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# Matrix metalloproteinase-sensitive thermogelling polymer for bioresponsive local drug delivery

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ABSTRACT

Development of a successful bioresponsive drug delivery system requires exquisite engineering of the materials so that they are able to respond to signals stemming from the physiological environment. In this study we propose a new Pluronic® based thermogelling system containing matrix metalloproteinase-2 (MMP2) responsive peptide sequences. A novel thermosensitive multiblock co-polymer comprising an MMP2-labile octapeptide (Gly–Pro–Val–Gly–Leu–Ile–Gly–Lys) was synthesized from a Pluronic® triblock co-polymer. The polymer was designed to form a thermogel at body temperature and degrade in the presence of MMP overexpressed in a tumor. The synthesized polymer was a multiblock co-polymer with ~2.5 U of Pluronic®. The multiblock co-polymer solutions exhibited reverse thermal gelation around body temperature. The gelation temperatures of the multiblock co-polymer solutions were lower than those of the corresponding Pluronic® monomer at a particular concentration. The cytotoxicity of the synthesized polymer was lower compared with the monomer. The solubility of the hydrophobic anticancer drug paclitaxel was enhanced in the polymer solutions by micelle formation. The synthesized polymer was preferentially degraded in the presence of MMP. Paclitaxel release was dependent on the enzyme concentration. These findings suggest that the synthesized polymer has potential as a controlled drug delivery system due to its unique phase transition and bioresponsive behavior.



## 1. Introduction

Advances in the technologies to engineer biomimetic materials has been an important breakthrough that will greatly influence human health care [1–3]. Careful fabrication of materials with precise consideration of the underlying cellular microenvironments has made this approach suitable for various biomedical applications. Biomimetic materials have been consistently considered for drug delivery and tissue engineering [4–6]. These materials have frequently been obtained by incorporating bioactive molecules such as proteins and peptides [1,7]. In particular, peptide conjugation has been proven as an approach to developing biomimetic materials fulfilling biomedical needs [8–10]. Several approaches have been tried to engineer these materials to either target diseased tissue or mimic the extracellular matrix [11–13].

Targeted delivery of anticancer drugs has been one of the pressing research areas using bioactive molecules. Anticancer therapeutic agents, such as methotrexate, paclitaxel and cisplatin, have been specifically delivered to tumor tissues by conjugating them to polymers using bioactive molecules [14–18]. However, most polymeric prodrugs carrying pendent cytotoxic drugs have been attached to polymer backbones via a peptide linker. Critical problems associated with polymeric prodrugs include low drug loading capacities and altered physico-chemical properties of the polymeric prodrug as a whole. Another drawback associated with polymeric prodrugs is the release of peptide fragment-modified drug rather than intact drug molecules, resulting in reduced activity of the parent drug [19]. An alternative to overcome the problems associated with these approaches would be local drug delivery systems with enhanced drug loading. In particular, local drug delivery systems based on amphiphilic thermosensitive polymers have been investigated in recent years [20–22]. Thermosensitive polymer solutions are free flowing fluids at room temperature, allowing easy administration into the body. The polymer solution forms a gel depot upon injection into the body, which acts as a drug reservoir [23]. In addition, amphiphilic polymers can improve the solubility of insoluble drugs. From a clinical perspective, injectable thermogels have great benefits because they allow minimally invasive drug delivery directly to the diseased tissue.

Different thermogels were prepared with aqueous solutions of various polymeric materials such as chitosan, hyaluronic acid,

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alginate, Pluronic® co-polymers and poly(*N*-isopropylacrylamide)-based co-polymers [24–26]. Among the reported thermosensitive polymers, Pluronic® copolymers, a class of polyethylene glycol–polypropylene glycol–polyethylene glycol (PEG–PPG–PEG) triblock co-polymers, have been extensively studied due to their drug loading capacity and acceptable biocompatibility [24,27,28]. It is worth mentioning that an anticancer micellar formulation based on Pluronic® L61 and F127 has reached phase II clinical trials [29]. Besides the ability of Pluronic® co-polymers to form micelles and thermogels, they are capable of sensitizing multidrug-resistant cells and increasing drug transport across cellular barriers [30]. To overcome the undesirable gel properties associated with Pluronic® triblock co-polymers, such as weak mechanical strength and rapid erosion, multiblock co-polymers made of PEG and PPG have been proposed due to their enhanced physico-chemical properties [31–35]. A family of disulfide multiblock co-polymers was synthesized from Pluronic® P65, P85 and P105 to prolong the gel lifespan and allow thiol-dependent degradation [32]. Interestingly, thermogels of the multiblock co-polymers degraded and released paclitaxel in a glutathione-responsive manner. Recently, PoligoGel®, a multiblock co-polymer of PEG–PPG–PEG or PEG–polybutylene glycol (PBG)–PEG connected via dicarboxylic linkages has been used for the delivery of rat mesenchymal stem cells [33,34]. The thermogel was shown to have potential as a scaffold for the retention of cells at the target site. Novel thermogelling polymers based on multiblock poly(ether ester urethane)s have been engineered from poly((R)-3-hydroxybutyrate) (PHB), PEG and PPG using hexamethylene diisocyanate as coupling agent [35]. The thermogel showed improved gelation and drug release properties compared with Pluronic® triblock co-polymers. Even though several different thermogelling systems have been prepared, drug release from the thermogels is mainly mediated by simple diffusion and non-specific hydrogel erosion. These obstacles necessitate uncovering a specific mechanism to control drug release from the thermogels. Matrix metalloproteinases (MMPs) can act as in situ controlling factors to regulate drug release from thermogels. MMPs are zinc-dependent endopeptidases that can cleave many of the extracellular matrix proteins. Many MMPs have been found to be overexpressed and active during the advanced stages of cancer while they are minimally expressed in normal tissue. In particular, MMP2 and MMP9 (type IV collagenases), which degrade extracellular matrices, have been shown to play a critical role in tumor progression, angiogenesis and metastasis [8]. Thus the combination of thermogelling and enzyme sensitivity provides a useful approach to engineer novel in situ forming polymers that are suitable for bioresponsive local drug delivery.

A peptide specifically degraded by MMPs is either incorporated into or conjugated to a thermosensitive polymer to make a thermogel system which is sensitive to MMPs. A MMP13 sensitive peptide, QPQGLAK, has been incorporated into a thermosensitive poly(*N*-isopropyl acrylamide) hydrogel matrix [11,12,36]. The peptide in the hydrogel system provides a cross-linking structure while maintaining the thermosensitive properties. In addition, the system showed concentration-dependent degradation by MMP2. Recently an enzymatically degradable temperature-sensitive polypeptide has been reported. The block co-polymer, poly(ethylene glycol)–block–poly (alanine–co-phenylalanine) (PEG–PAF), showed temperature sensitivity in water as well as degradability in the subcutaneous layer of rats [9]. Enzymes present in the subcutaneous layer, such as cathepsin B, cathepsin C and elastase, were assumed to be responsible for degradation of the polymer in vivo.

Here we report the synthesis of novel Pluronic®-based multiblock co-polymers connected via a MMP2-sensitive peptide, Gly–Pro–Val–Gly–Leu–Ile–Gly–Lys–$NH_2$ (GPVGLIGK-$NH_2$). The hypothesis behind this project is that the combination of thermosensitive Pluronic® and a MMP-sensitive peptide would result in a dual stimulus-sensitive polymer which can form a gel at body temperature and allow bioresponsive degradation in the presence of MMP. The physical properties and in vitro cytotoxicity of the polymers were studied to assess in vivo applicability as a local drug delivery system. Finally, release of a chemotherapeutic agent, paclitaxel (PTX), has been followed to demonstrate bioresponsive drug release from the thermogels.

## 2. Experimental

### 2.1. Materials

The peptide GPVGLIGK-$NH_2$ was synthesized by American Peptide Co. (Vista, CA). Pluronic® P85 and P104 were kindly provided by BASF Chemical Co. (Florham Park, NJ). Collagenase IV was purchased from Sigma (St. Louis, MO). PTX was obtained from LC Laboratories (Woburn, MA). Polystyrene molecular weight standards of 1, 4, 20, 50 and 100 kDa were purchased from Polysciences Inc. (Warrington, PA). All other chemicals were obtained from Fisher Scientific (Pittsburgh, PA) and used without further purification.

### 2.2. Polymer synthesis

#### 2.2.1. Activation of the triblock co-polymer

Pluronic® P85 or P104 (10 g) was dried by azeotropic distillation from 150 ml of anhydrous toluene. The dried Pluronic® was dissolved in 20 ml of anhydrous methylene chloride. About 2 ml of triethylamine (9.6 M equiv.) was added to the reaction mixture at room temperature. After cooling the reaction mixture in an ice bath, 4-nitrophenyl chloroformate (NPC) (8.0 M equiv.) was added. Initially, the mixture was allowed to react for 1 h at 0 °C. The reaction was continued overnight at room temperature under magnetic stirring. After all the solvent had been removed the activated Pluronic® (NPC–Pluronic®) was dissolved in 50 ml of anhydrous ethyl acetate and filtered to remove precipitated triethylamine HCl. The NPC–Pluronic® was reprecipitated from 50 ml of diethyl ether to remove unreacted NPC. This step was repeated twice to remove most of the unreacted NPC. Finally, the NPC–Pluronic® was dried under reduced pressure and kept in a desiccator until peptide coupling.

#### 2.2.2. Coupling of the peptide to the activated triblock co-polymer

The peptide (100 mg) and NPC–Pluronic® (1.1 M amount relative to the peptide) were dissolved separately in 1 ml of dimethyl acetamide (DMA). NPC–Pluronic® solution was added to the peptide solution at room temperature with magnetic stirring. Triethylamine (5.0 M amount relative to the peptide) was slowly added to the reaction mixture. The reaction was carried out for 1 day (at room temperature) or 2 days (first day at room temperature and second day at 50 °C) or 3 days (1 day at room temperature and 2 days at 50 °C). Dry nitrogen was flushed through to evaporate DMA from the reaction mixture. This crude multiblock co-polymer was dissolved in water and dialyzed (molecular weight cut-off 6000–8000, Spectra/Por®) against deionized water for 2 days with periodic medium changes. The final product was freeze-dried to obtain polymer powder. Two different multiblock co-polymers, MMP–P85 from Pluronic® P85 and MMP–P104 from Pluronic® P104, were synthesized at the yields of 80% and 89%, respectively.

### 2.3. Polymer characterization

$^1$H nuclear magnetic resonance ($^1$H NMR) was used to analyze the chemical structures of the synthesized polymers. The polymers were dissolved in either $CDCl_3$ or $D_2O$ and the spectra were

recorded in a 400 MHz NMR spectrometer (Bruker Ultrashield™ 400 PLUS, Germany). A Waters gel permeation chromatography (GPC) system (Waters, Milford, MA) equipped with a binary pump (Waters 1525), a refractive index detector (Waters 2414) and a Styragel HR4E column (300 × 7.8 mm inner diameter, 5 μm particle size) was used to obtain the molecular weights and polydispersities of the polymers. Tetrahydrofuran was eluted at a flow rate of 1 ml $min^{-1}$ at 25 °C. Polystyrene standards (1–50 kDa) were also run to obtain a calibration curve and the calibration curve was used to calculate the molecular weights of the polymers. Thermograms of the polymers were obtained by differential scanning calorimetry (DSC) (Diamond DSC, Perkin–Elmer, Waltham, MA). Melting temperatures of the polymers were determined from the thermograms. DSC was run in the temperature range 0–50 °C with a heating and cooling rate of 10 °C $min^{-1}$. Initially the samples were loaded in aluminum pans and equilibrated at 0 °C for 5 min. A dry nitrogen flow at a rate of 20 ml $min^{-1}$ was maintained throughout the analysis.

### 2.4. Critical micelle concentration (CMC) of MMP–P104

The CMCs of Pluronic® P104 and MMP–P104 were determined by a dye solubilization method using 1,6-diphenyl-1,3,5-hexatriene (DPH) [27]. Briefly, polymer solutions in the concentration range $1.0 \times 10^{-5}$–10 wt.% were prepared. DPH solution (10 μl, 0.4 mM in methanol) was added to 1.0 ml of each polymer solution. The polymer solutions were incubated at 25 °C for 24 h in the dark. The absorbance values of the test solutions were recorded on a Lambda EZ201 UV Spectrophotometer (Perkin–Elmer, Waltham, MA) at 377 and 391 nm. A graph was drawn plotting the difference between two absorbance values on the vertical axis and concentration on the horizontal axis.

### 2.5. DSC of polymer solutions

DSC thermographs of Pluronic® P104 and MMP–P104 solutions were obtained in the concentration range 1–25 wt.%. Sample solutions were prepared by dissolving a specific amount of polymer in deionized water with gentle stirring at 4 °C. Accurately weighed sample solutions (6 mg) were placed in aluminum cells and reference cells were made up with the same amount of deionized water. DSC was run in the temperature range −10 to 50 °C at a rate of 5 °C $min^{-1}$. Samples were thermally equilibrated at −10 °C for 15 min before each scan.

### 2.6. Circular dichroism (CD) spectroscopy

The CD spectra of MMP–P104 and peptide solutions were analyzed in the wavelength range 250–190 nm using a CD spectrometer (Olis® DSM 20 CD Spectrophotometer, Bogart, GA). MMP–P104 and peptide solutions were prepared at 0.1 and 0.01 wt.%, respectively. The sample solutions were equilibrated at 4 °C overnight before the analysis. A cuvette (path length 1 mm) was charged with a sample solution and CD spectra were recorded at 15, 25, 35 and 45 °C. Molar ellipticity of all the samples was calculated after a blank reference of water was subtracted from the raw data.

### 2.7. In vitro cytotoxicity

An in vitro cytotoxicity study was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) on HT1080 fibrosarcoma cells [37]. The cells were cultured in Roswell Park Memorial Institute (RPMI) medium. RPMI medium was supplemented with 10% fetal bovine serum (FBS) and antibiotics. The cultured cells in 96-well plates ($5 \times 10^3$ cells per well) were grown for 24 h at 37 °C. The culture medium was replaced with MMP–P104 solution (100 μl, 0.05–1.00 wt.%) in serum-free Dulbecco's modified Eagle's medium (DMEM). The cells were incubated for 4 h in serum-free DMEM (100 μl) followed by 12 h incubation in 200 μl of DMEM containing 10% FBS. After 16 h total incubation the test solutions were replaced with fresh medium (200 μl) and 5 mg $ml^{-1}$ MTT solution (20 μl). The cells were incubated for 4 h in MTT solution. Later the MTT solution was replaced with 400 μl of dimethyl sulfoxide to dissolve internalized purple formazan crystals. To measure the absorbance at 570 nm in a VICTOR3V™ Multilabel Counter (Perkin–Elmer, Waltham, MA), 150 μl of the solution was transferred from each well into a fresh 96-well plate. Cell viability was calculated compared with phosphate-buffered saline (PBS)-treated cells (100% survival).

### 2.8. Thermal gelation of polymeric solutions

#### 2.8.1. Test tube inversion method

Polymer solutions were prepared by dissolving them in deionized water at 4 °C. A series of polymer solutions at concentrations of 5, 10, 15, 20 and 25 wt.% were prepared. Scintillation vials (4 ml) were loaded with 1 ml of the polymer solution and incubated at 4 °C overnight. The sol–gel and gel–sol phase transition temperatures of each polymer solution were determined with increments of 2 °C [24,38]. The experiment was conducted in the temperature range 15–80 °C. The temperature at which a polymer solution stopped flowing upon tube inversion was recorded as the gelation temperature.

#### 2.8.2. Falling ball method

MMP–P104 solutions at concentrations of 10, 15, 20 and 25 wt.% were prepared in deionized water. An NMR tube, 4.0 mm in diameter, was filled with the polymer solution. The polymer solution was incubated at 10 °C for 20 min. A steel ball (diameter ($D$) 1.97 mm, density ($\rho_s$) 7.97 g $ml^{-1}$) was dropped through the polymer solution. The time ($t$) required for the steel ball to fall a specified distance ($d$ = 3 cm) was measured with a temperature increment of 2 °C per step for each polymer concentration. The polymer solutions were equilibrated for 20 min at each temperature. The intersection of extrapolated lines of travel time of the steel ball on a graph plotted of temperature against transit time gives the sol–gel transition temperature of the polymer solution [39]. The dynamic viscosity ($\mu$) was calculated using the formula.

$$\mu = (\gamma_s - \gamma_f)D^2/(18v)$$

where the velocity of the falling ball ($v$) = $d/t$, the specific gravity of the sphere ($\gamma_s$) = $\rho_s g$, the specific gravity of the polymer solution ($\gamma_f$) = $\rho_f g$, and the acceleration due to gravity ($g$) = 980 cm $s^{-2}$. The density of the polymer solution ($\rho_f$) was assumed to be 1.0 g $cm^{-3}$.

### 2.9. MMP–P104 degradation

A MMP–P104 solution was prepared at a concentration of 25 wt.% in deionized water. The polymer solution (50 μl) was placed in 1 ml vials and the vials were kept at 4 °C overnight. The polymer solutions were incubated at 37 °C for 15 min to induce gelation. Phosphate buffer (0.1 ml, 50 mM) was added to the first three vials (control). Phosphate buffer containing collagenase IV (250 μg $ml^{-1}$) was added to another three vials. Degradation of the gels was stopped by freezing them at predetermined time intervals. The frozen samples were then freeze-dried to obtain the degraded polymer. GPC was run to check the molecular weight distribution of the degraded polymer.

### 2.10. In vitro paclitaxel release

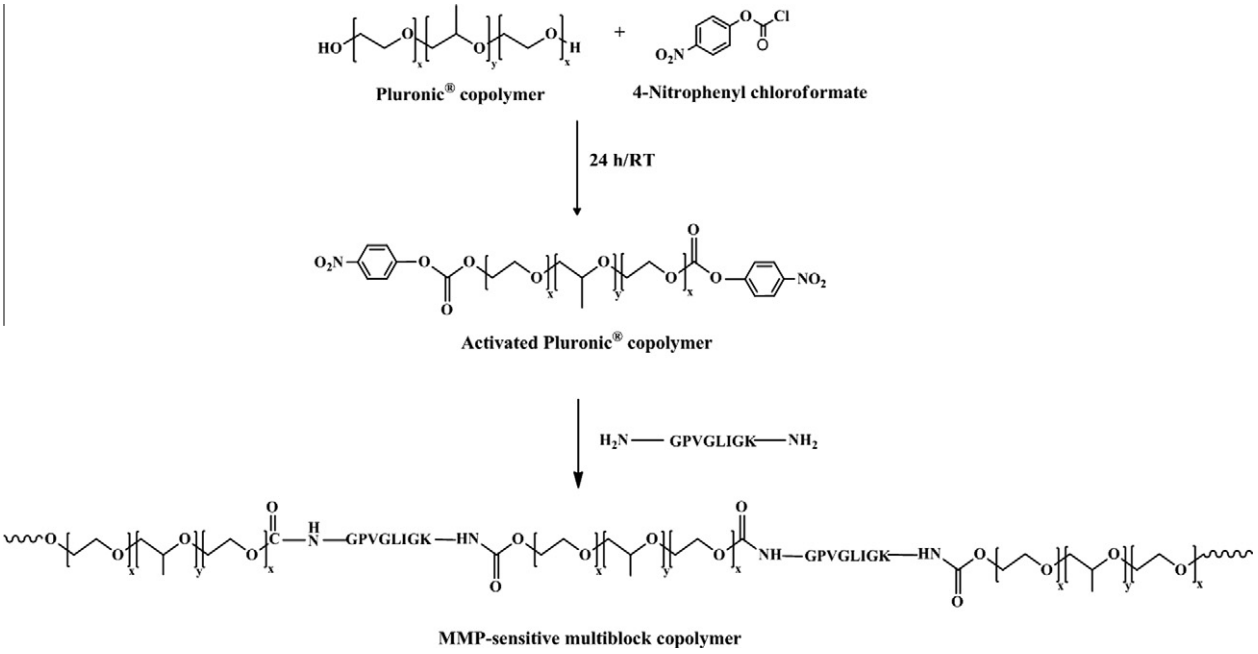
MMP–P104 (250 mg) was dissolved in 200 μl of acetone. After partial removal of the acetone by dry nitrogen flushing, 20 μl of acetone containing 500 μg PTX was added to the polymer solution. By continuously mixing the polymer–drug mixture the acetone was removed by dry nitrogen flushing. Finally, the polymer–drug mixture was vacuum dried for 4 h to completely remove the acetone. The MMP–P104–PTX mixture was dissolved in 750 μl of water with vigorous stirring at 4 °C. The polymer–drug solution (0.2 ml) was placed in 2 ml vials and allowed to gel at 37 °C. The gels were equilibrated at this temperature for 15 min. Phosphate buffer (1.6 ml, 50 mM) including 1% w/v Tween 80 was added to the first set of vials (control). Phosphate buffer (1.6 ml, 50 mM) containing collagenase IV (1, 10, 50 and 250 μg $ml^{-1}$), Tween 80 (1% w/v), calcium chloride (0.5 mM) and sodium azide (0.2 mg $ml^{-1}$) was added to another set of vials. During incubation at 37 °C samples were collected, while the total medium was replaced every 12 h to maintain perfect sink conditions. The samples were analyzed for PTX by high performance liquid chromatography (HPLC). A Waters HPLC system (Waters, Milford, MA) equipped with a binary pump, dual absorbance detector, and XTerra® RP 18, 5 μm column (4.6 × 150 mm). A mixture of acetonitrile and water in the ratio 55:45 was eluted at a flow rate of 1 ml $min^{-1}$ at 25 °C. The detection wavelength was set at 230 nm.

## 3. Results and discussion

Multiblock co-polymers (MMP–P85 and MMP–P104) were synthesized by conjugating thermosensitive Pluronic® (P85 and P104), a group of triblock co-polymers of PEG–PPG–PEG, to a MMP-sensitive peptide (GPVGLIGK-$NH_2$). The synthesis involves a two-step reaction as described in Scheme 1. The $^1H$ NMR spectra of Pluronic® P104 and purified NPC–Pluronic® are shown in Fig. 1. The activation of Pluronic® was confirmed by the characteristic proton peaks of 4-nitrophenyl carbonate ranging from 7.4 to 8.4 ppm in the $^1H$ NMR spectrum [40]. Fig. 1A shows the proton peak of hydroxyl protons (c) at each end of Pluronic®. The hydroxyl proton peak disappeared from the NMR spectrum (Fig. 1B) upon activation of Pluronic®. In addition, the appearance of the proton peak of the methylene group (f) at 4.45 p.p.m. confirmed successful activation of Pluronic®.

Several peptide sequences, such as GPLGIAGQ, GPQGPAGQ, PVGLIG and CGLDD, have shown MMP susceptibility [13,14,19]. An octapeptide sequence (GPVGLIGK-$NH_2$) has been synthesized to conjugate to the Pluronic® moiety. An MMP-sensitive hexapeptide (PVGLIG) [19] was modified at the ends with two amino acids (glycine and lysine) to provide amine functional groups on both ends. This modification allows conjugation reactions at the termini while maintaining MMP cleavage of the peptide at –GL– [41]. The terminal amino groups of the peptide allow linear oligomerization, between the peptide and NPC–Pluronic®. As Pluronic® of molecular weight ~5000 may not be readily accessible for oligomerization the reaction time and temperature were increased to determine the effect of these parameters on the final molecular weight of MMP–P104. Table 1 summarizes the molecular weights under MMP–P104 at different reaction conditions. The molecular weight of MMP–P104 increased slightly with increasing reaction time and temperature. Fig. 1C shows the $^1H$ NMR spectrum of MMP–P104. Oligomerization was confirmed by the characteristic proton peaks of the peptide and Pluronic® in the final polymer. The proton peaks at *g* to *v* show the presence of the peptide and its conjugation to Pluronic®.

The molecular characteristics of the Pluronic® co-polymers MMP–P85 and MMP–P104 are summarized in Table 2. The calculated molecular weights of MMP–P85 and MMP–P104 were found to be 15,290 and 23,540 g $mol^{-1}$, respectively. The molecular weight analysis indicates that MMP–P85 and MMP–P104 are multiblock co-polymers with 2.06 and 2.76 U of Pluronic® P85 and Pluronic® P104, respectively. Compared with previously reported Pluronic®-based multiblock co-polymers [24], multiblock co-polymers of relatively low molecular weight were obtained because of the relatively high molecular weight peptide (~740 g $mol^{-1}$), which might limit access of the nitro group at the end of Pluronic® to the amine group in the peptide during oligomerization. Accord-




**Scheme 1.** Two-step reaction scheme showing the synthesis of MMP-sensitive multiblock co-polymers from Pluronic® (P85 or P104) triblock co-polymers. Pluronic® and NPC were reacted at room temperature overnight to activate Pluronic®.

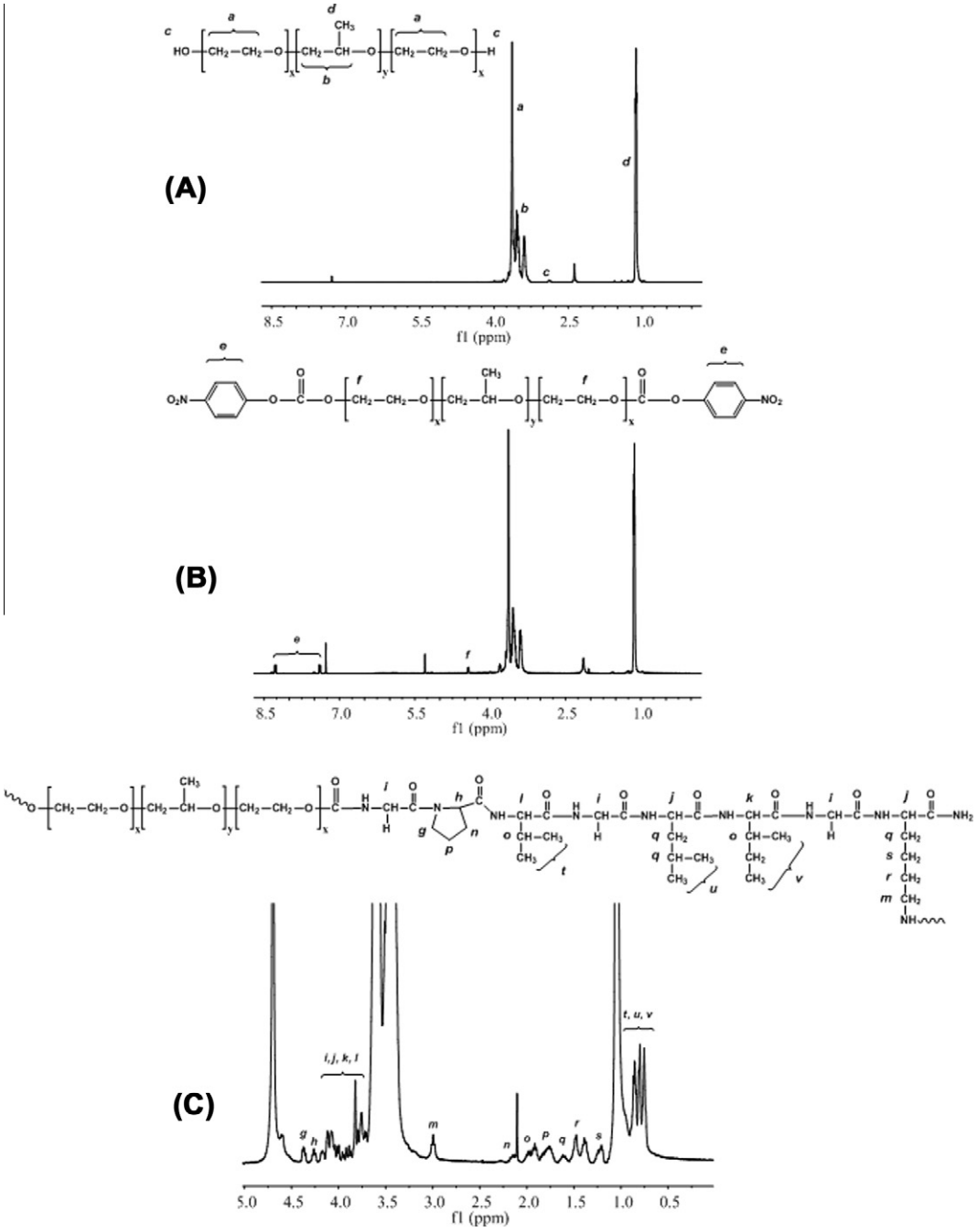



**Fig. 1.** $^{1}$H NMR spectra of (A) Pluronic® P104, (B) activated Pluronic® P104 and (C) MMP–P104. $CDCl_3$ was used to measure the NMR spectra of all polymers.

**Table 1**
Effect of different reaction conditions on the molecular weight of the final polymer.

| Polymer | Reaction conditions | $M_n$ (g mol$^{-1}$)[a] | $M_w/M_n$[b] |
|---|---|---|---|
| 1 | RT (1 day) | 17,570 ± 1060 | 1.34 |
| 2 | RT (1 day) + 50 °C (1 day) | 21,120 ± 960 | 1.47 |
| 3 | RT (1 day) + 50 °C (2 days) | 23,270 ± 1100 | 1.33 |

The $M_n$ values represent means ± SD for $n = 3$.
[a] Number average molecular weights determined by GPC.
[b] Polydispersities based on GPC measurements.

ing to the DSC data the melting temperatures ($T_m$) of MMP–P85 and MMP–P104 were 28.5 ± 1.8 and 30.9 ± 2.2 °C. The $T_m$ values of the multiblock co-polymers are lower compared with the $T_m$ values of Pluronic® monomers. These lower $T_m$ values of MMP–P85 and MMP–P104 may be due to interrupted polymer packing due to the incorporation of a peptide in the polymer structure [24].

The relatively high molecular weight of MMP–P104 made this multiblock co-polymer an interesting candidate compared with MMP–P85. Thus further characterization was mainly performed with MMP–P104. A hydrophobic dye (DPH) solubilization method was used to measure the CMC of MMP–P104 [24,27]. The absorbance of DPH increased sharply at the CMC of MMP–P104 (Fig. 2). The CMC was calculated by extrapolating the absorbance versus logarithmic concentration curve. The CMC of MMP–P104 was found to be 0.208 ± 0.012 wt.%, which is slightly lower than the CMC of Pluronic® P104 (0.300 ± 0.009 wt.%). This lowering of the CMC is mainly attributed to the increased molecular weight

**Table 2**
Characterization of multiblock co-polymers and triblock monomers.

| Polymer | $M_n$ (g mol$^{-1}$)[a] | $M_w/M_n$[b] | Number of repeating units | $T_m$ (°C)[c] |
|---|---|---|---|---|
| Pluronic® P85 | 7410 ± 120 (4600[d]) | 1.06 | | 38.9 ± 1.2 |
| Pluronic® P104 | 8520 ± 110 (5900[d]) | 1.05 | | 39.4 ± 0.9 |
| MMP–P85 | 15,290 ± 920 | 1.47 | 2.06 | 28.5 ± 1.8 |
| MMP–P104 | 23,540 ± 1100 | 1.33 | 2.76 | 30.9 ± 2.2 |

The $M_n$ and $T_m$ values represent means ± SD for $n = 3$.
[a] Number average molecular weights determined by GPC.
[b] Polydispersities based on GPC measurements.
[c] Melting temperatures determined by DSC.
[d] Molecular weights from BASF chemical company.

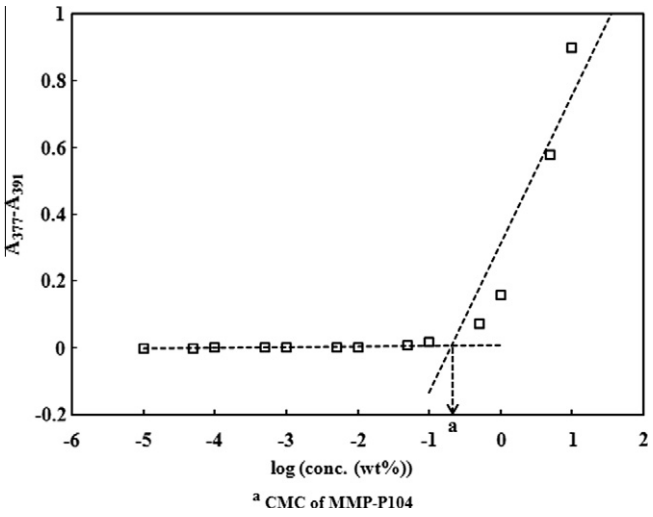



**Fig. 2.** CMC determination of MMP–P104 using a dye solubilization method. The CMC is defined as the point of intersection of two exponential lines. The CMC of MMP–P104 was found to be 0.208 ± 0.012 wt.%. The CMC value represents the mean ± SD of three experiments.

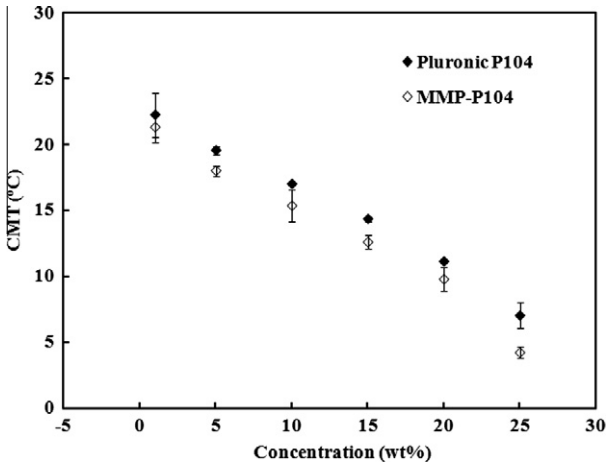



**Fig. 3.** Determination of CMT values of Pluronic® P104 and MMP–P104 solutions by DSC. The CMT value represents the mean ± SD of three experiments.

of MMP–P104. This can be explained in two ways. First, at a given PPO/PEO ratio higher molecular weight Pluronic® polymers form micelles more readily than lower molecular weight polymers [42]. Second, micelle formation is favored for multiblock co-polymers as covalently bonded PPGs facilitate easy hydrophobic core formation during micelle structuring [27].

The polymer solutions were further characterized for critical micellization temperature (CMT). Micelle formation is characterized by an endothermic peak during the heating cycle in DSC. The onset temperature at which micelles start to form is the CMT at a particular polymer concentration. Fig. 3 shows the comparison between CMT values of Pluronic® P104 and MMP–P104 solutions at different concentrations. The trend in CMT changes with polymer concentration in accordance with the phase transition of Pluronic® P104 [42,43]. The CMT of MMP–P104 was slightly lower than that of Pluronic® P104 at all polymer concentrations. This is mainly due to the higher molecular weight of the multiblock co-polymer compared with Pluronic® P104. High molecular weight block polymers are known to favor micelle formation more than low molecular weight polymers [42].

The secondary structures of MMP–P104 and the peptide in solution were analyzed using CD spectroscopy as a function of temperature. Formation of α-helices (one positive band centered at ~195 nm and two negative bands between 205 and 225 nm), β-sheets (one positive band centered at ~195 nm and one negative band between 210 and 220 nm) or random coils (one negative band centered at ~195 nm and one positive band centered at ~215 nm) show characteristic CD spectra [8]. The spectra of both the peptide and MMP–P104 suggested a random coil arrangement of the MMP-sensitive peptide in the multiblock co-polymer in an aqueous environment. Pluronic® did not appear to have a significant effect on the random coil arrangement of the peptide in water. Fig. 4 shows the CD spectra of MMP–P104 at 15, 25, 35 and 45 °C. No visible changes in the CD spectra were observed as the temperature of the polymer solutions was increased. The effect of temperature on the random coil arrangement of the peptide was also minimal (Supporting Information Fig. S4), indicating that the

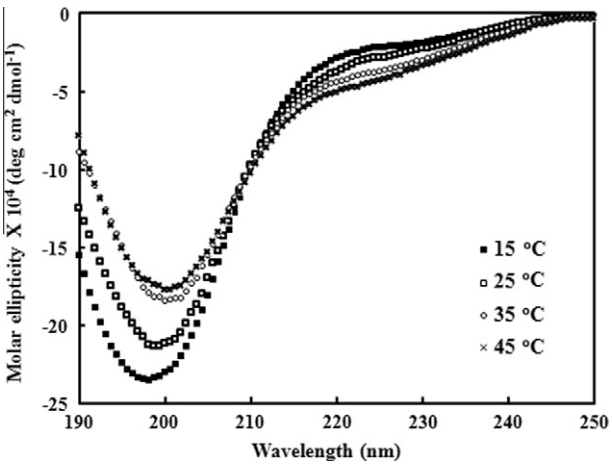



**Fig. 4.** CD spectra of MMP–P104 at different temperatures. Molar ellipticity is plotted on the vertical axis. All the solutions showed a negative band at ~195 nm and a positive band at ~215 nm.

temperature does not affect the secondary structural arrangement of the octapeptide in MMP–P104.

The cytotoxicity of Pluronic® P104 and MMP–P104 was assessed by MTT assay using HT1080 fibrosarcoma cells. HT1080 cells were chosen for the assessment of the cytotoxicity as they are known to express different types of MMPs, especially MMP2 and MMP9 [44,45]. Fig. 5 shows the results of MTT assays for the polymer concentration range 0.05–1.00 wt.%. It can be seen that MMP–P104 resulted in greater cell viability compared with monomeric Pluronic® P104. The difference in cytotoxicity can be attributed to the molecular weight difference between MMP–P104 and Pluronic® P104. Previously a Pluronic® P104-based multiblock co-polymer (MBCP 2) of molecular weight ~40,000 showed a cell viability of >80% [24]. Thus an increase in polymer molecular weight could be a useful way to improve the biocompatibility of MMP–P104. Biocompatible MMP–P104 of increased molecular weight could provide an ideal cancer drug delivery platform, with bioresponsive drug release as well as enhanced cytotoxicity to cancer cells due to the sensitizing effect of released Pluronic® P104.

The fundamental properties, such as the sol–gel and gel–sol transition temperatures of Pluronic® P104 and MMP–P104 polymer solutions were evaluated by the test tube inversion and falling ball methods. Fig. 6 shows the phase diagrams of MMP–P104 and Pluronic® P104 at different polymer concentrations. The sol–gel transition temperatures determined by the test tube inversion method are in good agreement with the values determined by the falling ball method, although a slight difference in gelation temperature was observed between two methods due to an extended equilibration time for the falling ball method. MMP–P104 solutions were free flowing liquids at room temperature which could be easily injected through a 25 gauge needle at all polymer concentrations tested for gelation. Upon increasing the temperature to 37 °C the solution formed a gel, depending on the polymer concentration. Fig. 7 shows the change in viscosity with temperature of MMP–P104 solutions at different polymer concentrations. The viscosities were below 500 g $cm^{-1}$ $s^{-1}$ for the "sol" state, whereas they ranged between 1000 and 9000 g $cm^{-1}$ $s^{-1}$ for the "gel" phase for all concentrations. MMP–P104 solutions in the range 10–25 wt.% showed sol–gel–sol transitions on increasing the temperature, however, the 25 wt.% Pluronic® P104 solution showed sol–gel–sol–gel–sol transitions. Formation of isotropic or cubic crystalline phases is responsible for gelation at low temperature, whereas formation of multiple phases (cubic/hexagonal/lamellar) cause gelation at high temperature [46]. The sol–gel transition temperatures of MMP–P104 were lower compared with Pluronic® P104 at any particular concentration. For example, at 25 wt.% the gelation temperature of Pluronic® P104 was 68.5 °C, whereas the gelation temperature of MMP–P104 was 30.7 °C. In addition to the sol–gel transition, the critical gelation concentration (CGC) of MMP–P104 was also lower compared with the CGC of Pluronic® P104. The alteration in gelation properties was mainly attributed to the increase in micelle size due to increased molecular weight of MMP–P104 compared with Pluronic® P104 (Supporting Information Table S2). Thus the larger micelle size of the multiblock co-polymer might be the reason for the lower CGC with facile and/or extensive polymer interactions compared with Pluronic® P104 micelles.

Fig. 8 shows GPC chromatographs of degraded MMP–P104 in the presence and absence of MMP. An MMP concentration of 250 μg $ml^{-1}$ was chosen to study polymer degradation under facilitated conditions. MMP–P104 was rapidly degraded in the presence of MMP showing complete polymer degradation within 24 h, while it was degraded much more slowly in the absence of MMP. Complete polymer degradation was observed at the end of day 12 without the enzyme. GPC was also run on Pluronic® P104 for comparison. The chromatograph of completely degraded MMP–P104 was similar to that of Pluronic® P104, indicating that the multiblock co-polymer was degraded into its monomeric units.

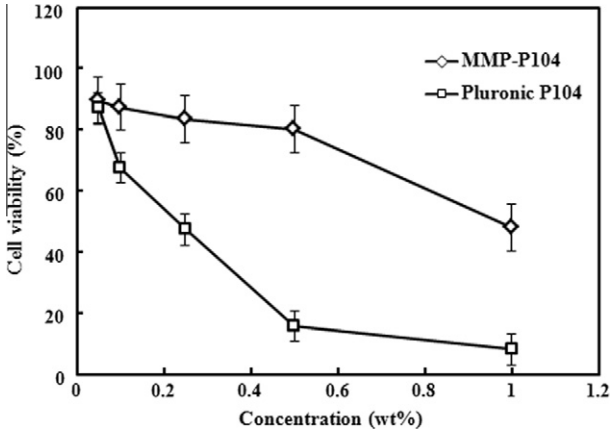



**Fig. 5.** Cell viability as a function of concentration was measured for Pluronic® P104 and MMP–P104 on HT1080 cells. The results represent the mean ± SD for $n = 3$.

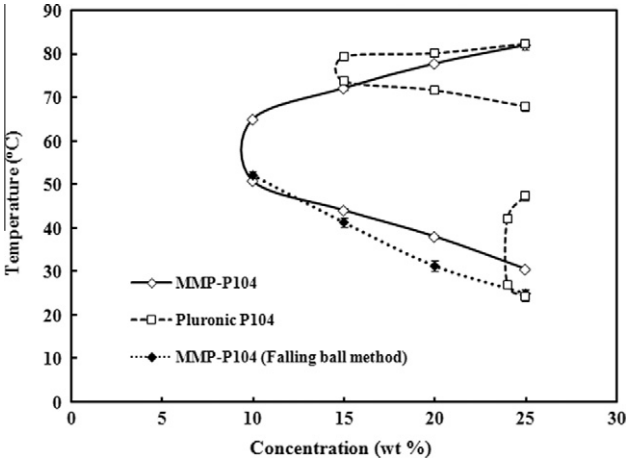



**Fig. 6.** Phase diagrams of Pluronic® P104 and MMP–P104 aqueous solutions constructed by both the test tube inversion method and the falling ball method. The results represent mean ± SD for $n = 3$.

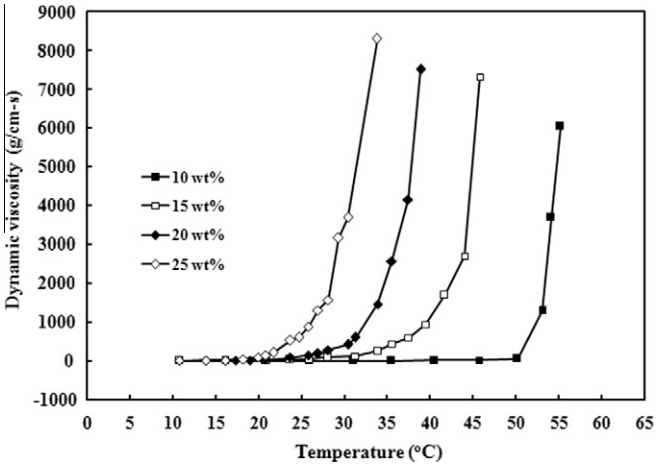



**Fig. 7.** Dynamic viscosity measurement of MMP–P104 solutions as a function of temperature. Dynamic viscosity was measured using the falling ball method for different polymer concentrations.

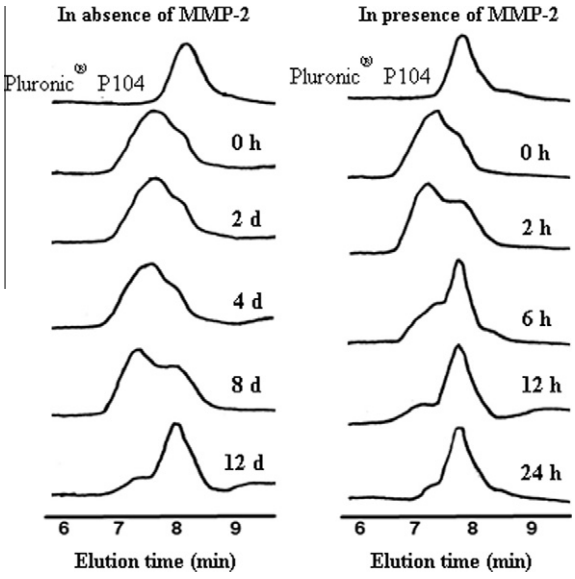



**Fig. 8.** GPC chromatographs showing MMP–P104 polymer degradation in the presence and absence of MMP. The multiblock co-polymers were degraded into lower molecular weight monomers. At 0 h a small amount of unreacted Pluronic® P104 was detected in the chromatographs of MMP–P104.

Considering the MMP-cleavable site in the peptide, the probable degradation product of the multiblock co-polymer is Pluronic® monomers with four amino acids on the two termini. However, remaining peptide segments will eventually be degraded to amino acids by proteases in the body.

The MMP–P104 solutions demonstrated rapid gelation, which occurred within 1 min at below body temperature at a polymer concentration of 25 wt.%. Based on this observation 25 wt.% polymer was selected for the in vitro PTX release experiments. The PTX concentration in 25 wt.% MMP–P104 solution was found to be 0.847 ± 0.019 mg ml$^{-1}$. The PTX concentration was nearly 200-fold greater than in water, with a PTX concentration increase from 0.004 to 0.847 mg ml$^{-1}$ in the presence of the polymer. PTX release from the MMP-sensitive thermogel was investigated at four different MMP concentrations, as well as in MMP-free medium. The release profiles are shown in Fig. 9. PTX release continued for 2, 5 and 8 days at MMP concentrations of 50, 10 and 1 μg ml$^{-1}$, respectively. Rapid drug release due to erosion of the gels was observed

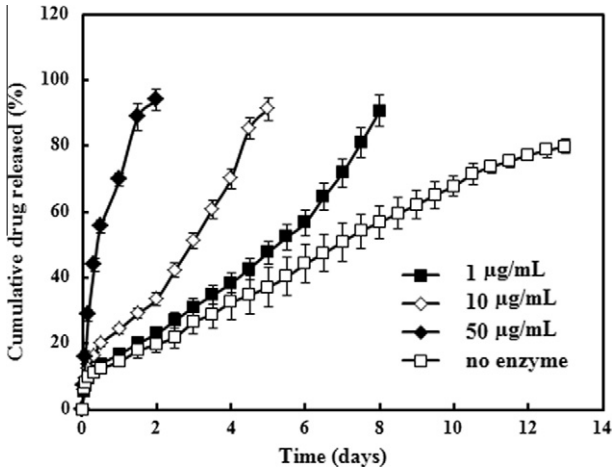



**Fig. 9.** PTX release at different MMP concentrations (0, 1, 10 and 50 μg ml$^{-1}$). The results represent mean ± SD for $n = 3$.

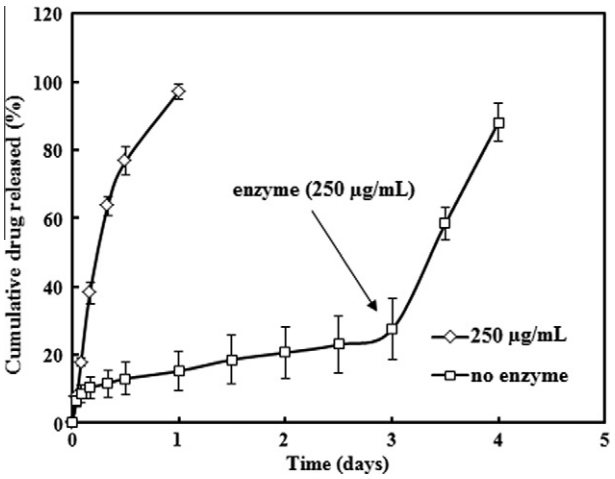



**Fig. 10.** PTX release from MMP–P104 thermogels with an alternating challenge of MMP-free medium followed by MMP-containing medium. The addition of MMP to the release medium at a concentration of 250 μg ml$^{-1}$ triggered raid drug release. The results represent mean ± SD for $n = 3$.

in presence of MMP. Based on the agreement between PTX release and polymer degradation, it is notable that erosion played a major role in drug release in the presence of MMP. On the other hand, both erosion and diffusion were implicated in sustained drug release for up to 13 days in the absence of MMP.

Precise drug release from an in situ formed hydrogel depot to achieve a high local therapeutic drug concentration is a difficult task since there is no known mechanism to control drug release once it forms a depot. The hydrogel depot is expected to monotonously release incorporated drug at a predetermined rate until the incorporated drug is exhausted. An extra mechanism that is able to control drug release after in situ depot formation and result in therapeutic levels of the drug is highly desirable for successful local drug delivery, particularly for diseases such as cancer. MMP-sensitive thermogels were tested for cooperative drug release by diffusion and enzymatic hydrogel degradation, which may be potentially useful in the achievement of effective local drug delivery for cancers. The thermogel was challenged with alternating medium changes to simulate the elevated MMP concentration associated with some cancers. As shown in Fig. 10, PTX release from the thermogel immediately increased upon replacement of MMP-free medium by MMP-containing medium. Upon addition of MMP at a concentration of 250 μg ml$^{-1}$ cumulative PTX release increased dramatically from 27% to complete drug release within 24 h. The MMP-sensitive PTX release demonstrated in Figs. 9 and 10 is in accordance with the polymer degradation presented in Fig. 8. The marked increase in drug release from the MMP-sensitive thermogel suggests its potential as a bioresponsive local drug delivery platform, particularly applicable for cancer drug delivery.

## 4. Conclusions

MMP-sensitive thermogelling polymers have been successfully synthesized for potential drug delivery applications by incorporating an MMP-sensitive peptide into an amphiphilic multiblock co-polymer. An aqueous solution of the synthesized polymer underwent gelation at body temperature. In addition, the gelation temperature of the polymer solutions varied depending on the polymer concentration. The cytotoxicity of the synthesized polymer was lower compared with the corresponding monomer. The secondary structure of the peptide inside the thermogel was confirmed to be a random coil arrangement. The polymer was rapidly degraded in the presence of MMP. The synthesized polymer greatly

enhanced the solubility of a hydrophobic cancer drug, PTX. The polymer gels released the incorporated drug in a bioresponsive manner.

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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.actbio.2011.02.005.

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