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Chitosan *in situ* gelation for improved drug loading and retention in poloxamer 407 gels

Tofeeq Ur-Rehman, Staffan Tavelin[1], Gerhard Gröbner*

Department of Chemistry, Umeå University, 90187 Umeå, Sweden

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ABSTRACT

A method for the *in situ* gelation of poloxamers and the mucoadhesive polymer chitosan has been developed by exploiting the tendency of poloxamer solution to form gel at physiological temperatures and of chitosan (CT) to form ionotropic gel structures in the presence of sodium tripolyphosphate (TPP). Novel poloxamer gels containing CT–TPP complex formed *in situ* during the administration were prepared by mixing poloxamer–CT and poloxamer–TPP solutions in double syringes. The micellization and gelation of poloxamer 407 in the presence of chitosan and/or TPP were studied using differential scanning calorimetry and tube inversion; both additives were found to reduce the critical micellization temperature and critical gelation temperature of poloxamer aqueous solution. The poloxamer gels containing CT–TPP complex formed *in situ* were found to exhibit reduced dissolution rate and superior release characteristics with three different drugs – metoprolol, doxycycline and flufenamic acid. Furthermore, by varying the compositions of the two solutions independently, it is possible to control the pH in a way to suit the solubilization of a drug as well as the specific environment of a particular application site. By varying the concentrations of chitosan, TPP and poloxamer, the delivery system can be fine-tuned to afford gels with specific properties, ranging from nanoparticle suspensions to semisolid gels. These *in situ* gels have the potential to increase the utility of thermo-reversible poloxamers in drug delivery.

1. Introduction

In certain solvents, poloxamers can form structures with micellar or gel-like features in a concentration and temperature dependent way (Alexandridis and Hatton, 1995; Wanka et al., 1994; Yu et al., 1992). Because of their low toxicity and biocompatibility, poloxamer-based gels have the potential to be of great utility in drug and gene delivery, and they have been studied extensively in these contexts (Escobar-Chavez et al., 2006; Kabanov et al., 2002). Poloxamer 407 (P407) is one of the most studied member of this family of block polymers (structure shown in Fig. 1a), which is also known as Pluronic®F127. P407 gels have been studied as a formulation adjuvant in a number of applications, ranging from solutions for ocular, nasal, periodontal, vaginal, rectal, transdermal and subcutaneous drug delivery, to the local delivery of anticancer drugs (Amiji et al., 2002; Barichello et al., 1999; El-Kamel, 2002; Liu et al., 2009; Pillai and Panchagnula, 2003; Ryu et al., 1999; Scherlund et al., 2000b).

However, the viability of poloxamer gel based delivery strategies is restricted by the gels' relatively fast dissolution under physiological conditions (Dumortier et al., 2006). Various potential solutions for this issue have been explored, focusing on improving the strength, mucoadhesiveness, and residence time of poloxamer systems either by modifying their structures or by incorporating mucoadhesive polymer substances such as carbophil, sodium alginate, polycarbophil, carageenan, or chitosan (Cho et al., 2003; Gratieri et al., 2010; Jones et al., 2009; Kim et al., 2007; Liu et al., 2009; Niu et al., 2009; Ryu et al., 1999; Sosnik et al., 2003). In particular, studies aiming to improve the utility of poloxamer gels in drug delivery systems have focused on the mucoadhesive polymers like chitosan, (structure shown in Fig. 1b) because of its biocompatibility and biodegradability properties. The addition of chitosan alone has been found to improve the mucoadhesiveness and gel strength of poloxamer gels (Gratieri et al., 2010). However, the scope for improving the residence time of chitosan-modified poloxamer gels and modulating their controlled release properties via *in situ* ionotropic gelation has not yet been examined. This paper describes a new approach, in which sodium tripolyphosphate (TPP) is used in conjunction with a double syringe (see Supplemental

Abbreviations: P(a), P407 aqueous solution (a% of P407); PC(a:b), P407 aqueous solution with chitosan (a% of P407: b% of chitosan); PT(a:c), P407 aqueous solution with TPP (a% of P407: c% of TPP); PCT(a:b:c), *in situ* mixture of PC and PT through double syringe (a% of P407:b% of chitosan:c% of TPP); S, drug aqueous solution.

* Corresponding author.
E-mail address: gerhard.grobner@chem.umu.se (G. Gröbner).
[1] Present address: Department of Pharmacology and Clinical Neuroscience, Umeå University, 90185 Umeå, Sweden.

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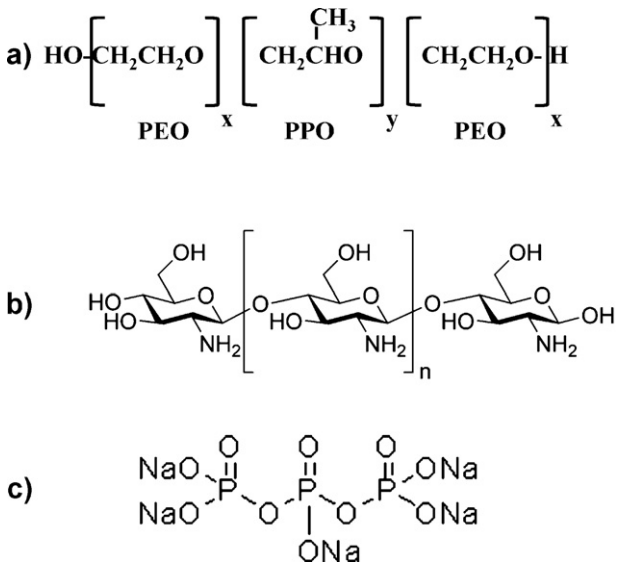

Fig. 1. (a) Chemical structure of the poloxamer P407, which contains 95–105 monomeric ethylene oxide (x) subunits and 54–60 propylene oxide (y) subunits; (b) chemical structure of chitosan; and (c) chemical structure of sodium tripolyphosphate (TPP).

Fig. 1) for the *in situ* gelation of chitosan in P407gels. TPP is a polyanion (structure shown in Fig. 1c) used in the preparation of chitosan nanoparticles (Calvo et al., 1997) and is categorized as being GRAS (generally recognized as safe) by the FDA.

The main objective of this study was to identify the optimal formulation combinations of P407-chitosan and P407-TPP mixtures, suitable for ocular, vaginal, orthodontal, and local parenteral administration of model drugs. Since additives have pronounced effects on the micellization, gelation and release profile of poloxamer aqueous solutions (Armstrong et al., 1996; Gilbert et al., 1987; Kwon et al., 2001; Pandit and Kisaka, 1996; Scherlund et al., 2000a; Su et al., 2003; Ur-Rehman et al., 2010), we investigated the impact of chitosan and TPP on the critical micellization temperature (CMT), the critical gelation temperature (CGT), and the dissolution behaviour of P407 aqueous solutions using differential scanning calorimetry (DSC), tube inversion and gravimetric methods. *In situ* gels were characterized in terms of syringeability, injectability, and gelation time with the aim to extend their use in preclinical animal studies. To investigate the drug release pattern from gels, three model drugs (metoprolol, doxycycline and flufenamic acid) were used which have different physicochemical and/or pharmacological properties (see Supplementary Table 1). The drug release from poloxamer gels has been studied using membrane-less or a membrane based experimental approach (Dumortier et al., 2006); both approaches were applied here to test the applicability of gel formulations in multiple routes of administration such as ocular, vaginal, dermal and subcutaneous route. Finally, the scope for adapting the method of *in situ* formation of nanoparticles using a dual syringe was also examined to broaden the drug delivery applications of poloxamer gels.

2. Materials and methods

2.1. Materials

P407 (Pluronic F127, culture-tested, manufactured by BASF, USA) with an average molecular weight of 12,600 Da, low molecular weight chitosan (50,000–190,000 Da, 75–85% deacetylated), doxycycline (as doxycycline hyclate) and flufenamic acid were purchased from Sigma (Sweden); metoprolol (as metoprolol tartrate) was a gift from Astra Zeneca (Sweden). Sodium tripolyphosphate, acetic acid, citric acid, NaOH and Na_2HPO_4 were of analytical grade.

2.2. Sample preparation

Poloxamer solutions were prepared according to the "cold method" first described by Schmolka (Irving, 1972). Briefly, a weighed amount of P407 was added to water or an aqueous solution of chitosan or TPP that had been equilibrated at 4–8 °C before use. The poloxamer solution was kept for another 24–36 h in an ice bath or refrigerator with occasional shaking until a clear solution was obtained. Chitosan was dissolved in glacial acetic acid (0.6–2.5%) or citric acid (2.1%) to prepare stock solutions, which were further diluted with water to achieve the desired concentration. For micellization studies, 1% P407 solutions were prepared by diluting the stock solution with appropriate quantities of chitosan or TPP solutions or with water. When studying the impact of chitosan and TPP on gelation temperature and gel dissolution, an appropriate amount of P407 was added to CT/TPP solutions. Samples for drug release studies were prepared by adding drugs to water or chitosan/TPP solutions (at concentration and pH that allowed complete dissolution of drug) followed by addition of calculated amount of P407. All the samples were prepared on a w/w basis and reported as wt%.

2.3. Differential scanning calorimetry

Calorimetric experiments were carried out using a Microcal VP-DSC micro calorimeter (MicroCal USA) equipped with VP Viewer software. For sample analysis, the reference cell was filled with water, the sample and reference cell pressure was adjusted to 15 psi, and thermograms were acquired using heating and cooling cycles between 2 and 65 °C at temperature scan rates of 90, 60, or 30 °C/h. The pre/post scan equilibration time was set to 5 min. Data were analyzed using Microcal Origin to obtain onset temperature (T_{onset}); the area under the peak; the peak temperature (T_{peak}); and the endset temperature (T_{endset}) of endothermic peak.

2.4. Gelation temperature

The gelation temperatures of the examined formulations of P407 were determined by the "Visual Tube Inversion Method", as previously described (Ur-Rehman et al., 2010). Briefly, glass vials with a diameter of 13 mm containing 1 g of sample were placed in a water bath. The temperature of the bath was slowly increased; the temperature at which the sample solution stopped flowing on tilting was noted as the gelation temperature (t_1). Similarly, samples were placed in a hot bath, which was slowly cooled; the temperature at which the gel started flowing was noted as the gel melting temperature (t_2). The thermocouple of a digital thermometer (Fluke, USA) was placed in a 13 mm vial containing 1 ml of water. The vial containing the thermocouple was situated next to sample vial in the water bath. Three measurements of t_1 and t_2 were made on each sample; each sample was prepared and analyzed in duplicate. The critical gelation temperature was then calculated as the mean ± S.D. of the measured t_1 and t_2 values.

2.5. Syringeability/injectability and gelation time

The flow behaviour of the gel formulations was characterized in terms of their syringeability and injectability when using needles identical to those used in clinical studies on smaller laboratory animals such as mice and rats (Baumnas et al., 2001). The syringeability of the poloxamer formulations was assessed by withdrawing 1 ml of the refrigerated poloxamer formulation through a hypodermic needle of dimensions 0.4 mm × 19 mm (27G) or 0.6 mm × 25 mm

(23G). The injectability of the chitosan/TPP-loaded poloxamer solutions was assessed by administering them through a 27/23G needle attached to standard 1 ml syringe (BD 1 ml syringe with Luer-Lok tip). The injectability of aqueous CT–TPP mixtures prepared *in situ* (with or without added poloxamer) was assessed using a pre-filled refrigerated double syringe (2.5 ml, 1:1, with mixer and Luer-Lock, purchased from Medimix, Switzerland). In terms of their injectability, the mixtures were classified as being one of the following: (i) injectable suspension (a free-flowing suspension of fine suspended particles that passes easily through a 27G needle without noticeable resistance); (ii) injectable gel (a solution that passes through a 27G needle with some resistance and emerges as a stream of coagulated gel); (iii) administrable gels (a solution that is difficult to push through a 27G needle and emerges in the form of gel droplets rather than a stream); or (iv) semisolid gel (a gel solution that cannot be injected through a 27G needle but passes easily through a 23G needle, emerging as a thick gel). All gel formulations were stored at 4–8 °C prior to syringeability/injectability testing.

The gelation time of poloxamer gels with chitosan or TPP or mixtures thereof was noted by administering the refrigerated gel into vials maintained at 36–37 °C as described in Section 2.4. The formulations were divided into three categories on the basis of their gelation times: less than 15 s, between 15 and 30 s, and more than 30 s.

2.6. Gel erosion and dissolution

Dissolution profiles of chitosan (0–1.2%) and TPP (0–2%) containing P407-based gels (17%, 18%, 20%) in aqueous environments were determined by the gravimetric method, as described previously (Ur-Rehman et al., 2010). Double syringe with 1:1 mixing was used for *in situ* PCT gels samples. For this purpose, a preweighed glass vial of 13 mm diameter containing 0.6 g of the gel was equilibrated at 37 °C and 0.3 ml of water (upon prior equilibration at 37 °C) was layered on top. After pre-determined time intervals the liquid medium was removed, and the weight of dissolved gel was calculated from the change in the weight of the vial. The whole process was carried out in an incubation room maintained at 37 °C.

2.7. In vitro drug release

Drug release studies were performed in an incubation room (37 °C) using metoprolol, doxycycline and flufenamic acid as model drugs in combination with different gel formulations. The *in vitro* drug release setups were either membrane-free or using permeable polycarbonate membranes which were separating the gels from the release medium. The drug content was 0.1% of the final formulation (for composition of formulations see Supplementary Table 2). All release experiments were performed as triplicates.

2.7.1. Membrane free model

For the determination of the release profile of metoprolol, a membrane-free experimental set-up was used to study the drug release pattern of gels in water rich environments such as eyes etc.; method described previously (Ur-Rehman et al., 2010). Briefly, metoprolol loaded P(18), PC(18:0.8), PT(18:2), PCT(18:1:2), P(20), PC(20:0.8), and PCT(20:0.64:1.6) gels were prepared and treated as described in Section 2.6; the released medium, collected at predetermined time intervals, was diluted with water to a final volume of 1 ml before analysis.

2.7.2. Membrane model

The polycarbonate membrane models are commonly used in studies of drug permeability across epithelial cell monolayers such as Caco-2 (Artursson and Karlsson, 1991). Transwell® permeable inserts (12 mm diameter with 3 μm pore size (Corning Incorporated, USA)) fitted on well plates were used in this study. The membrane inserts were loaded with 0.5 ml/gm of doxycycline solution in either water or in P407 gel without or with addition of TPP and/or chitosan. PC gel with 0.2% doxycycline was mixed *in situ* with drug-free PT gel using a double syringe (1:1 mixing). The gels and water were equilibrated for 0.5 h at 37 °C followed by addition of 1.5 ml of preheated water to each well and then whole system was placed on a tumbler at 20 rpm. At specified time intervals, 0.5 ml of the solution from individual well was withdrawn and replaced with pre-heated water.

Flufenamic acid was dissolved in 1 M NaOH and diluted with water/TPP solution for S, P(20) and PT(20:0.9). For PCT(20:1.1:0.9) gels, drug free PC(20:1.1) gel and drug loaded PT(20:0.9) gels were mixed *in situ* with double syringe (1:1 mixing). For release studies of flufenamic acid, experimental set-up was identical to that described above, with the exception that the release medium was citrate-phosphate buffer at pH 7 or pH 4.2 instead of water, and that 1 ml of the release medium was removed and replaced with fresh media.

2.7.3. Determination of released drug

The amount of drug in release medium was determined by measuring the absorbance using a 5 mm cuvette and a Cary 5000 UV–Vis spectrophotometer (Varian; USA). Absorbance at 274 nm and linear calibration curve (Abs = 0.00191*conc-0.0036; R^2 = 0.9999; standard solution 5–400 μg/ml) were used for metoprolol estimation. Metoprolol samples from PCT experiments were centrifuged at 6700 g for 2 min (Mini spin plus; Eppendorf, Germany) to settle the suspending gel particle prior to analysis. A standard calibration curve (Abs = 0.01473*con; R^2 = 0.9997; standard solution 1–75 μg/ml; λ = 274 nm) was used for determination of doxycycline. Similarly absorbance of flufenamic acid samples at 288 nm was converted to concentration with a linear equation (Abs = 0.0276*Con; R^2 = 0.9998) based on its standard solutions (0.5–35 μg/ml). Water for standard solutions and respective regent blank for samples were used for the base line correction. If concentrations of analyte were found beyond the linear range, the samples were further diluted.

2.8. In situ particles formation

2.8.1. Chitosan–TPP nanoparticles

A modification of the method of Calvo et al. (1997) was employed. Thus, solutions of chitosan (0.08–0.2%) and TPP (0.07–0.1%) were loaded separately into a double syringe system (2.5 ml, 4:1 ratio, purchased from Medimix, Switzerland). Upon extrusion through mixer and 27G needle into a vial, the mixture was stirred for 10 min. The particles were then centrifuged for 30 min at 14,000 g on a glycerol bed at 25 °C prior to analysis (Microfuge 18; Beckman Coulter, USA).

2.8.2. In situ formation of chitosan–TPP nanoparticles in P407 gels

The nanoparticles were produced using a method similar to that described above: 17% solutions of P407 containing chitosan on the one hand and TPP on the other were mixed using a double syringe. After dilution of the mixture, the particles were centrifuged at 5 °C.

For comparison chitosan and TPP solution were also mixed in a vial, stirred for 30 min and processed as described above. The size distribution of the particles was determined using a Zeta sizer, nano-S (Malvern industries, USA); Transmission electron micro-

scopic (TEM) characterization of the particles was carried out using JEM-1230 (Japan) with 2% phosphotungstic acid staining.

3. Results

3.1. Critical micellization temperatures of P407 solutions

DSC was used to study the effects of chitosan and TPP on the micellization behaviour of aqueous poloxamer systems and to determine the CMT of the mixture. Fig. 2a shows a selection of illustrative DSC profiles: that of a P(1); PC(1:0.65); and PT(1:0.7) solutions. All three profiles feature a clear endothermic peak indicative of micellization. This peak arises from the dehydration of the hydrophobic polypropylene oxide block (PPO) of the P407 molecules in the course of their micellization, as previously described by various authors (Alexandridis and Holzwarth, 1997; Hecht and Hoffmann, 1995). The thermogram produced by PC(1:0.65) exhibits lower values of $T_{onset,}$ T_{peak} and T_{endset} relative to that of P(1), and also shows a reduced peak height. Henceforth, T_{peak} is always referred to as the CMT. The thermogram of PT(1:0.7) also exhibits lower values of $T_{onset,}$ CMT and $T_{endset,}$ but in this case the height of the endothermic peak is increased relative to that of P(1). However, the effects of added chitosan and TPP on the micellization of P407 are comparatively minor, as they cause only small reductions in the CMT. In a second series of experiments (see Fig. 2b), the effects of varying the amounts of added chitosan (0–1.8%) and TPP (0–1.8%) on the phase behaviour of aqueous P407 systems (1%) were investigated. In general, chitosan has a less pronounced effect on the CMT of the P407 system than TPP. The effect of the chitosan–TPP complex on the CMT was studied by using a double syringe to mix PC(1:0.1–0.25) and PT(1:0.1–0.45%) solutions immediately before injecting the mixture into the DSC cell. The thermogram for PCT (1:0.1–0.25:0.1–0.45) solutions are similar to the one obtained with individual additives; with all of them having lower CMTs in comparison to the P(1) value and between those values observed for PC or PT solutions. The thermo-reversible nature of poloxamer micellization in the presence of the chitosan–TPP complex was confirmed by performing a DSC upscan immediately followed by a downscan (DSC profile not shown).

3.2. Critical gelation temperature of P407 gels

To identify the optimum composition which would form gel in the physiological temperature region and would flow reasonably freely during administration, we measured the CGTs of different poloxamer solutions (see Fig. 3). The effects of addition of chitosan (0–1.6%) on the CGT of 17%, 18%, and 20% aqueous solutions of P407 are shown in Fig. 3a. It should be noted that, as described in Section 2.2, the chitosan solutions used in these experiments contain acetic acid. Therefore, for consistency, the quoted CGT values for poloxamer solutions with 0% chitosan refer to solutions containing 0.25% of acetic acid. For comparative purposes, the CGTs of acetic acid free P(17), P(18) and P(20) gels are also shown (open symbols). In the case of the 20% poloxamer gel, the CGTs of mixtures containing 0%, 0.2% and 0.5% chitosan are slightly increased compared to P(20) solution; as more chitosan is added, the CGT declines to that of the P(20) solution. Overall, chitosan in acetic acid causes only minor changes (0.1–1 °C) in the gelation temperature of the 20% aqueous poloxamer solution. The addition of acetic acid to 18% and 17% poloxamer solutions causes a more pronounced increase in the CGT (3–4 °C); a behaviour which is attenuated with an increasing in chitosan concentration.

The effect of increasing concentrations of TPP on the CGT of P407 aqueous solutions of various concentrations (17%, 18%, 20%) is shown in Fig. 3b. It is readily apparent that the addition of TPP reduces the CGT of poloxamer solutions to a much greater extent than chitosan. The addition of more than 2% of TPP in 20% P407 aqueous solutions leads to the formation of a gel phase below room temperature. The gelation temperatures of freshly injected PCT gels were also determined (results not shown).

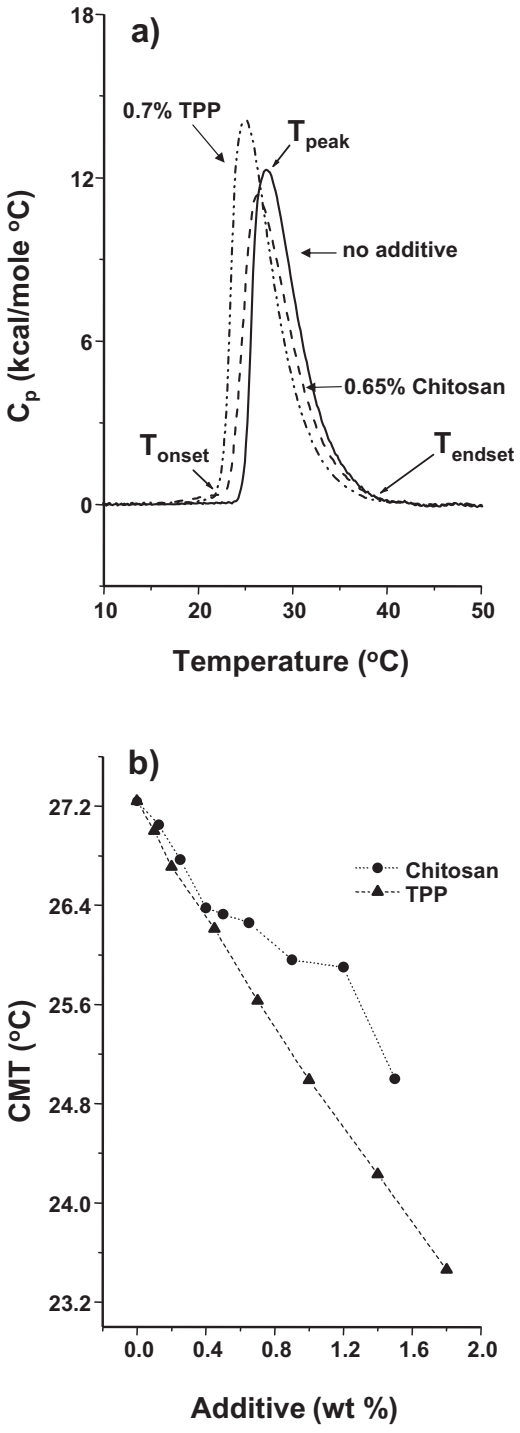

Fig. 2. Results of differential scanning calorimetry analyses of aqueous poloxamer (P407) solutions, showing the effects of chitosan and TPP on their critical micellization temperatures. (a) Representative thermograms of 1% P407 aqueous solutions containing no additive (solid line), 0.65% chitosan (dashed line), and 0.7% TPP (dash-dotted); (b) a plot of the critical micellization temperature (CMT) of aqueous solutions of 1% P407 as a function of the concentrations of chitosan (●) and TPP (▲).

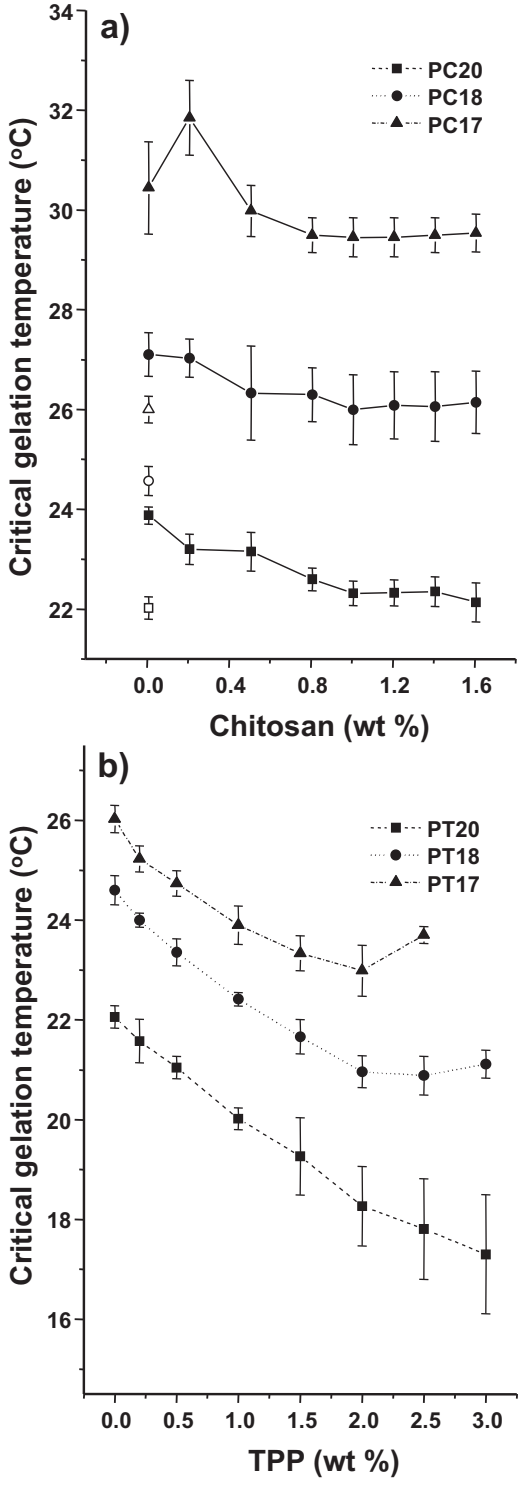

Fig. 3. Changes in the critical gelation temperature of aqueous solutions of P407 as function of the concentration of chitosan and TPP. (a) The effects of varying the chitosan concentration on the CGT of 17% (▲), 18% (●), and 20% (■) P407 solutions. Because acetic acid was used to solubilize the chitosan, the value quoted for the solution containing 0% chitosan in fact refers to a solution of poloxamer with 0.25% acetic acid, For comparative purposes, the critical gelation temperature of respective aqueous poloxamer solutions without acetic acid are also shown as respective open symbols (△, ○ and □); (b) the effects of varying the TPP concentration on the gelation temperature of 17% (▲), 18% (●) and 20% (■) P407 solutions (The critical gelation temperature of each sample was measured three times, and each sample was analyzed in duplicate; the values quoted thus represent the means of six measurements ± S.D.).

3.3. Syringeability/injectability and gelation time

It was found that refrigerated poloxamer formulations with concentrations above 20% lost syringeability within 2–3 min at room temperature. Therefore, detailed formulation studies were only conducted on mixtures with P407 concentrations of 20% or less. All these formulations could be withdrawn through a 23G needle but only the PC(18:0.35) could be withdrawn with 27G needle (see Fig. 4b and c).

The injectability of the chitosan ionotropic gel formed by *in situ* mixing of aqueous solutions of chitosan (0.2–1.8%) and TPP (0.2–2.5%) without poloxamer is shown in Fig. 4a. It is apparent that mixtures of lower concentrations (0.2–0.5%), are in category of "injectable suspensions" which may be suitable for parenteral application. At medium concentrations (0.5–1%), the particles coagulate, making it necessary to apply little force to inject the solution through a 27G needle and these were labeled as "injectable gels". Further increase in the concentrations of chitosan/TPP (to 1–2.5%) makes the gel difficult to inject through a 27 G needle. In this case, it is necessary to apply extra pressure which causes the gel to emerge as droplets. Formulations of this type are labeled as "administrable gels" in Fig. 4a. The injectability profiles of selected combinations of chitosan (0–1.5%), TPP (0–2.5%), and P407 gel (either 18% or 20%) are also shown in Fig. 4b and c. It is apparent from Fig. 4b that the gels with 18% P407 are "injectable" when TPP is less than 1.25% and "administrable" when using TPP concentrations above 1.25%. Fig. 4c shows that 20% poloxamer gels remain "injectable" with chitosan concentrations of up to 1% and TPP concentrations of up to 0.35%. 20% P407 gels with TPP concentrations in excess of 1.25% are in category of "semisolid" gels. The 20% poloxamer gels with the CT-TPP complex shows better injectability than poloxamer–chitosan or poloxamer–TPP alone.

The gelation time of the 18% formulation without CT-TPP complex is more than 30 s; in the presence of the CT–TPP complex, it is reduced to less than 25 s. Similarly, the 20% gel containing the CT–TPP complex has a gelation time of less than 15 s at 37 °C.

3.4. Dissolution of poloxamer gels

Fig. 5a shows the dissolution profile of P(18), PC(18:0.8), PT(18:2) and PCT(18:1:2) gel as determined by gravimetric method. Both, TPP and chitosan accelerated the dissolution of 18% poloxamer gel; however, the incorporation of CT–TPP complex prepared *in situ* causes a slight reduction in the rate of dissolution of the 18% poloxamer gel. Similarly, Fig. 5b presents the dissolution pattern of P(20), PC(20:0.8) and PCT(20:0.64:1.6) gels. As seen for the 18% gel, the addition of chitosan also accelerates the dissolution of the 20% gel, but the CT-TPP complex formed *in situ* slows down the dissolution. The gels having 17% or less of P407 dissolved quickly (within 4–5 h) and large variation in the dissolution pattern was observed within the same group.

3.5. In vitro drug release from poloxamer gels

3.5.1. Release of metoprolol with membrane free setup

Fig. 6 shows the effects of adding chitosan, TPP, or the CT-TPP complex (formed *in situ*) on the release of metoprolol from poloxamer gel. Metoprolol release curves from P(18), PC(18:0.8), PT(18:2) and PCT(18:1:2) gels are shown in Fig. 6a, whereas drug release results obtained from P(20), PC(20:0.8) and PCT(20:0.64:1.6) are shown in Fig. 6b. In both cases (18% and 20% P407), it was found that the metoprolol release patterns were straight and more inclined in the initial phase and slightly curved during the last phase which is different from dissolution profile of the respective gel. The gels containing either chitosan or TPP

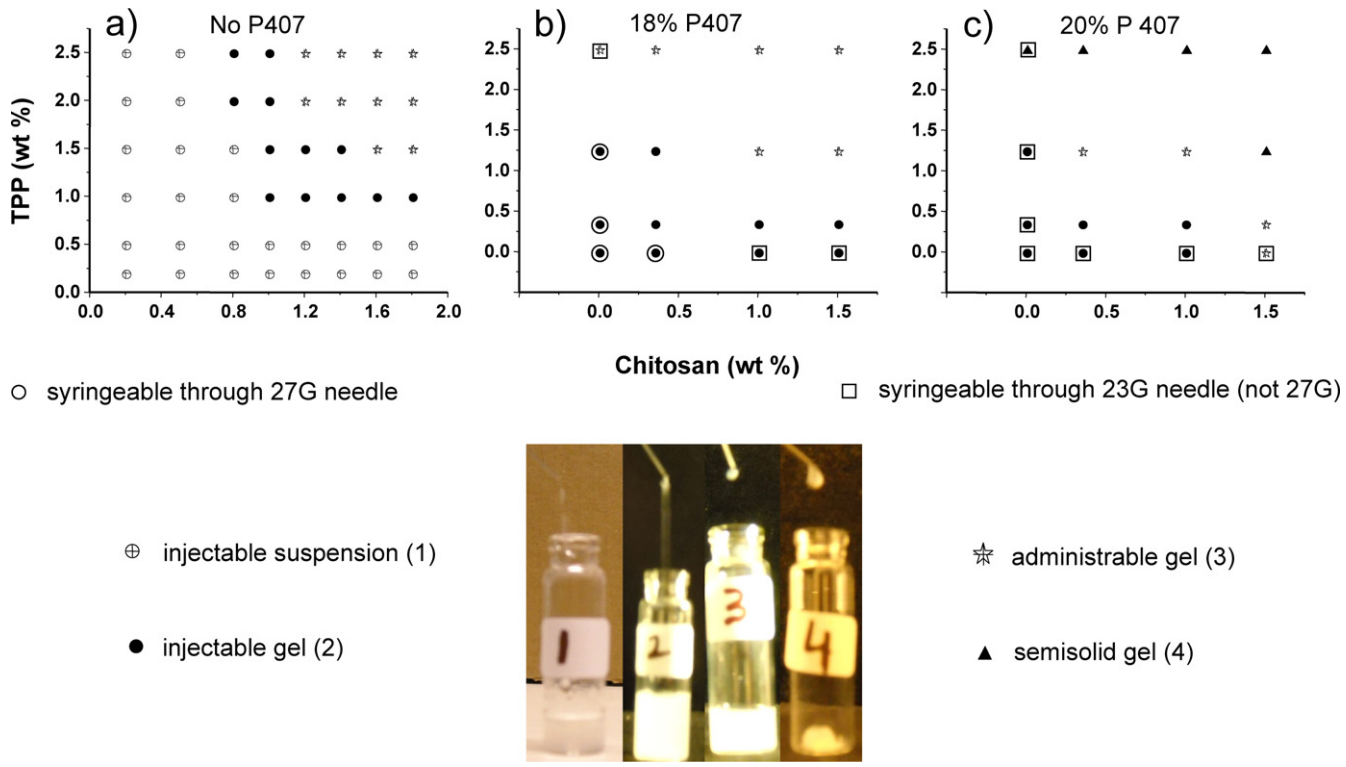

Fig. 4. Flow behaviour of CT-TPP gels in terms of their syringeability and injectability through 27G and 23G needles. (a) The injectability of a mixture of aqueous chitosan (0.2–1.8%) and aqueous TPP (0.2–2.5%) using a double syringe; (b) Syringeability and injectability of 18% P407 solution containing chitosan (0–1.5%) and TPP (0–2.5%); (c) syringeability and injectability of 20% P407 solution containing chitosan (0–1.5%) and TPP (0–2.5%). Legend: (○) poloxamer gel that is syringeable through a 27G needle; (□) poloxamer gel that is syringeable through a 23G but not through a 27G needle; (⊕) "Injectable suspension", a free flowing suspension of fine suspended particles that can be injected easily through a 27 G needle; (●) "Injectable gel", a gel that can be injected through a 27G needle with some resistance and emerge as a stream of coagulated gel; (☆) "Administrable gel" a gel that is difficult to be injected through a 27G needle and emerge in the form of gel droplets; (▲) "Semisolid gel" a gel that cannot be injected through 27G needle but passes easily through a 23G needle, emerging as thick gel. Inserted photograph: Visualization of different gels (as numbered).

released the drug slightly faster than poloxamer gels without additives, whereas gels incorporating chitosan–TPP complex formed *in situ* exhibited a slightly sustained drug release profile. We did not observed any effect of drug loading in one or both barrels of double syringe on release pattern (data not shown).

3.5.2. Release of doxycycline across membrane

The release profile of doxycycline from S, P(20), PC(20:0.9) and PCT(20:0.9:1.6) is shown in Fig. 7a. Apparently, the release of doxycycline from PC gels is slower than the one observed for the P gel and release rate of doxycycline from PCT gel is considerably slower than that from PC gel. Although addition of chitosan (0.9%) to 20% P407 gels has shown restricted release, the presence of CT–TPP complex has a more pronounced effect on the sustained release of doxycycline from P407 gel.

3.5.3. Release of flufenamic acid across membrane

In Fig. 7b the release profile of flufenamic acid is shown when drug is migrating from solution and gels into pH 7 buffer. The transfer of flufenamic acid across the membrane from the P(20) gel is considerably slower than that from an aqueous solution of the drug. The pronounced sustained release of flufenamic acid from PCT(20:1.1:0.9) gel is apparent from the fact that after 12 h, only 25% of the drug contained in the PCT gel have diffused across the membrane; the corresponding value for the P(20) gel is around 70%. We also examined the release of the drug under more acidic conditions, mimicking vaginal administration of similar compounds (i.e. compounds with limited aqueous solubility at low pH). The amounts of flufenamic acid released from aqueous solution, P(20) and PCT(20:1.1:0.9) gels into a pH 4 release medium are shown in Fig. 7c. In this case the release rate of flufenamic acid from both gels is considerably faster than from the drug solution. Under pathological conditions, the pH of the vaginal fluids increases; to mimic this effect, the acidic release medium was replaced with pH 7 buffer at the 5th time point; a rapid increase in the release rate of the drug from the aqueous solution at this point is visible in the Fig. 7c. In all of the experiments with membrane, almost all of P(20) gel was dissolved by the end of the experiment. However, none of the PCT gels dissolved completely over the same period.

3.6. In situ nanoparticles

First we examined the formation of nanoparticles by mixing aqueous solutions of chitosan and TPP of different concentrations and acidities, in the absence of P407 using a single or a 4:1 double syringe. It was found that the nanoparticles formed by simple mixing or injection with a dual syringe are not of uniform shape and size (Fig. 8a and b)—their diameter range from 150 nm to 850 nm, and in some cases particles larger than 1 μm were also observed. However, some of the samples prepared from solutions containing flufenamic acid exhibited relatively narrow size distributions (Fig. 8e). Analysis of micrographs obtained at higher magnifications established that the particles formed *in situ* are not generally spherical and compact, as can be seen in Fig. 8c. Nanoparticle formation was also observed when PC(17:0.18) gel and PT(17:0.08) gel were mixed and diluted before centrifugation. To visualize the *in situ* nanoparticle dispersed in P407 gel, relatively low concentration of chitosan was used and TEM micrograph of nanoparticles formed from mixing of PC(17:0.084) gel and PT(17:0.07) gel are shown in Fig. 8d.

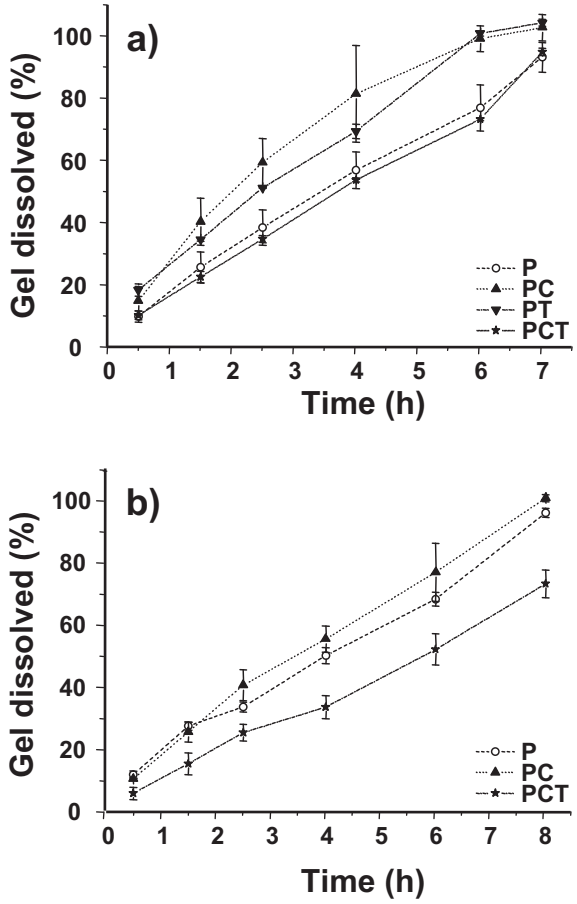

Fig. 5. Dissolution profiles of P407 gels at 37 °C in water, showing the influence of added chitosan, TPP, and of CT-TPP complex formed *in situ*. (a) Dissolution profiles of P(18) gel (○); PC(18:0.8) gel (▲); PT(18:2) gel (▼); PCT(18:1:2) gel (★); (b) dissolution profiles of P(20) gel (○); a PC(20:0.8) gel (▲); and a PCT(20:0.64:1.6) gel (★) ($n = 3$, mean ± S.D.).

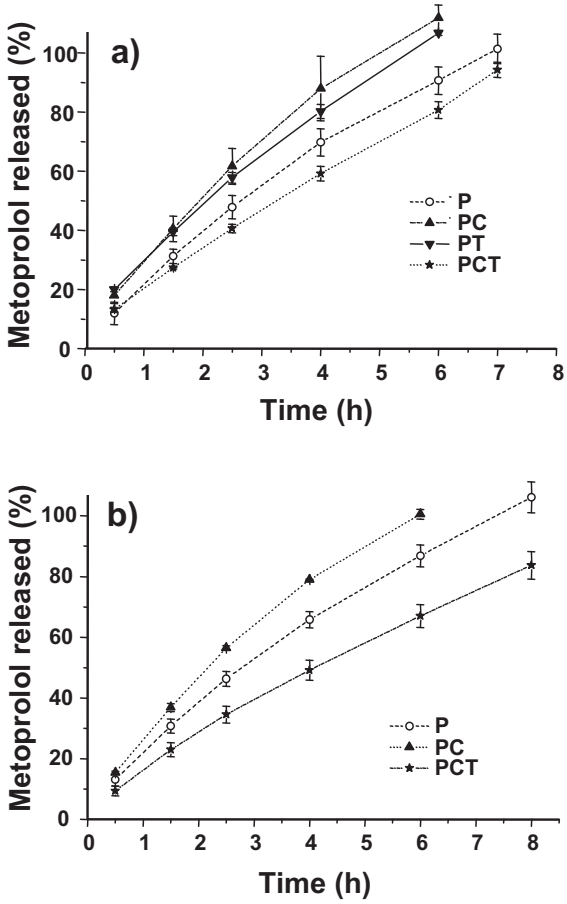

Fig. 6. Release profile of metoprolol from different poloxamer gels as determined using the membrane-free method at 37 °C: (a) Release profile of metoprolol from P(18) gel (○); from PC(18:0.8) gel (▲); from PT(18:2) gel (▼); and from PCT(18:1:2) gel (★); (b) release profile of metoprolol tartrate from an P(20) gel (○); from a PC(20:0.8) gel (▲); and from a PCT(20:0.64:1.6) (★) ($n = 3$, mean ± S.D. for all except PC(18:0.8) where $n = 2$).

4. Discussion

We have developed a method for the *in situ* ionotropic gelation of chitosan inside poloxamer gels in conjunction with a double syringe system. The primary objective of this study was to develop thermo-reversible poloxamer gels with enhanced strength; such gels would be expected to exhibit increased resistance to dissolution by body fluids and an enhanced drug loading capacity. The key concept of the project is the ability of the cationic polymer chitosan to form ionotropic gels on mixing with the anions such as TPP. *In situ* mixing of PC and PT solution, by using double syringe, should generate a solution with favorable properties that forms a stronger gel at physiological temperatures.

4.1. Effect of chitosan and/or TPP on basic properties of P407

It is important to ascertain that basic properties of P407 are not affected adversely in the presence of working concentration of chitosan and TPP, since the micellization and gelation ability of poloxamers can be lost with some additives as reported previously (Pandit and Kisaka, 1996).

DSC studies of dilute solutions of P407 with both additives revealed that this poloxamer undergoes micellization (which is the initial step in gel formation) at lower temperatures in the presence of chitosan, TPP, or their *in situ* mixture. The behaviour of chitosan can be explained in terms of the high charge density of its $-NH_2$ groups, which is likely to affect the formation of water channels around the PPO block of P407. A similar effect has been observed with the cationic surfactant CTAB (Hecht and Hoffmann, 1995). The effect of TPP is comparable to that of salts such as NaCl, $NaHPO_4$, NaH_2PO_4, and Na_3PO_4 (Alexandridis and Holzwarth, 1997; Anderson et al., 2002). Fortunately, both additives namely CT and/or TPP do not alter the micellization behaviour significantly in the working concentration range as established by us.

As seen in Fig. 3a, the presence of chitosan resulted in a slight increase in the CGT relative to that observed with aqueous solution of P407 without chitosan. However, this effect is largely due to the acidity of the medium (pH 3.4) in which the chitosan was dissolved. The effect of pH on the gelation of aqueous poloxamer solutions has been studied by various groups, who described an inverse correlation between gelation temperatures and pH (Choi et al., 1999; Scherlund et al., 2000b). In our experiments, the presence of acetic acid alone proved to be sufficient to cause an increase in the CGT. The addition of increasing amounts of chitosan to this acidic P407 solution resulted in a slight decrease in CGT. The addition of TPP was found to reduce the CGT of P407 solutions (see Fig. 3b). In a similar way, CGT-lowering behaviour has been observed when using other salts as additives in poloxamer gels, including NaCl, $CaCl_2$ $NaHPO_4$ or Na_3PO_4 (Gilbert et al., 1987; Malmsten and Lindman, 1992; Pandit and Kisaka, 1996).

The rheological behaviour of poloxamer gels has been studied by various groups (Edsman et al., 1998; Jones et al., 2009). However, there have been comparatively few reports of studies

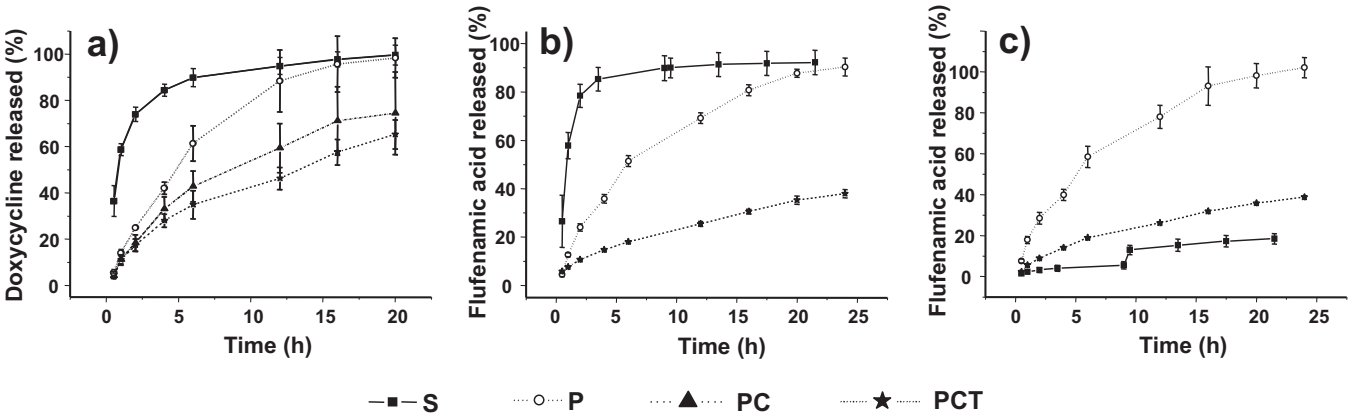

Fig. 7. Release profile of model drugs across a polycarbonate membrane at 37 °C from different formulations ($n = 3$, mean ± S.D.): (a) Release of doxycycline into water from aqueous drug solution (■); from P(20) gel (○); from PC(20:0.9) gel (▲); and from PCT(20:0.9:1.6) gel (★); (b) release of flufenamic acid across a polycarbonate membrane into neutral release medium (citrate-phosphate buffer at pH 7), from drug aqueous solution (■); P(20) gel (○); and PCT(20:1.1:0.9) gel (★); (c) release of flufenamic acid across a polycarbonate membrane into acidic release medium (citrate-phosphate buffer at pH 4); from drug aqueous solution (■); from P(20) gel (○); and from PCT(20:1.1:0.9) gel (★).

addressing the practical issues of flow behaviour during administration and handling of gels (Jones et al., 1997; Kelly et al., 2004; Liu et al., 2010; Rungseevijitprapa and Bodmeier, 2009). Because of their impressive *in vitro* profile, P407 gels are now being evaluated in pre-clinical animal studies (Dumortier et al., 2006; Escobar-Chavez et al., 2006). Therefore one objective in our study was the characterization of the practical aspects of the *in situ* dual ionotropic gel system in terms of the solution injectability and syringeability. Typically, smaller animals such as mice and rats are used in pre-clinical studies, necessitating the use of small volumes of gel solution and fine 23G or 27G needles (Baumnas et al., 2001; Shimizu, 2004). Although higher poloxamer concentrations (above 20%) have been shown to have excellent controlled release properties, we have observed that while such highly concentrated

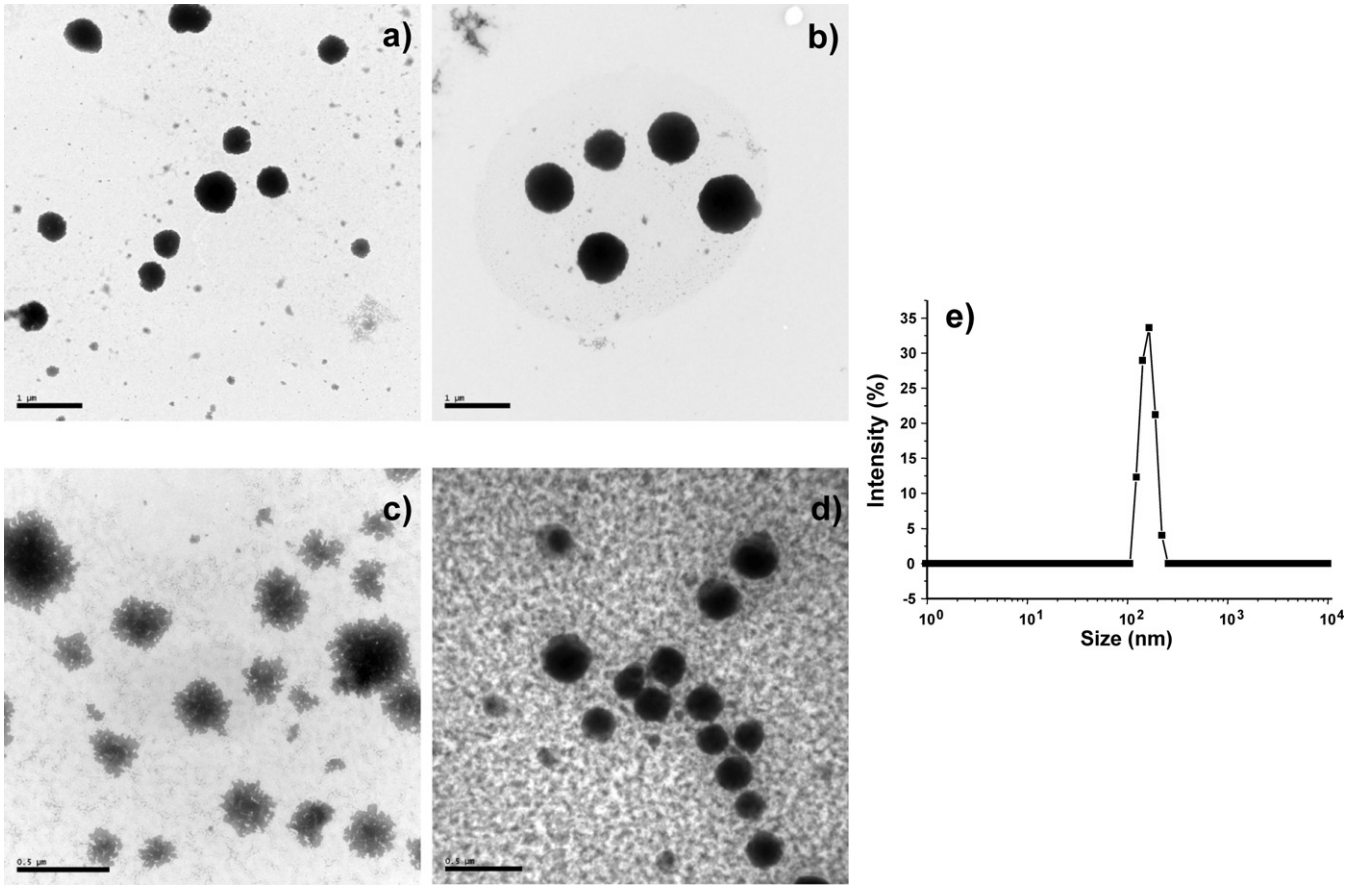

Fig. 8. *In situ* nanoparticle formation using a double chamber syringe. TEM micrograph of chitosan nanoparticles formed by mixing of 0.084% TPP with (a) 0.2% chitosan, pH 3.4 in a vial; (b) 0.2% chitosan, pH 3.4 using double syringe; and (c) 0.2% chitosan, pH 4.5 using double syringe. Fig. 8d shows a TEM micrograph of chitosan nanoparticle dispersed in P407 gel, which were formed by mixing of PC(17:0.09) gel and PT(17:0.07) gel via double syringe (4:1) approach. The particle size distribution of one flufenamic acid formulation is shown in Fig. 8e.

solutions flow freely when refrigerated, they become highly viscous after 2–3 min exposure to typical animal house temperatures, making their administration impossible (unpublished observations). In principle, it might be possible to use these cold formulations, but the internal administration of a cold fluid (less than 4 °C) may be painful to the animals and would raise ethical issues. However, such cold formulations may be suitable for burn dressings, which need to be highly viscous so that the gel is not dispersed from the location of the burn. The different formulations examined in this study were classified as being suitable for different applications on the basis of their syringeability and injectability (see Fig. 4). Our results suggest that 18% poloxamer gels containing CT–TPP at concentrations of up to 1:1% may be suitable for subcutaneous administration to small animals. Conversely, formulations containing 20% P407 are impossible to withdraw into a syringe in an animal house. However, this issue can be circumvented by supplying refrigerated solutions of the gel in pre-filled syringes/drops and 20% P407 gels having maximum 1% CT with 0.35% TPP are "injectable". Refrigerated "administrable gel" of either 18% or 20% poloxamer may be suitable for vaginal, nasal, or ocular administration. Similarly, refrigerated "semisolid gels" may be useful for oral (orthodontic and buccal) or rectal administration or application to outer skin as in case of burns, but gel of such a high viscosity would not be suitable for parenteral, ocular or nasal administration. It is also important to characterize the gelation time of gel formulations at physiological temperatures. If the gelation time is too long, the gel solution will be diluted by body fluids prior to gelation, but immediate gelation may not be desirable if spreading of gel is required at the application site such as vaginal cavity. From the results it was evident that P(20) gels have shorter gelation time as compared to P(18) gels and that the CT–TPP complex has minor effect on their gelation times. On the basis of the CGT and injectability experiments, we focused on aqueous solutions containing 20% or less of P407, and 2% or less of TPP in subsequent studies on the viability of these gels in drug delivery applications at physiological temperatures and on their safe handling during storage and administration at room temperature.

4.2. Improved drug loading of P407 gels with in situ gelation of chitosan

Mostly hydrophilic drugs are incorporated into poloxamer formulations due to their high water content and low solubilizing capacity. However, the majority of compounds currently being screened as drug candidates have limited water solubility. Here, we have shown that hydrophilic as well as hydrophobic (polar) drugs can be incorporated in poloxamer gels without the use of organic co-solvents. These results suggest that by using a double syringe system/double chamber tube (PC/PT) one can adjust the relative concentrations and acidities of the two solutions independently, which upon mixing generates a gel with an optimal pH and properties tailored for a specific administration site. For example, ocular formulations should have nearly a neutral pH value. Thus, one may use a chitosan solution prepared using a relatively low concentration of acid (acetic, citric, or hydrochloric acid) to generate a pH of around 5.5 while maintaining the viscosity of the chitosan solution at a level that would not cause problems during handling. This system can be combined with a TPP solution containing a relatively high level of Na_2HPO_4 which would neutralize the acidity of the chitosan solution upon mixing. On the other hand, application sites such as the vagina, are relatively acidic with a pH of around 4 ± 0.5 (Boskey et al., 2001). For formulations targeting this administration site, one can therefore use a more acidic CT-TPP solution.

Due to the ability of nanocarriers to penetrate the mucosal barrier, nanoparticle formulations are being developed extensively for mucosal delivery of proteins, peptides and low molecular weight drugs (Lai et al., 2009; Moghimi et al., 2005). Previously, drug loaded nanoparticles have been prepared and loaded into poloxamer gels for sustained delivery and to enhance the stability of entrapped drugs such as insulin (Barichello et al., 1999; Gou et al., 2008; Le Renard et al., 2010; Zhang et al., 2005). In general, ionotropic gelation of cationic chitosan with anionic TPP can result in spontaneous particle formation under specific conditions such as concentration, pH or stirring (Ali et al., 2010; Gan et al., 2005; Hasanovic et al., 2009). Typically, chitosan nanoparticles are prepared by injecting or dripping a solution of TPP or some other anionic species into a solution containing chitosan and the target drug. However, this method inevitably results in the disposal of some of the drug compound because not all of the drug in solution will be entrapped in the growing particles. Consequently, this technique may not be used in the early stages of drug development, when often only small quantities of the compounds are available. Here, we presented results of the *in situ* formation of chitosan nanoparticles using the double syringe approach with chitosan (0.09–0.2%) and TPP (0.06–0.1%) solutions (see Fig. 8). The technique of *in situ* ionotropic gel formulation described in this paper is unique in the way that it also facilitates the formation of nanoparticles and microparticles dispersed in the poloxamer gels; this can enhance the entrapment of potent drugs within the poloxamer gel and facilitate their sustained delivery over extended periods of time. *In situ* chitosan gelation in P407 gel is more efficient in a way that any drug that is not entrapped within the forming particles will instead be dispersed in the poloxamer gel, and will be released shortly thereafter. As the gel dissolves, the drug enclosed within the nanoparticles will be released slowly. The method is not without limitations: the size distribution of the particles formed *in situ* is comparatively broad, and the particles are not particularly compact. Nanoparticles dispersed in poloxamer gels may be beneficial in mucosal delivery as the particles are likely to exhibit enhanced penetration and will facilitate the delivery of different types of drugs, ranging from conventional small molecules to proteins. Nevertheless, further experiments are needed to quantify the drug loading in the particles and to ascertain the release profile of various drugs.

4.3. Effect of chitosan in situ gelation on dissolution and drug release profile of P407 gels

The dissolution experiments showed that PC gels have increased rates of dissolution as compared to P and PCT gels. This observation deviates from an earlier report where chitosan-P407 formulations have been shown to improve hardness, retention time and mucoadhesiveness of P407 gels (Gratieri et al., 2010). In our opinion chitosan enhances the mucoadhesiveness due to its cationic nature. However, the acidic nature of the formulation would adversely affect the gelation of P407 as evident from elevated CGTs and dissolution rates. PCT gels exhibited a decreased rate of dissolution, particularly in the case of the 20% gels. The gel network formed by chitosan in the presence of TPP may interpenetrate with the network of the P407 gel. This probably decreases the rate at which water penetrates into the P407 gel, thereby delaying the unpacking of the poloxamer micelles and the subsequent dissolution of the gel. To improve gel residence time, similar approaches of cross-linking chitosan inside P407 gel have been investigated previously using chemical agents such as glutaraldehyde (Chung et al., 2009; Kim et al., 2007). However, the toxicity of these cross-linking agents renders such gels unsuitable for further clinical trials.

The drug release from poloxamer gel is controlled by the rate of dissolution of the gel and the diffusion of the drug; dissolution of gel is a predominant factor if it is placed in an aqueous environment (as is the case in ophthalmic, rectal, or parenteral administration), while diffusion is the main mechanism of drug release if the gel is confined by a membrane, as in transdermal and some parenteral

applications (Dumortier et al., 2006; Moore et al., 2000; Ricci et al., 2005). In the membrane-free experimental model, the release rate of the drug from the gel was reduced after longer time intervals as impact of the diffusion is reduced due to a smaller concentration gradient. To mimic the conditions encountered in the vagina, rectum, subcutaneous space and nose and in the transdermal local application of gels to thermal wounds, release experiments were also conducted with membranes of large pore size (3 μm) under slow agitation of the release medium. Under these conditions both drug and poloxamer may diffuse across the membrane and dissolve into the release medium. As dissolution of gel was restricted in this setup, the release of drug was predominantly controlled by the diffusion of drug (see Fig. 7). With both experimental setups, we observed that PCT gels were not dissolved completely at the end of experiments as compared to P gels and the release of drug (metoprolol, doxycycline or flufenamic acid) from PCT gels was more sustained and controlled as compared to P or PC gels. Therefore we can conclude that the presence of CT–TPP complex restricts the dissolution of poloxamer gel and therefore improves its sustained release property. Metoprolol tartrate is an antihypertensive drug which is freely soluble in water, P407 solution, chitosan solution, and TPP solutions. Metoprolol loaded PCT gels may be suitable as ocular formulations for the treatment of glaucoma; ocular delivery of timolol, a similar antihypertensive, has been evaluated previously (El-Kamel, 2002). Flufenamic acid is an anti-inflammatory drug suitable for ocular, periodontal, buccal and dermal applications. Flufenamic acid is freely soluble in ethanol or basic solutions, but exhibits limited solubility in water at neutral pH or under acidic conditions. Our results suggest that administering flufenamic acid in a PCT gel has two beneficial effects: the release rate is reduced and, it does not precipitate, even when the gel is exposed to a release medium of pH 7 or even pH 4 (Fig. 7b and c). Solubility of flufenamic acid in different solvents (Supplementary Table 1) and this observation support that, the P407 micelles remain intact at 37 °C, even when the gel has been significantly diluted (by a factor of 30–50 in this case). In this temperature range the critical micellization concentration of P407 is reported rather low (0.0021–0.005%) (Kabanov et al., 2003; Wanka et al., 1994; Wei et al., 2009), which explains the enhanced solubility of hydrophobic drug.

The properties of the poloxamer gels used in the formulations described in this paper are crucial to their success. First, their gelation at physiological temperatures modulates the burst release typically observed with ionotropic gels. Second, they protect the body from immediate exposure to unbound chemicals. Third, the poloxamer traps the chitosan and TPP solutions during their mixing and extends their contact time by preventing the rapid diffusion of TPP into the surrounding medium; the rate at which TPP can be lost is limited by the comparatively slow rate of erosion of the gel surface. Fourth, the poloxamer micelles at physiological temperature enhance the solubility. In turn, chitosan greatly enhances the mucoadhesion of the poloxamer gel, while the interleaving of the poloxamer gel with CT-TPP greatly enhances the gel's viscosity, strength and residence time. The role of TPP in this system is twofold. First, its presence induces gelation of the chitosan, increasing the overall viscosity of the gel system. Secondly, it counteracts the increase in the CGT of the poloxamer gel caused by the acetic acid used to solubilize the chitosan and the attendant reduction in gel strength; the effects of acids on the strength of poloxamer gels have been described by other authors (Choi et al., 1999). Double syringes have previously been used in the periodontal application and for the dressing of wounds (Balakrishnan et al., 2005) and recently reported for *in situ* gelation of alginate for delivery of magnetic particles for local hyperthermia (Le Renard et al., 2010). Similarly, dual chamber syringes, eye drops, nasal drops, and vaginal or rectal applicators may be developed to facilitate the uptake of these *in situ* gels for efficient drug delivery.

5. Conclusion

This study presents the *in situ* ionotropic gelation of the natural polymer chitosan with TPP as a suitable method to enhance the drug delivery applications of poloxamer based thermoreversible gels. The use of double syringe system, to keep the poloxamer–chitosan and poloxamer–TPP gels separated until the last possible moment, facilitated drug loading and handling during administration. Furthermore, *in situ* ionotropic gelation of chitosan can enhance the strength and mucoadhesiveness of the poloxamer gel. In addition, our *in situ* chitosan gelation process can generate poloxamer gels with restricted dissolution and sustained drug release profiles, and which are also tunable to deliver drugs at specific application site. This approach can also be adapted for the *in situ* formation of chitosan nanoparticles dispersed in P407 gels, an area of rapidly growing interest. Besides the CT-TPP system discussed in this paper, other ionotropic systems such as alginate-$CaCl_2$ and pectin-$CaCl_2$ can be explored to extend the utility of poloxamer gel involving three-component systems.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2011.02.017.

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