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

Keywords

thermosensitive; hydrogel; Pluronic[®]; biodegradable; matrix metalloproteinase-2

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1. INTRODUCTION

The advance in technologies to engineer biomimetic materials has been an important breakthrough that would greatly influence human health care [1–3]. Careful fabrication of materials with precise consideration of the underlying cellular microenvironments 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]. Particularly, peptide conjugation has been proven as a vital approach to develop the biomimetic materials to suit the biomedical needs [8–10]. Several approaches have been tried to engineer these materials either to target the diseased tissue or to 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. The critical problems associated with the polymeric prodrugs include low drug loading capacity and altered physicochemical properties of the polymeric prodrug as a whole. Another drawback associated with the polymeric prodrug is the release of peptide fragment-modified drug rather than intact drug molecules, resulting in the reduced activity of parent drug [19]. An alternative to overcome the problems associated with these approaches would be local drug delivery systems with enhanced drug loading. Particularly, the local drug delivery systems based on amphiphilic thermosensitive polymers has been investigated in recent years [20–22]. The thermosensitive polymer solution is a free-flowing fluid at room temperature allowing easy administration into the body. The polymer solution turns into gel depot upon injecting into the body, where the gel depot acts a drug reservoir [23]. In addition, the amphiphilic polymer can improve the solubility of insoluble drugs. From a clinical perspective, the injectable thermogels have a great benefit 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, alginate, Pluronic® copolymers and poly(N-isopropylacrylamide)-based copolymers [24–26]. Among the reported thermosensitive polymers, Pluronic® copolymers, a class of polyethylene glycol-polypropylene glycol-polyethylene glycol (PEG-PPG-PEG) triblock copolymers, were extensively studied due to their drug loading capacity and acceptable biocompatibility [24, 27, 28]. It is worth mentioning about an anticancer micellar formulation based on Pluronic® L61 and F127, which has reached phase II clinical trials [29]. Besides the ability of the Pluronic® copolymers to form micelles and thermogels, they are capable of sensitizing multi-drug resistant cells and increasing drug transport across cellular barriers [30]. To overcome the undesirable gel properties associated with Pluronic® triblock copolymers such as weak mechanical strength and rapid erosion, multiblock copolymers made of PEG and PPG have been proposed for enhanced physicochemical properties [31–35]. A family of disulfide multiblock copolymers was synthesized from Pluronic® P65, P85 and P105 to prolong the gel duration and to undergo thiol-dependent degradation [32]. Interestingly, the thermogels of multiblock copolymers degraded and released paclitaxel in a glutathione-responsive manner. Recently, PoligoGel®, a multiblock copolymer 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 a coupling agent [35]. The thermogel showed improved gelation and drug release properties comparing to Pluronic® triblock copolymers. Even though several different thermogelling systems have been prepared, the 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 tools to regulate drug release from the thermogels. MMPs are zinc-dependent endopeptidases that can cleave many of the extracellular matrix proteins. Many of the MMPs are found to be overexpressed and active during advanced stages of the cancer while they are minimally expressed in the normal tissue. Particularly, MMP2 and 9 (Type IV collagenases) which degrade extracellular matrices have been known to play a critical role in tumor progression, angiogenesis and metastasis [8]. Therefore, the combination of thermogelling property and enzyme sensitivity would provide a vital 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 thermosensitive poly(N-isopropyl acrylamide) hydrogel matrix [11, 12, 36]. The peptide in the hydrogel system provided a cross-linking structure while maintaining thermosensitive properties. In addition, the system showed concentration dependent degradation towards MMP2. Recently, an enzymatically degradable temperature sensitive polypeptide has been reported. The block copolymer, poly (ethylene glycol)-*block*-poly (alanine-co-phenyl alanine) (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 the degradation of polymer in vivo.

Here we report the synthesis of novel Pluronic®-based multiblock copolymer connected via a MMP-2-sensitive peptide, Gly-Pro-Val-Gly-Leu-Ile-Gly-Lys-NH² (GPVGLIGK-NH₂). The hypothesis behind this project is that the combination of thermosensitive Pluronic® and MMP-sensitive peptide would result in a dual-stimuli-sensitive polymer which can form gel at body temperature and allow bioresponsive degradation in presence of MMP. Physical properties and in vitro cytotoxicity of the polymers were studied to assess in vivo applicability as a local drug delivery system. Finally, a chemotherapeutic agent, Paclitaxel (PTX), has been released to demonstrate the bioresponsive drug release from the thermogels.

2. EXPERIMENTAL

2.1. Materials

The peptide, GPVGLIGK-NH₂, was synthesized by American Peptide Company (Vista, CA). Pluronic® P85 and P104 were kindly provided from BASF Chemical Company (Florham Park, NJ). Collagenase-IV was purchased from Sigma (Saint Louis, MO). PTX was obtained from LC Laboratories (Woburn, MA). Polystyrene standards of molecular weights 1, 4, 20, 50 and 100K 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 triblock copolymer—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 molar 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 molar 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 was 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 activated triblock copolymer—The peptide (100 mg) and NPC-Pluronic[®] (1.1 molar amount 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 by magnetic stirring. Triethylamine (5.0 molar amount to the peptide) was slowly added to the reaction mixture. The reaction was carried out for one day (at room temperature) or two days (first day at room temperature and second day at 50°C) or three days (one day at room temperature and two days at 50°C). Dry nitrogen was flushed to evaporate DMA from the reaction mixture. This crude multiblock copolymer was dissolved in water and dialyzed (molecular weight cut off: 6000–8000, Spectra/Por[®]) against deionized water for two days with periodic media changes. The final product was freeze-dried to obtain polymer powder. Two different multiblock copolymers, MMP-P85 from Pluronic[®] P85 and MMP-P104 from Pluronic[®] P104, were synthesized with the yields of 80% and 89%, respectively.

2.3. Polymer characterization

¹H-nuclear magnetic resonance (¹H-NMR) was used to analyze the chemical structures of the synthesized polymers. Polymers were dissolved in either CDCl₃ or D₂O and the spectra were recorded on a 400MHz NMR spectrometer (Bruker Ultrashield™ 400 PLUS, Germany). 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 I.D., 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 at 25°C. Polystyrene standards (1000–50000 Da) 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 from differential scanning calorimeter (DSC) (Perkin Elmer Diamond DSC, Waltham, MA). Melting temperatures of the polymers were determined from the thermograms thereafter. DSC was run in the temperature range of 0 to 50°C with a heating and cooling rate of 10°C/min. Initially, the samples were loaded in aluminum pans and equilibrated at 0°C for 5 min. Dry nitrogen flow at a rate of 20 mL/min 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 1.0×10^{-5} to 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 a dark place. 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 vertical axis and concentration on horizontal axis.

2.5. DSC of polymer solutions

DSC thermographs of Pluronic® P104 and MMP-P104 solutions were obtained in the concentration range of 1 to 25 wt%. Sample solutions were prepared by dissolving 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 same amount of deionized water. DSC was run in the temperature range of –10 to 50°C at a rate of 5°C/min. Samples were thermally equilibrated at –10°C for 15 min before the scanning.

2.6. Circular dichroism (CD) spectroscopy

The CD spectra of MMP-P104 and peptide solutions were analyzed in the wavelength range of 250 to 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-1mm) 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

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 medium (RPMI). The RPMI was supplemented with 10% fetal bovine serum (FBS) and antibiotics. The cultured cells in 96-well plates (5×10^3 cells/well) were grown for 24 h at 37°C. The culture medium was replaced with MMP-P104 solution (100 μ L, 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 μ L) followed by 12 h incubation in 200 μ L DMEM containing 10% FBS. After 16 h of total incubation, the test solutions were replaced with fresh media (200 μ L) and 5 mg/mL MTT solution (20 μ L). The cells were incubated for 4 h in MTT solution. Later, the MTT solution was replaced with 400 μ L dimethyl sulfoxide to dissolve internalized purple formazan crystals. To measure the absorbance at 570 nm on a VICTOR3 V™ Multilabel Counter (Perkin Elmer, MA, USA), 150 μ L of the solution was transferred from each well into a fresh 96-well plate. Cell viability was calculated as compared to phosphate buffered saline (PBS)-treated cells (100 % survival).

2.8. Thermal gelation of polymeric solutions

2.8.1. Test tube inverting 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 (4mL) were loaded with 1mL of the polymer solution and incubated at 4°C overnight. The sol-gel and gel-sol phase transition temperatures of the each polymer solution were determined with increments of 2°C [24, 38]. The experiment was conducted in the temperature range of 15 to 80°C. The temperature at which a polymer solution stopped flowing upon a tube inversion was recorded as 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. NMR tube of diameter 4.0 mm 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 (ρ_s) = 7.97 g/mL) 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 between temperature and the transit time gives the sol-gel transition temperature of the polymer solution [39]. The dynamic viscosity (μ) was calculated using the following formula

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

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

2.9. MMP-P104 degradation

The MMP-P104 solution was prepared at a concentration of 25 wt% in deionized water. The polymer solution (50 μL) was placed in 1mL vials and the vials were kept at 4°C overnight. The polymer solutions were incubated at 37°C for 15 min for gelation. Phosphate buffer (0.1mL, 50mM) was added to first three vials (control). In another three vials, phosphate buffer containing collagenase-IV (250 $\mu\text{g/mL}$) was added. The degradation of the gels was stopped by freezing the gels at predetermined time intervals. The frozen samples were then freeze-dried to get the degraded polymer. GPC was run to check the molecular weight distribution of the degraded polymer.

2.10. In vitro paclitaxel release

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

3. RESULTS & DISCUSSION

Multiblock copolymers (MMP-P85 and MMP-P104) were synthesized by conjugating thermosensitive Pluronic[®] (P85 and P104), a group of triblock copolymer of PEG-PPG-PEG, to a MMP-sensitive peptide (GPVGLIGK-NH₂). The synthesis involves a two-step reaction as described in Scheme 1. The ¹H-NMR spectra of Pluronic[®] P104 and purified NPC-Pluronic[®] were shown in Figure 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 ¹H-NMR spectrum [40]. Figure 1A shows the proton peak of hydroxyl protons (c) at each end of the Pluronic[®]. The hydroxyl proton peak was disappeared in the NMR spectrum (Figure

1B) upon the activation of Pluronic[®]. In addition, the appearance of proton peak of the methylene group (f) at 4.45ppm confirmed the successful activation of the Pluronic[®].

Several peptide sequences such as GPLGIAGQ, GPQGPAGQ, PVGLIG, and CGLDD have shown MMP susceptibility [13, 14, 19]. An octapeptide sequence (GPVGLIGK-NH₂) 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. The modification in the peptide would allow the conjugation reaction to the terminals while maintaining MMP-sensitive cleavage of the peptide at –GL– [41]. The terminal amino groups of the peptide allowed the linear oligomerization between the peptide and NPC- Pluronic[®]. As Pluronic[®] of molecular weight ~ 5000 may not be easily accessible for the oligomerization, the reaction time and temperature were increased to check the effect of these parameters on the final molecular weight of MMP-P104. Table 1 summarizes the molecular weights of MMP-P104 at different reaction conditions. The molecular weight of MMP-P104 slightly increased on increasing the reaction time and temperature. Figure 1-C shows the ¹H-NMR spectrum of MMP-P104. The oligomerization was confirmed by the characteristic proton peaks of peptide and Pluronic[®] in the final polymer. The proton peaks at g to v shows the presence of peptide and its conjugation to Pluronic[®].

Determined molecular characteristics of Pluronic[®] copolymers, MMP-P85 and MMP-P104 were summarized in Table 2. The calculated molecular weights of MMP-P85 and MMP-P104 were found to be 15290 and 23540 g/mol respectively. The molecular weight analysis indicates that MMP-P85 and MMP-P104 are multiblock copolymers with 2.06 and 2.76 units of Pluronic[®] P85 and Pluronic[®] P104 respectively. Comparing to previously reported Pluronic[®]-based multiblock copolymers [24], multiblock copolymers with relatively low molecular weights were achieved because of relatively high molecular weight peptide (~740 g/mol) which might limit the access of the nitro group at the end of the Pluronic[®] to the amine group in the peptide during oligomerization. According to DSC data, the melting temperatures (T_m) of MMP-P85 and MMP-P104 were found to be 28.5 (±1.8) and 30.9 (±2.2) °C. The T_m values of multiblock copolymers are lowered comparing to the T_m values of Pluronic[®] monomers. This lowering of the T_m values of MMP-P85 and MMP-P104 may be due to the interrupted polymer packing by the incorporation of a peptide in the polymer structure [24].

The relatively high molecular weight of MMP-P104 made this multiblock copolymer an interested candidate comparing to MMP-P85. So, the 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 has sharply increased at CMC of the MMP-P104 (Figure 2). The CMC was calculated by extrapolating the absorbance versus logarithmic concentration. 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 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 the lower molecular weight polymers [42]. Second, the easy micelle formation is favored for multiblock copolymer as covalently bonded PPGs facilitate easy hydrophobic core formation during micelle structuring [27].

The polymer solutions were further characterized for critical micellization temperature (CMT). The micellization process is characterized by endothermic peak during the heating cycle in DSC. The onset temperature at which micelles start to form is considered as the CMT at the particular polymer concentration. Figure 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 was in accordance with phase transition of Pluronic® P104 [42, 43]. The CMT of MMP-P104 slightly lowered than that of Pluronic® P104 at each polymer concentration. This is mainly due to the higher molecular weight of the multiblock copolymer comparing to Pluronic® P104. High molecular weight block polymers are known to be more favorable for micelle formation than low molecular weight polymers [42].

Secondary structures of MMP-P104 and 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 205–225 nm) or β - sheets (one positive band centered at ~ 195 nm and one negative band between 210–220 nm) or random coils (one negative band centered at ~ 195 nm and one positive band centered at ~ 215 nm) will show characteristic CD spectra [8]. Spectra of both peptide and MMP-P104 suggested the random coil arrangement of MMP-sensitive peptide in the multiblock copolymer in an aqueous environment. Pluronic® did not show a considerable effect on the random coil arrangement of peptide in water. Figure 4 shows the CD spectra of MMP-P104 at 15, 25, 35 and 45°C. As the temperature of polymer solutions increased, no visible change in the CD spectra was observed. Also, the effect of temperature on the random coil arrangement of peptide is very minimum (supporting information: Figure S4) indicating that the temperature does not affect the secondary structural arrangement of the octapeptide in MMP-P104.

Cytotoxicity of Pluronic® P104 and MMP-P104 was assessed by MTT assay using HT1080 fibrosarcoma cells. As HT1080 cells are known to express different types of MMPs, especially MMP 2 and 9, they were chosen for the assessment of the cytotoxicity [44, 45]. Figure 5 shows the results of MTT assay in the polymer concentration ranging from 0.05 to 1.00 wt%. It is shown that MMP-P104 resulted in a greater cell viability comparing to monomeric Pluronic® P104. The difference in cytotoxicity can be attributed to molecular weight difference between MMP-P104 and Pluronic® P104. Previously, a Pluronic® P104 based multiblock copolymer (MBCP 2) of molecular weight ~ 40000 showed cell viability of $>80\%$ [24]. Therefore the increase in polymer molecular weight would be a vital means to improve the biocompatibility of MMP-P104. Biocompatible MMP-104 with increased molecular weight would provide an ideal cancer drug delivery platform which can achieve bioresponsive drug release as well as enhanced cytotoxicity to cancer cells with the sensitizing effect from released Pluronic® P104.

The fundamental properties such as sol-gel and gel-sol transition temperatures of Pluronic® P104 and MMP-P104 polymer solutions were evaluated by test tube inverting method and falling ball method. Figure 6 shows the phase diagram of MMP-P104 and Pluronic® P104 at different polymer concentrations. The sol-gel transition temperatures determined by test tube inverting method are in good agreement with the values determined by falling ball method. Due to an extended equilibration time during the execution of falling ball method, a slight difference in gelation temperature was observed between two methods. At all polymer concentrations tested for gelation, MMP-P104 solutions were free flowing liquids at room temperature which could be easily injected through 25-gauge needle. Upon increasing the temperature to 37°C, the solution immediately turned into gel depending on the polymer concentration. Figure 7 shows the change in viscosities with temperature of MMP-P104 solutions at different polymer concentrations. The viscosities are below $500 \text{ g/cm} \cdot \text{sec}^{-1}$ during the “sol” state where as they ranged between $1000\text{--}9000 \text{ g/cm} \cdot \text{sec}^{-1}$ during the “gel” phase for all the measured concentrations. MMP-P104 solutions showed sol-gel-sol transition in the range of 10–25 wt%. But, the Pluronic® P104 (25wt%) solution showed sol-gel-sol-gel-sol transition on increasing the temperature. Formation of isotropic or cubic crystalline phases is responsible for gelation at low temperature whereas formation of multi-

phases (cubic/hexagonal/lamellar) cause gelation at high temperature [46]. The sol-gel transition temperatures of MMP-P104 were lowered comparing to Pluronic® P104 at 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 lowered comparing to the CGC of Pluronic® P104. The alteration in the gelation properties was mainly attributed to the increase in the micelle size due to increased molecular weight of MMP-P104 comparing to Pluronic® P104 (supporting information: Table S2). Therefore, the larger micelle size resulting from multiblock copolymer might be the reason for lower CGC with facile and/or extensive polymer interactions comparing to Pluronic® P104 micelles.

Figure 8 shows the GPC chromatographs of degraded MMP-P104 in presence or absence of MMP. An MMP concentration of 250 µg/mL was chosen to study the polymer degradation in a facilitated condition. MMP-P104 degraded rapidly in presence of MMP showing complete polymer degradation within 24 h while the polymer degraded much slower 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 to compare the changes in chromatographs. The chromatograph of completely degraded MMP-P104 was similar to that of Pluronic® P104 indicating that the multiblock copolymer degraded into its monomeric units. Considering the MMP-cleavable site in the peptide, the possible degradation products of multiblock copolymer would be the Pluronic® monomers with four amino acids on both the terminals. However, remaining peptide segments will be eventually degraded into amino acids by proteases in the body.

MMP-P104 solutions demonstrated quick gelation which occurred within a minute below body temperature at a polymer concentration of 25 wt%. Based on this observation, 25 wt% was selected for in vitro PTX release. The achieved PTX concentration in 25 wt% MMP-P104 solution was found to be 0.847 (±0.019) mg/mL. The achieved PTX concentration was nearly 200-fold increase in PTX solubility in water with a PTX concentration increase from 0.004 mg/mL to 0.847 mg/mL 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 media. Release profiles have been summarized in Figure 9. PTX release lasted for 2, 5 and 8 days at MMP concentrations of 50, 10, and 1 µg/mL, respectively. Rapid drug release due to the erosion of the gels was observed in presence of MMP. Based on the consistency between PTX release and polymer degradation, it is notable that erosion played a main role in the drug release in presence of MMP. On the other hand, both erosion and diffusion have caused sustained drug release up to 13 days in the absence of MMP.

Precise drug release from an in-situ formed hydrogel depot to achieve a local therapeutic drug concentration is a difficult task since there is no viable 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 incorporated drug is completely exhausted. The presence of an extra mechanism that is able to control drug release even beyond in-situ depot formation and secure a therapeutic level of drug will be highly desirable for successful local drug delivery particularly for detrimental diseases such as cancers. MMP-sensitive thermogels were tested for cooperative drug release by diffusion and enzymatic hydrogel degradation which may be useful for the achievement of effective local drug delivery potentially for cancers. The thermogel was challenged with an alternating medium change to simulate an MMP-elevation in cancer. As shown in Figure 10, the thermogel immediately increased PTX release upon a medium replacement from MMP-free medium to MMP-containing medium. Upon an addition of MMP at a concentration of 250 µg/mL, cumulative PTX release was dramatically increased from 27% to complete drug release within 24 hr.

The MMP-sensitive PTX release demonstrated in Figures 9 and 10 is completely in accordance with the polymer degradation presented in Figure 8. Marked increase in drug release from the MMP-sensitive thermogel suggests the capability of MMP-sensitive thermogel as a bioresponsive local drug delivery platform which would be 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 copolymer. The 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 comparing to 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 presence of MMP. The synthesized polymer greatly enhanced the solubility of a hydrophobic cancer drug, paclitaxel. The polymer gels exhibited the release of incorporated drug in a bioresponsive manner.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
 ^1H -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.

Figure 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 $\pm$ SD of three experiments.

Figure 3. Determination of CMT values of Pluronic® P104 and MMP-P104 solutions by DSC. The CMT value represents the mean $\pm$ SD of three experiments.

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

Figure 5. Cell viability as a function of concentration was measured for Pluronic[®] P104 and MMP-P104 on HT1080 cells. The results represent the mean $\pm$ SD for n=3.

Figure 6. Phase diagrams of Pluronic® P104 and MMP-P104 aqueous solutions constructed by both test tube inverting method and falling ball methods. The results represent mean $\pm$ SD for $n=3$.

Figure 7.
Dynamic viscosity measurement of MMP-P104 solutions as a function of temperature. Dynamic viscosity was measured using falling ball method for different polymer concentrations.

Figure 8. GPC chromatographs showing MMP-P104 polymer degradation in presence or absence of MMP. The multiblock copolymers 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.

Figure 9. PTX release at different MMP concentrations such as 0, 1, 10 and 50 µg/mL. The results represent mean $\pm$ SD for n=3.

Figure 10. PTX release from MMP-P104 thermogels with an alternating challenge of MMP free medium following MMP containing medium. The addition of MMP in the release medium at a concentration of 250 µg/mL triggered a fast drug release. The results represent mean $\pm$ SD for n=3.

Scheme 1.

Two step reaction scheme showing the synthesis of MMP-sensitive multiblock copolymers from Pluronic[®] (P85 or P104) triblock copolymers. Pluronic[®] and NPC were reacted at room temperature for overnight to activate Pluronic[®].

Table 1

Effect of different reaction conditions on the molecular weight of the final polymer. The M_n values represent mean $\pm$ SD for n=3.

Polymer	Reaction conditions	M_n (g/mol) ^a	M_w/M_n ^b
1	RT (one day)	17570 ($\pm$ 1060)	1.34
2	RT (one day) + 50°C (one day)	21120 ($\pm$ 960)	1.47
3	RT (one day) + 50°C (two days)	23270 ($\pm$ 1100)	1.33

^a Number average molecular weights determined by GPC

^b Polydispersities based on GPC measurements

Table 2

Characterization of multiblock copolymers and triblock monomers. The M_n and T_m values represent mean $\pm$ SD for $n=3$.

Polymer	$M_n(\text{g/mol})^a$	M_w/M_n^b	Number of repeating units	$T_m(^{\circ}\text{C})^c$
Pluronic® P85	7410 ($\pm$ 120) (4600 ^d)	1.06	-	<u>38.9 ($\pm$ 1.2)</u>
Pluronic® P104	8520 ($\pm$ 110) (5900 ^e)	1.05	-	<u>39.4 ($\pm$ 0.9)</u>
MMP-P85	15290 ($\pm$ 920)	1.47	2.06	<u>28.5 ($\pm$ 1.8)</u>
MMP-P104	23540 ($\pm$ 1100)	1.33	2.76	<u>30.9 ($\pm$ 2.2)</u>

^a Number average molecular weights determined by GPC

^b Polydispersities based on GPC measurements

^c Melting temperatures determined by DSC

^{d,e} Molecular weights from BASF chemical company