at higher concentrations of chitosan. Chondroitin 6 sulphate, a biodegradable polysaccharide renders good mucoadhesive property and hence the drug release is enhanced. At 0.05% concentration of CS and 0.05% of chitosan i.e PC2, the drug release is the highest. As the chitosan concentration is increased alongside, the drug release is found to be decreasing. And hence, even when the concentration of CS is 0.3 but when chitosan concentrations are 0.05 and 0.1% respectively, the drug release is mediocre. But when chitosan concentration is 0.3 at a 0.3% concentration of CS, the drug release is lowest.

DISCUSSION

Fig. (1) shows that the gels prepared with 15% Pluronic and 0.05% chitosan consisting of varying concentrations of CS show Newtonian flow in non-physiologic state (pH 4 and 25ºC) while in physiological conditions (pH 7.4 and 37ºC) they show psuedoplastic flow. Other concentrations of chitosan with the same percentage of Pluronic behaved similarly. Although 20% concentration of Pluronic with all combinations of chitosan with varying concentrations of CS show Newtonian flow in non-physiologic state and psuedoplastic flow in physiological conditions (Fig. 2), but as Table 3 shows the phase change temperature (PCT) of these gels even before dilution with artificial tear is less than 37ºC which indicates that they are not applicable as eye drop. At higher concentration of Pluronic i.e., 25% in all combinations of chitosan with varying concentrations of CS, a pseudoplastic flow was observed both in physiologic and non-

physiologic conditions (Fig. 3) that means this concentration is not useful too. An alteration (gelation) in the rheological behavior of the formulation, from a liquid to a semisolid (i.e. gel) happens. This would result in a change in the rheological behavior and an increase in the viscosity of the formulation at the thermogelation point. Hence, as a result of the increase in the viscosity, the resulting gel could remain in contact within the eye for a longer period of time and prolongs the precorneal residence time of a drug that improves ocular bioavailability of the drug. Fig. (4) and (5) typically shows the changes of shearing stress with temperature and behavior of gels in PCT. Fig. (4a) shows a rapid phase change around 37ºC for gel containing 15% Pluronic and 0.05% chitosan and 0.05% CS. However, Fig. (5) shows a rapid phase temperature occurs around 20ºC for all gels containing 25% of Pluronic and 0.1% of chitosan(Fig. 5b). For other mixtures of these two polymers lower PCTs after dilution (Fig. 5a and 5c) indicates that they are not suitable as their viscosity will increase and change to solid flow before use in the eye and cannot be dripped. As Table 3 indicates Pluronic will lose the gelation ability after dilution by lacrimal fluid since when it is not combined with chitosan its PCT will change significantly ($p<0.05$) from 40ºC to 43ºC after dilution and its concentration is not enough anymore for gelling. However, formulations prepared with a combination of a specific concentration of Pluronic with chitosan don't show significant difference ($p>0.05$) between their PCT before and after dilution (for example PC2) while there is statistical significant difference ($p<0.05$) between PCT of different concentrations of Pluronic after dilution. This means that PCT is more affected by the concentration of Pluronic. This table also shows that the highest GT relates to PC2, PC5 and PC8 while, the lowest ones are PC22, PC25 and PC28 and the best concentration from rheological behavior point of view and PCT before and after dilution of gels are PC2, PC5 and PC8. Block copolymer gels of Pluronic F127 are thought to be formed by hydrogen bonding in aqueous systems, caused by the attraction of the Pluronic ether oxygen atom with protons of water[13]. If the hydrogen bonding is supplemented by adding compounds with hydroxyl such as cellulose derivatives or NH groups of chitosan, the desired gel strength may be achieved with reduced Pluronic concentration. It was seen in one of the studies that GT of poloxamer solutions containing 18- 25% of P407 alone or 30% of P188 was 13-25 °C and 48 °C, respectively[14]. Their results indicated that P407 or P188 alone could not provide the suitable GT. In the cases of P407 and P188 mixtures, several formulations gelled at the physiological temperature. As the concentration of P407 increased, the mixtures needed smaller amounts of P188 to gel at the desirable GT. The w/w percentage ratios of P407/P188 with GT in the range of 30-36 °C were 9/25%, 12/20% and 15/15-15/20%[15]. The results of release test are shown in Figs. (7, 8). As these profiles indicate increasing the CS concentration (Fig. 7) or chitosan concentration (Fig. 8) in all gels reduces the release rate of timolol maleate as the penetration rate of water decreases in higher viscosities of the gels. All the drug release curves seem to follow a biphasic pattern, with a faster rate of drug release over the first 2h, followed by a slower and steadier rate of drug release for the remaining 6h that may be related to the drug trapped between the tortuous ways of the gels which takes longer time to be released. The greatest release efficiency is seen in PC2 gel and its MRT also shows a sustained release pattern. At 0.05% concentration of CS and 0.05% of chitosan i.e PC2, the drug release is the highest. As the chitosan concentration is increased alongside, the drug release is found to be decreasing. And hence, even when the concentration

of CS is 0.3 but when chitosan concentrations are 0.05 and 0.1% respectively, the drug release is mediocre. But when chitosan concentration is 0.3 at a 0.3% concentration of CS, the drug release is lowest. These results indicate that the structure of the gel functioned as an increasingly resistant barrier to drug release as the concentration of chitosan increased. The mechanism for such enhanced resistance may be due to reduction in the number and dimension of water channels and to the increase in the number and size of micelles within the gel structure. The shorter intermicellar distance leads to greater numbers of cross-links between neighboring micelles leading to higher viscosity and lower rate of drug release [16,17]. The concentration of Pluronic 127 also has an important effect on the viscosity of the gels. This assumption may be potentiated by the rheology studies that indicate direct proportionality between gel concentration and viscosity. The slowest rate of drug release was obtained from the formula containing 0.3% of chitosan and 0.3% of CS. This could be due to the formation of micelle junction zones between chitosan and polyethyloxylene-polypropyloxylene block copolymers (Pluronic) similar to the formation of these physical cross-links between Pluronic and methylcellulose mentioned by Fu et al[18]. Chitosan is a copolymer consisting of 2- amino-2-deoxy-D glucose and 2-acetamido-2-deoxy-Dglucose units linked with β-(1-4) bonds. Although the polymers differ in chemical structure, both have hydrophobic regions in their chains: the D glucose residues of chitosan and polypropyloxylene block of Pluronic. Water molecules structured around the hydrophobic regions of polymer chains in a sol become disordered with increases in temperature. Newly exposed hydrophobic regions attract one another to form bonds, whereas hydrophilic areas rearrange to maximize their contact with the aqueous medium. The resulting structures are micelles, which continue to grow in size and number at higher temperatures, leading eventually to more rigid gel structure. And subsequently drug release is retarded.

CONCLUSION

Timolol maleate (0.5% w/v), a non-selective beta-adrenergic receptor blocking agent used for the treatment of open-angle glaucoma, was successfully formulated in thermoresponsive *In situ* gel forming eye drop using 15% Pluronic F127 as the gelling agent and 0.05% chitosan plus 0.05% chondroitin 6 sulpahte as a viscosity enhancing agent. The formulation was liquid in non-physiologic conditions (pH 4 and 25^{o} C) which transformed into the gel form on exposure to physiologic conditions (pH 7.4 and 37^{o} C). The PCT of in situ gel did not change upon dilution and gave a sustained release over an 8 hr period. Owing to prolongation in precorneal residence time and sustaining drug release, the developed formulation is a viable alternative to conventional eye drop and thereby serving to be a better treatment option for open angle glaucoma.

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