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ORIGINAL ARTICLE

Synthesis and characterization of Poloxamer 188-grafted heparin copolymer

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Abstract

Background: Poloxamer 188 is a safe biocompatible polymer that can be used in protein drug delivery system. *Aim*: In this study, a new heparin–poloxamer 188 conjugate (HP) was synthesized and its physicochemical properties were investigated. HP structure was confirmed by Fourier transform infrared spectroscopy (FTIR) and Hydrogen-1 nuclear magnetic resonance spectroscopy (^{1}H-NMR). Content of the conjugated heparin was analyzed using Toluidine Blue. The critical micelle concentration (CMC) of the copolymer was determined by a fluorescence probe technique. The effect of HP on the gelation of poloxamer 188 was characterized by the rheological properties of the HP–poloxamer hydrogels. Solubility and viscosity of HP were also evaluated compared with poloxamer 188. *Results*: From the results, the solubility of the conjugated heparin was increased compared with free heparin. The content of heparin in HP copolymer was 62.9%. The CMC of HP and poloxamer 188 were 0.483 and 0.743 mg/mL, respectively. The gelation temperature of 0.4 g/mL HP was 43.5°C, whereas that of the same concentration of poloxamer 188 was 37.3°C. With HP content in poloxamer 188 solution increasing, a V-shape change of gelation temperature was observed. *Conclusion*: Considering the importance of poloxamer 188 in functional material, HP may prove to be a facile temperature-sensitive material for protein drug-targeted therapy.

Key words: *Critical micelle concentration; heparin; poloxamer; synthesis; temperature sensitive*

Introduction

Heparin, including low molecular weight heparin and other types of heparin, is one of the most intensively studied glycosaminoglycans because of its anticoagulant properties. Heparin can be combined with a number of protein and peptides to form a complex, such as growth factors and cytokines. With this important bioactivity, protein drugs can be stabilized in the complex and the release behavior can be controlled. In addition, bioactivity of these proteins released from the complex does not change[1]. Based on these reports, vectors grafted with heparin can be used as carriers for some proteins to regulate their release in vivo and simultaneously play an essential role of anticoagulation.

Compared with heparin that was embedded directly into the blend, the immobilized heparin enjoys some advantages, such as the property of high stability and slow release of the heparin, which could prevent the initial burst release and thus reduce side effects or adverse reactions. The activity of the anticoagulant could be controlled by adjusting the length of the spacers or linkers.

Over the last decade, studies of properties and applications about material grafted with heparin were focused on the aspect of anticoagulant. In recent years, the potential for material grafted with heparin that could be used as drug carriers arouse increasing interest. Several polymaterials grafted with heparin have been developed, including PLGA[2], poloxamer[3], and its analogues[4–6], as well as glycosaminoglycan[7]. Some of these copolymers showed

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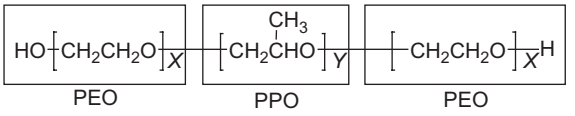


Figure 1. Structure of poloxamer 188 ($X = 79$, $Y = 28$).

good stability and sustained-release behavior, which had the potential to be used in drug delivery systems.

Poloxamer, in other name as Pluronic® block copolymer, is triblock copolymer of poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO), often denoted by PEO–PPO–PEO. Pluronic® compounds have gained special attention because of their ability and low in vivo toxicity[8].

Poloxamer 407 (or called as Pluronic® F-127) has been applied for the delivery of protein and peptide drugs, such as insulin[9], urease[10], interleukin-2[11], and growth factors[12]; typically it showed sustained-release kinetics over several hours. Recent studies have synthesized poloxamer 407-grafted heparin copolymer for protein drug delivery[3].

Poloxamer 188 (Figure 1, $X \approx 79$, $Y \approx 28$), an FDA-approved, commercially available biocompatible triblock copolymer, has the least toxicity among Pluronic® compounds and has been used as solubilizer, emulsifier, and carriers of genetic drugs in many drug delivery systems.

Therefore, a new copolymer poloxamer 188 grafted directly with heparin was synthesized, not the same photo-crosslinked process as in Yoon's research[3]. In this study, some physicochemical properties of the new poloxamer 188 copolymer were investigated to show its potential application in protein drug delivery system.

Materials

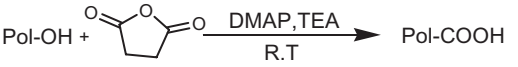
Poloxamer 188 was purchased from BASF (Shanghai, China). Stannous octoate, succinic anhydride, 4-dimethylaminopyridine, triethylamine, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), and *N*-hydroxysuccinimide (NHS) were purchased from Aldrich Chemical Company (Shanghai, China). Low molecular weight heparin sodium salt (from porcine intestinal mucosa, 140 unit/mg) was supplied by Freda Biochem Company (Jinan, China). All other chemicals were used as received without further purification.

Methods

Synthesis of heparin–poloxamer conjugate

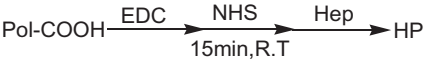
Preparation of COOH-terminated poloxamer 188 (Pol-COOH)

Poloxamer 188, succinic anhydride, 4-dimethylaminopyridine, and triethylamine were dissolved in 30 mL anhydrous 1,4-dioxane and stirred overnight at room temperature. 1,4-Dioxane was removed under a vacuum. The residue was dissolved in chloroform, precipitated into an excess of diethylether, and then filtered. With repeating the process, the residue was finally dried overnight under vacuum.



Synthesis of heparin–poloxamer 188 conjugate

Heparin–poloxamer 188 conjugate HP was prepared according to EDC/NHS method[13]. Carboxylated poloxamer 188 was coupled with low molecular weight heparin by EDC and NHS in MES buffer for 24 hours at room temperature. The resultant was dialysed against distilled water by using a dialysis bag (MWCO = 7 K) for 3 days and finally lyophilized to gain the product.



Characterization and determination of the conjugate properties

Characterization of the conjugate

The structure of HP copolymer was characterized using Fourier transform infrared spectroscopy (FTIR) (670 FT-IR, Nicolet, Madison, WI, USA). Hydrogen-1 nuclear magnetic resonance spectroscopy (^{1}H-NMR) (AVANCE III 600 MHz, Bruker, Fällanden, Switzerland) spectroscopy measurement was carried out to confirm the change of chemical structure of HP.

Solubility test

The solubility tests of heparin, poloxamer 188, and HP copolymer were carried out at room temperature in various solvents, such as deionized water, methanol (MeOH), dimethylsulfoxide (DMSO), *N*,*N*-dimethylformamide (DMF), tetrahydrofuran (THF), and *n*-hexane. Each sample (10 mg) was dispersed in 10 mL of each solvent and then the mixtures were shaken for 2 days in 25°C water bath. The solubility of samples in different solvents was evaluated by observation of the mixture appearance and clarity.

Determination of conjugated heparin

The content of the conjugated heparin was analyzed by the colorimetric method using Toluidine Blue[14,15]. Three milliliters of the 0.005% Toluidine Blue solution, which was dissolved in 0.9% NaCl was added into each

of test tube. One milliliter of the different concentration of standard heparin solution was added. Each tube was agitated by a Vortex mixer for 30 seconds and then bathed in 37°C water for 2 hours. Two milliliters of hexane was added to each tube and the tubes were shaken vigorously for another 30 seconds to separate the heparin–dye complex. After standing quietly for 20 minutes, the aqueous layers of all the tubes were removed and each residue in the tubes was diluted to 1:10 (v/v) with 0.9% NaCl. By measuring the absorbance at 629 nm, a standard curve was obtained to represent the relationship between absorbance and concentration.

Sixty micrograms per milliliter of HP copolymer was used for the determination of conjugated heparin. According to the same process mentioned above, the content of immobilized heparin in HP copolymer was determined based on the standard curve.

Determination of critical micelle concentration of HP

Fluorescent probe is commonly used for studying of micelle formation and critical micelle concentration (CMC), such as probe of pyrene[16]. The solubility and intensity will be increased suddenly and significantly as soon as micelles are formed in water. There are some changes when micellization occurs. The vibrational fine structure changes, as the transfer of pyrene from a polar environment to a nonpolar one suppresses the acceptance of the symmetry-forbidden (0, 0) band. This change can be described in terms of the ratio I_1/I_3 and the intensities of the first and third bands in the pyrene fluorescence spectrum[17]. Based on previous study, we set the emission wavelength at 372 nm. The micellization of poloxamer 188 and HP at room temperature was characterized by employing the ratio of excitation intensity (I_{339}/I_{333}).

Poloxamer 188 and HP copolymer were dissolved in distilled water to give 50 mg/mL micelle solution, respectively. Transferred into several volumetric flasks, each micelle solution was added to a certain volume of pyrene methanol solution. After the evaporation of methanol, solution was diluted to a final scale with distilled water. HP solutions were made with different concentration, ranging from 10^{-5} to 50 mg/mL, all of which contained 2.42×10^{-8} mol/L pyrene. All the micelle solutions were sonicated for 30 minutes, incubated in 50°C water bath for 2 hours, and then cooled to room temperature for determination using fluorescence spectrometer (RF-5301PC, Shimadzu Co., Tokyo, Japan).

Determination of the viscosity

A certain amount of poloxamer 188 and HP were dissolved in 1 mL distilled water. The viscosity and its change against the temperature were determined using the viscometer (DV-I Prime, Brookfield, CT, USA) with rotor CPE40. Each sample was tested three times to obtain the average of viscosity at the temperature. The gelatification temperature of each material was identified at the point where the viscosity suddenly increased.

Effect of HP on the gelation of poloxamer 188

The interaction between HP and poloxamer 188 was characterized by the rheological property of HP–poloxamer hydrogels. After poloxamer 188 solutions (0.35, 0.4, and 0.45 g/mL) were prepared, the required amount of HP was added to the solutions (the content of HP was 0%, 1%, and 5%, w/w, respectively). The partially dissolved solutions were then transferred into a cold chamber at 4°C till thoroughly mixed. After that the viscosity of each sample was determined to obtain the temperature at the point in which the viscosity suddenly increased.

Results and discussion

Characterization of HP conjugate

As shown in Figure 2, FTIR spectra demonstrated that HP had the same carbonyl stretching vibration at about 1600 cm^{-1} as heparin. In addition, HP was characterized by a new absorption peak at 1736 cm^{-1}, which belongs to carbonyl vibration of HP complex. A new band at 3300–3600 cm^{-1} was also observed in HP, which can be identified as hydroxyl groups of conjugated heparin. As shown in the ^{1}H-NMR spectra (Figure 3), there were characteristic chemical shifts of PPO and PEO (δ = 1.14, 3.4, and 3.5 ppm for –CH_3,–CH,–CH_2 in group of PPO, δ = 3.65 ppm for –CH_2 of PEO). From these spectral results, HP copolymer was successfully synthesized by EDC/NHS method.

Solubility test

Heparin, poloxamer 188, and HP were used for the solubility test in several solvents including water. Heparin is insoluble in organic solvents, because of its strong polarity. However, HP is soluble in both water and DMSO because of incorporated poloxamer moiety as shown in Table 1. From the result, HP showed more flexibility in preparation compared to heparin.

Determination of conjugated heparin

The standard curve for the determination of the content of conjugated heparin was shown in Figure 4. From the curve, heparin concentration had a negative correlation with the absorbance. The linear equation was

$$Y = -0.0017X + 0.4932 \ (r^2 = 0.9962)$$

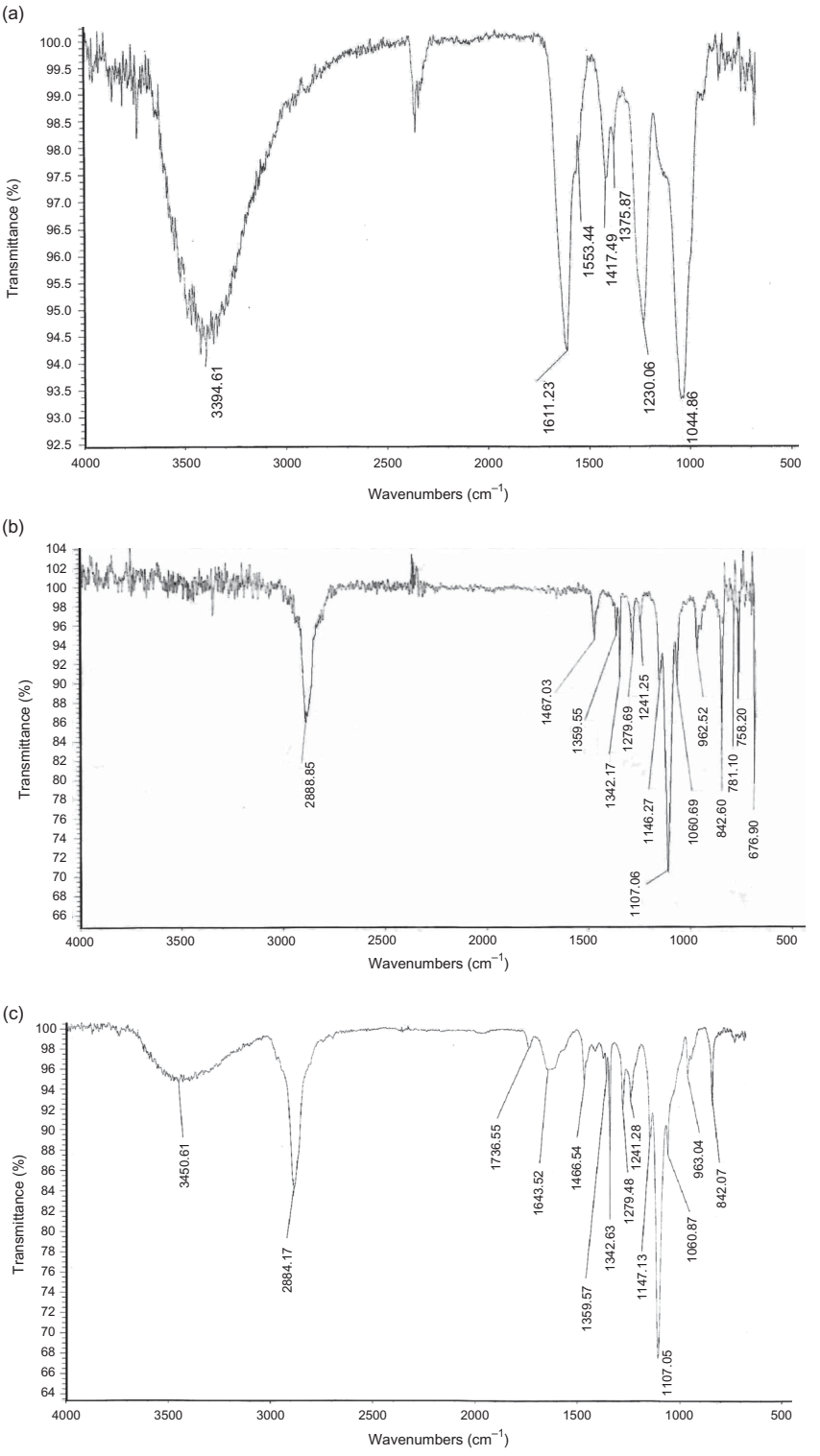


Figure 2. FTIR spectra of heparin (a), poloxamer 188 (b), and HP (c).

From the experiment, the absorbance (A_{629}) of 60 μg/mL HP copolymer was 0.429. The concentration of conjugated heparin in HP copolymer was 37.76 μg/mL according to the standard curve. Therefore, the content of heparin in HP copolymer was 62.9%. From the result, most of the heparin was conjugated with poloxamer 188.

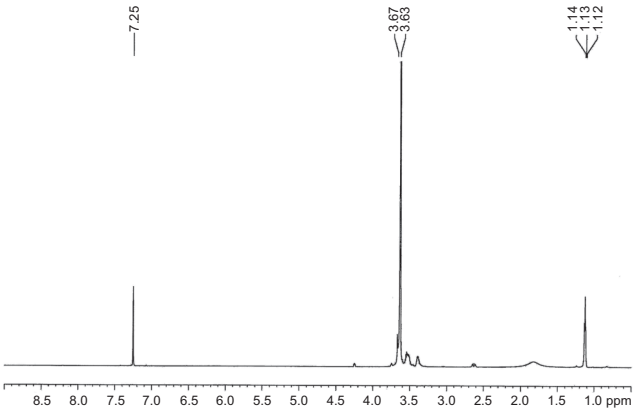


Figure 3. ^{1}H-NMR spectra of HP.

Table 1. Solubility of heparin, poloxamer 188, and HP.

Sample	Water	MeOH	DMSO	DMF	THF	*n*-Hexane
Heparin	❍	×	×	×	×	×
Poloxamer 188	❍	❍	❍	❍	❍	×
HP	❍	×	❍	×	×	×

'❍' soluble, '×' insoluble.

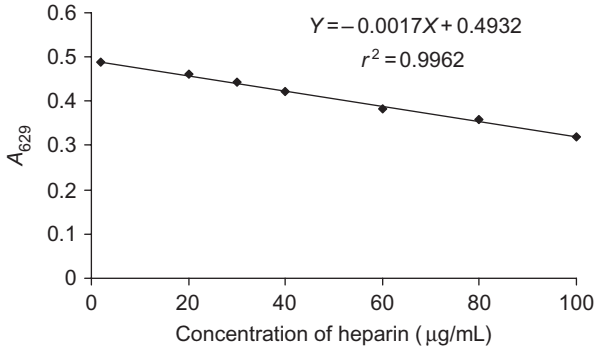


Figure 4. Relationship of heparin concentration with UV absorbance.

Determination of CMC

Poloxamer 188 and HP showed similar characteristics in Figure 5. As shown in Figure 5a for Poloxamer 188, the intersection point of smooth curve and wind-up curve was (–0.129, 0.656), compared to that of HP (–0.316, 0.644) (Figure 5b). After calculation, CMC values of poloxamer 188 and HP complex were 0.743 and 0.483 mg/mL, respectively. From the results, CMC was decreased when heparin grafted to poloxamer 188, which indicated that micelle of HP would be more stable than that of poloxamer 188.

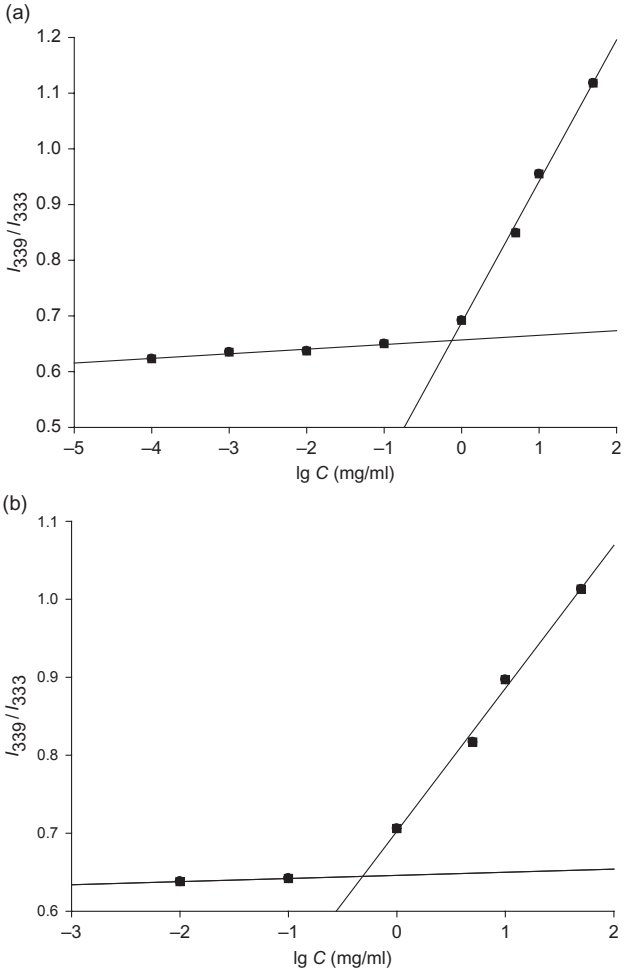


Figure 5. Fluorescence intensity (I_{339}/I_{333}) ratio of pyrene as a function of logarithm poloxamer 188 (a) and logarithm HP (b) concentration.

Determination of the viscosity

Poloxamer 188 (0.4 g) and HP (0.4 g) were dissolved in 1 mL distilled water. The results of each average viscosity were shown in Figure 6. From the profile, the gelation temperature of 0.4 g/mL HP was 43.5°C, whereas that of the same concentration of poloxamer 188 was 37.3°C. It suggested that HP might be developed as a temperature-sensitive material with proper content of heparin grafted on the terminal of PEO in poloxamer 188.

Effect of HP on the gelation of poloxamer 188

The gelation temperature of poloxamer solutions (0.35, 0.4, 0.45 g/mL) was characterized by increasing content of HP (0%, 1%, or 5%, w/w) in poloxamer 188 solution. As shown in Figure 7, a V-shape change of gelation temperature was obtained as increasing of HP content in poloxamer 188 solution. Similar phenomenon was

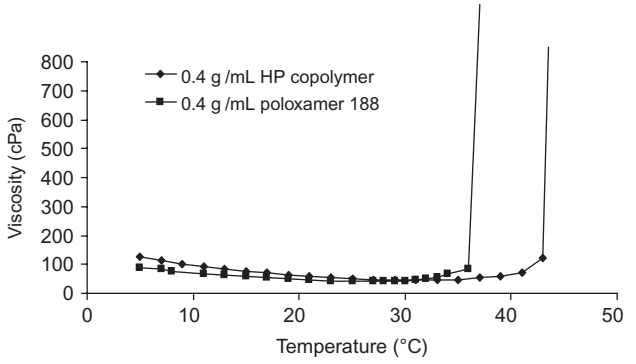


Figure 6. Changes of viscosity with temperature increasing.

also observed in a recent study[18]. According to quadric equation fitting, the value on the axis of abscissas corresponding with the valley bottom for three V-shape profiles was about 2.75%. In other words, minimum gelation temperature was obtained when HP content in poloxamer 188 solution reached 2.75%. It suggested that there was an interaction between HP and poloxamer 188, which control poloxamer 188 micelle–micelle formation. With proper HP addition in poloxamer 188, the gelation temperature could be adjusted even lower than pure poloxamer 188, which may provide the potential of temperature-sensitive material for drug-targeted therapy.

Summary

In this article, a new heparin–poloxamer 188 conjugate was synthesized and its unique nature was investigated compared with poloxamer 188. Through these experiments, we want to develop a new functional material that could be used in heparin-binding proteins. Some phenomena were observed in this study; for example, HP showed obviously lower CMC value compared with poloxamer 188. With hydrophilic heparin grafted to PEO of poloxamer 188, the length of hydrophilic groups in new copolymer was raised, which tended to form micelles in water easily. This property might effectively facilitate HP resisting the dilution of body fluids and increase the in vivo stability of HP micelle protein-loaded future. In addition, the gelation temperature of HP was higher than poloxamer 188. With proper HP addition in poloxamer 188, the gelation temperature could be adjusted even lower than pure poloxamer 188. Considering the importance of poloxamer 188 in functional material, HP may provide us a facile poloxamer-based temperature-sensitive material for protein drug delivery system.

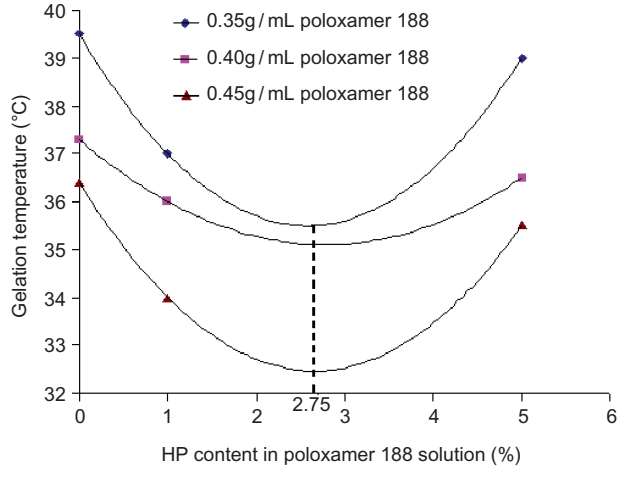


Figure 7. The effect of HP content in poloxamer 188 solution on the gelation temperature.

Acknowledgments

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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