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Release of Ciprofloxacin from Chondroitin 6-Sulfate-Graft-Poloxamer Hydrogel In Vitro for Ophthalmic Drug Delivery

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ABSTRACT The system was designed to use Poloxamer as a vehicle for ophthalmic drug delivery using in situ gel formation property. To enhance the wound healing and cell adhesion as well as transparency of Poloxamer hydrogel, chondroitin 6-sulfate (C6S) was introduced into Poloxamer. For this purpose, mono amine-terminated Poloxamer (MATP), which was end-capped with ethylene amine group only in one side of terminal hydroxyl groups of Poloxamer, was synthesized. Subsequently, C6S-graft-Poloxamer copolymer (C6S-*g*-Poloxamer) was prepared by reaction between the amine groups of MATP and carboxyl groups of C6S in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carboimide (EDC). The coupling of MATP with C6S was clarified by ^{1}H-NMR and FT-IR spectroscopy. The gelation temperature of graft copolymers was determined by measuring the temperature at which immobility of the meniscus in each solution was first noted. Release behavior of ciprofloxacin from C6S-*g*-Poloxamer hydrogel in vitro was investigated as a function of C6S content in the graft copolymer by a spectrophotometric assay at 287 nm using an UV spectrophotometer. Differences in the adhesion and morphology of human lens cell between Poloxamer- and C6S-*g*-Poloxamer-coated surfaces were also investigated. The gelation temperatures of C6S-*g*-Poloxamer copolymers were lowered with increasing of the concentration of the copolymer and decreasing of C6S content. The release of ciprofloxacin from the graft copolymer was sustained compared with Poloxamer itself and decreased with increasing the content of C6S in the copolymer due to the in situ gel formation of the copolymer and viscous properties of C6S. Human lens cells (B3) adhered to C6S-*g*-Poloxamer-coated surface were observed as transformed shapes after 2 days. The bioadhesive and thermally gelling of these graft copolymers will be expected to be an excellent drug carrier for the prolonged delivery to surface of the eye.

KEYWORDS Chondroitin 6-sulfate, Poloxamer 407, Ophthalmic drug delivery, Mucoadhesive, In situ gel formation

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INTRODUCTION

Recently, in situ gel formation of polymers has extensively been studied to enhance ocular bioavailability and duration of the drug activity. Edsman et al. (1998) used Poloxamer 407 as an in situ gel to increase ocular residence time. Sechoy et al. (2000) reported that carteolol was slowly released in the rabbit eyes by using in situ gel formation of alginate in the eye. It was also reported that thermosensitive poly(*N*-isopropylacrylamide) hydrogel was potential in controlled release of antiglaucoma drugs (Hsiue et al., 2002). In situ polymeric gels are interesting because these are conveniently dropped as a solution into the conjunctival sac (Lee, 1990), where they undergo a transition into a gel with their favorable residence time. The sol–gel transition of polymers occurs as a result of a chemical/physical change induced by the physiological environment. Among them are Poloxamer 407 (Desai, 1998; Miller & Donovan, 1982) and tetronics (Spancake et al., 1991; Vadnere et al., 1984), whose solution viscosity increases on increasing the temperature to that of the eye.

Poloxamer 407, a nontoxic poly(ethylene oxide)/poly(propylene oxide)/poly(ethylene oxide) (PEO/PPO/PEO) triblock copolymer with a weight-average molecular weight of 12,600, contains 70% hydrophilic ethylene oxide units and 30% hydrophobic propylene oxide units. It has the ability to form a clear gel in aqueous media at a concentration of approximately 20% (w/w) or more and exhibits the unique property of reversible thermal gelation; this latter is achieved at a higher temperature (e.g., body temperature) and is reversible on cooling (e.g., at refrigerator temperature), thereby yielding a low viscosity solution (Cafaggi et al., 2005). As a result of this reverse thermal gelation and extremely low toxicity, the administered solution containing drug turns into a gel in situ and renders slow release characteristics to the drug delivery system in the pharmaceutical fields (Edsman et al., 1998; Katakam et al., 1997; Lin & Sung, 2000; Moghimi & Hunter, 2000).

Chondroitin 6-sulfate (C6S) is a naturally occurring polysaccharide composed of alternating units of β-1, 3-linked glucuronic acid, and (β-1,4) *N*-acetyl-galactosamine (GalNAc) and sulfated on 6-position of the GalNAc residues. The C6S serves critical roles such as a basement membrane component (MaCarthy et al., 1989), wound healing (Kirker et al., 2002), cell adhesion and cell proliferation (Wight et al., 1992), cell migration (Faassen et al., 1993), and modulation of growth factor activity (Ruoslahti & Yamaguchi, 1991). In addition, C6S plays an important role in corneal strength and transparency (Davies et al., 1999).

In a previous study, Poloxamer was grafted onto hyaluronic acid (HA), and the HA-g-Poloxamer formed a gel on warming to body temperature and was applied for ophthalmic drug delivery in vitro (Cho et al., 2003).

In this study, we report grafting of Poloxamer onto C6S for application of ophthalmic drug delivery system. Graft copolymer was prepared by coupling monoamine-terminated Poloxamer (MATP) backbone with C6S using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and *N*-hydroxylsuccinimide (NHS) as coupling agents. This C6S-g-Poloxamer hydrogel will be expected to slow down drug elimination by lacrimal flow, both by undergoing in situ gel formation and by interacting with the mucus.

MATERIALS AND METHODS

Materials

Poloxamer 407, EDC, and diaminoethylene were purchased from Aldrich Chemical Co. (Milwaukee, WI). NHS, 4-nitrophenyl chloroformate, and ciprofloxacin were provided by Sigma (St. Louis, MO). C6S (from shark cartilage, cat. no. 400675) was provided by Seikagaku Kogyo (Tokyo, Japan). All other chemicals were of extra pure reagent grade and were used as received.

Methods

Synthesis of MATP

MATP was prepared by two step reactions as shown in Fig. 1. In the first step, 0.40 mM of Poloxamer was reacted with 0.50 mM of 4-nitrophenyl chloroformate dissolved in methylene chloride in the presence of triethylamine at room temperature for 4 h to yield a 4-nitrophenyl formate-derivatized intermediate. This intermediate was recovered by extraction, three times using petroleum ether. In the second step, 0.34 mM of the intermediate was reacted with 1.02 mM of diaminoethylene dissolved in methylene chloride at room temperature overnight. After reaction, the mixture was extracted three times with petroleum

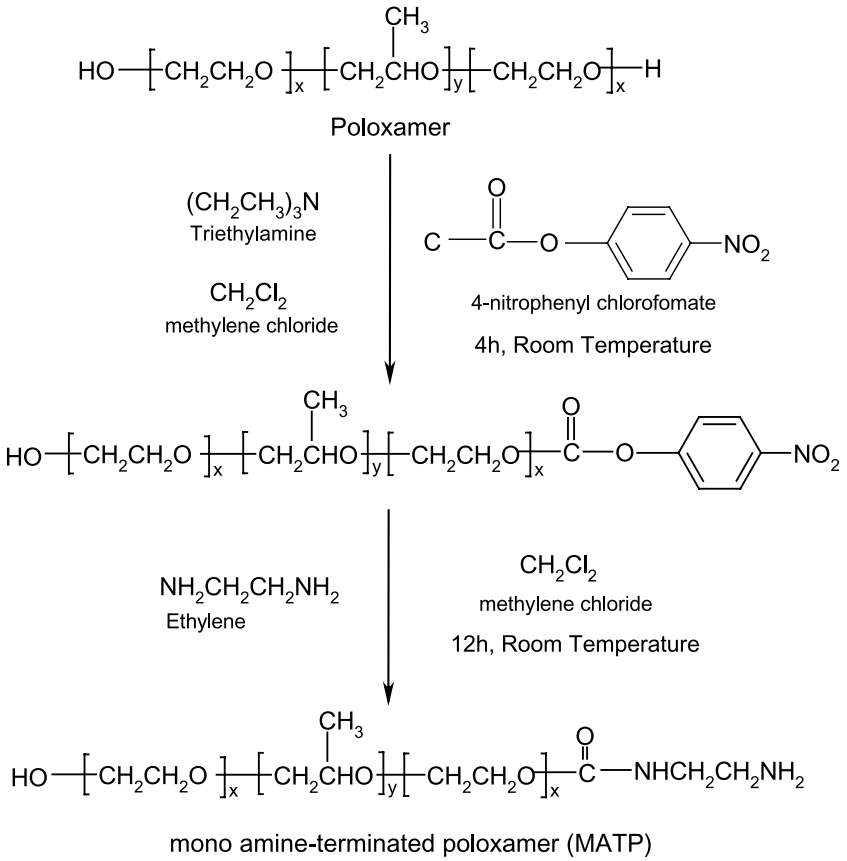

FIGURE 1 **Preparation of Amino-Terminated Poloxamer from Poloxamer.**

ether and then dialyzed against distilled water using a membrane with a molecular weight cutoff of 3500 for 3 days at room temperature, and then lyophilized to result in the product.

Grafting of Poloxamer onto C6S

C6S-*g*-Poloxamer was prepared by coupling MATP with the C6S (the molar ratio of C6S:MATP, 1:1 and 4:1) via an active ester intermediate using EDC. Briefly, 65.5 mg (0.159 mmol) of C6S was dissolved in 25 mL of distilled water, and then the carboxyl group of C6S was activated with a mixture of NHS (36.5 mg) and EDC (152 mg). EDC was fivefold molar excess over C6S and NHS/EDC molar ratio was 2:5. Subsequently, 2.0 g of MATP (0.159 mmol) was added into the activated C6S solution at an equivalent molar ratio to C6S.

The reaction was carried out at room temperature for 48 h. Specifically, reaction between the amine groups of Poloxamer and carboxyl ones of C6S in the presence of EDC resulted in amide bond formation as shown in Fig. 2. After reaction, the mixture was dialyzed against distilled water using a membrane with a molecular weight cutoff of 12,000–14,000 for 3 days at room temperature and then lyophilized to result in the product. Another graft copolymer that the molar ratio of C6S:MATP was adjusted at 4:1 was prepared by the same method above mentioned. That is, 262 mg (0.636 mmol) of C6S dissolved in 25 mL of distilled water was activated with a mixture of NHS (146 mg) and EDC (608 mg). Subsequently, 2.0 g of MATP (0.159 mmol) was added into the activated C6S solution and allowed to react with stirring for 48 h at room temperature.

^{1}H-NMR Spectroscopy Measurement

Proton nuclear magnetic resonance (^{1}H-NMR) spectra were measured at 25°C by using AVANCE 600 spectrometer operating at 600 MHz. ^{1}H-NMR spectra of graft copolymer were measured in D_2O to estimate the copolymer composition.

FT-IR Spectroscopy Measurement

The KBr pellets of C6S-*g*-Poloxamer and C6S were prepared, respectively. The infrared absorption (IR)

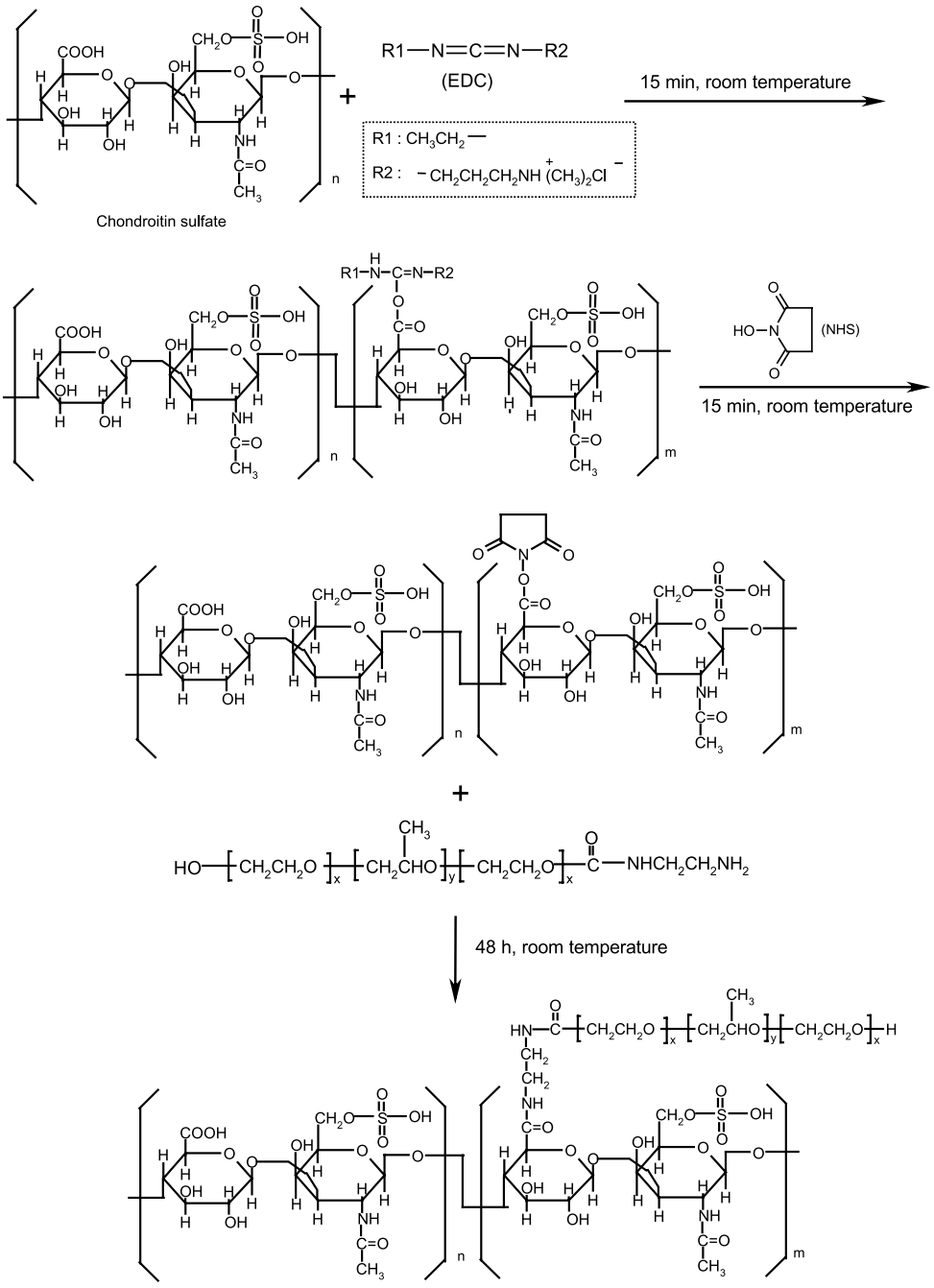

FIGURE 2 **Preparation of Poloxamer grafted onto Chondroitin 6-Sulfate (C6S-*g*-Poloxamer).**

spectra were recorded by using Midac M series FT-IR spectrometer (Costa Mesa, CA, USA).

Measurement of Gelation Temperature

A 5-mL transparent vial containing 1 mL of aqueous Poloxamer or graft copolymer solution at the concentration of 18, 19, and 20 wt % was placed in a water bath and heated at a rate of 0.5 °C min^{-1}. Gel formation was indicated by a lack of movement of the meniscus on tilting the vial; the temperature at which immobility of the meniscus in each vial was first noted was taken to be the sol–gel transition temperature at that concentration (Miyazaki et al., 1998).

In Vitro Release Studies

In vitro drug release from graft copolymer was carried out by filling 1 mL of ciprofloxacin (1.75 wt %)-loaded graft copolymer solution (20 wt %) into cylindrical vial in triplicate. The above vials were placed

for gelation at 34°C in an incubator. The vials were then filled with 1 mL of simulated artificial tear fluid. The tear fluid consisted of 0.670 g sodium chloride, 0.200 g sodium bicarbonate, and 0.008 g calcium chloride·$2H_2O$ in 100 mL of distilled water (Rediske et al., 2002). The temperature and stirring rate were maintained at 34°C and 50 rpm, respectively. Aliquots (1 mL) were withdrawn from the release medium under sink condition and replaced by an equal volume at each sampling time. The aliquot was diluted with the simulated tear fluid, and the amount of drug released was determined by a spectrophotometric assay at 287 nm by using an UV spectrophotometer.

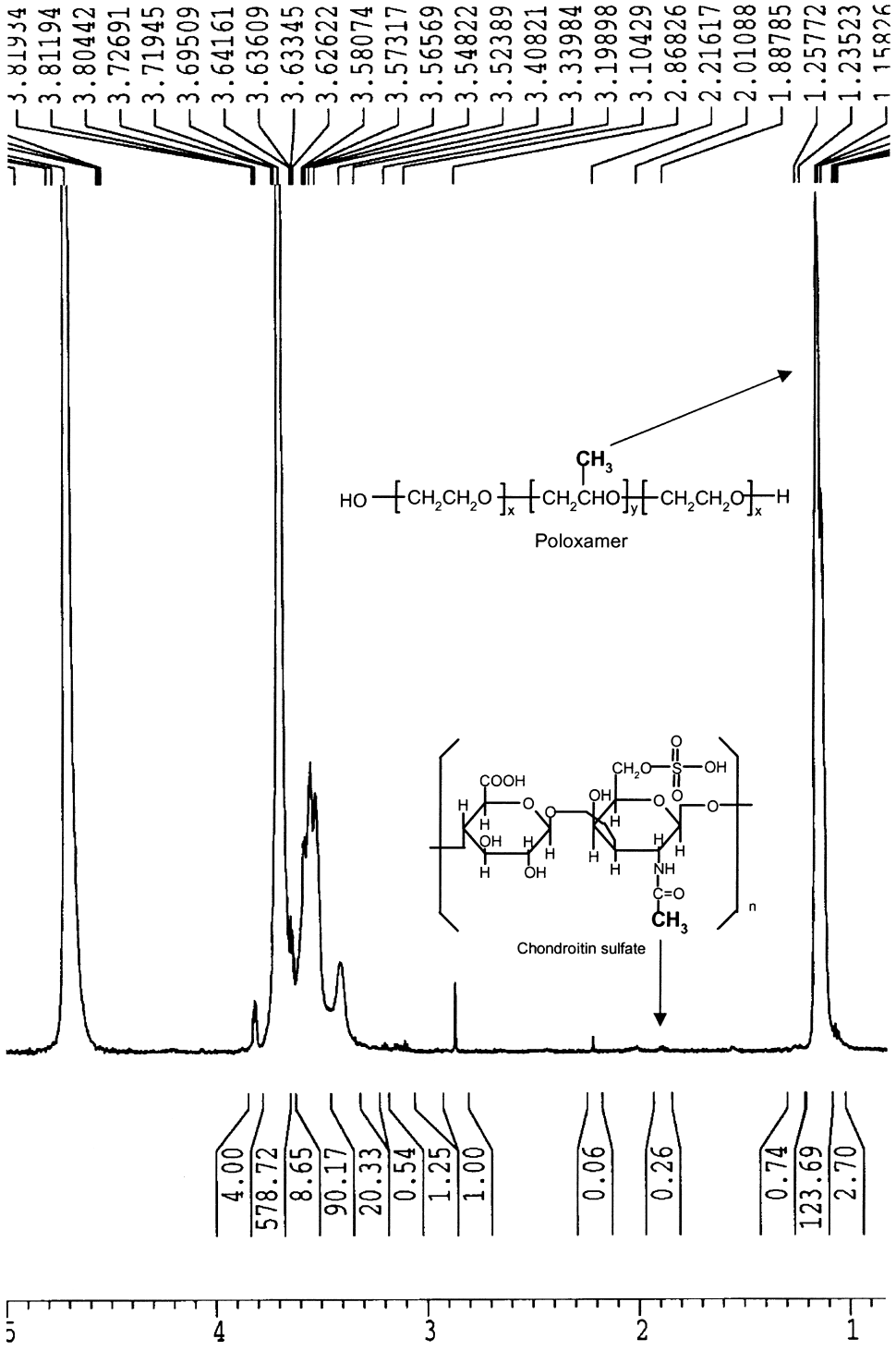

FIGURE 3 ^{1}H-NMR spectrum of Poloxamer grafted onto Chondroitin 6-Sulfate (C6S-g-Poloxamer).

Human Lens Cell (B3) Attachment

Human lens cells (B3) isolated from human eyeball were seeded in 24-well polystylene (PS) dish coated with C6S-*g*-Poloxamer at cell density of 1×10^6 cells/well. The cells were cultured with MEM medium supplemented with 20% FBS and gentamycin (50 μg/mL) at 37°C in a humidified air/CO_2 incubator (95/5 vol %).

RESULTS AND DISCUSSION

^{1}H-NMR Spectra of Graft Copolymer

Graft copolymer was prepared by coupling of MATP with C6S back bone at room temperature for 48 h by EDC/NHS method previously reported (Chung et al., 2002), and its composition was clarified by ^{1}H-NMR spectroscopy. Figure 3 shows ^{1}H-NMR spectrum of graft copolymer that the feed molar ratio of C6S to Poloxamer is equivalent. A typical signal of methyl groups (CH_3) of polypropylene oxide in Poloxamer was detected at 1.2 ppm. An apparent *N*-acetylate proton peak ($NCOCH_3$) in C6S was detected at 1.9 ppm. The other proton peaks that ranged from 3.5 to 4.0 ppm were undistinguished by the ^{1}H-NMR spectroscopy, because of the overlap of glucose unit peak of C6S and methylene peak of Poloxamer. Therefore, the compositon of graft copolymer was calculated by the integration of a methyl group peak of Poloxamer and *N*-acetylate proton one of C6S. From the estimation of NMR measurement, 0.517 and 2.42 wt % of C6S was grafted to Poloxamer, respectively. Unreacted Poloxamer was removed after dissolving reacted mixture in dichloromethane.

FTIR Spectroscopy

The amide bond formation resulted from the reaction between amine groups of the MATP, and carboxyl groups of C6S were clarified by FTIR spectroscopy. Figure 4 shows FTIR spectra of C6S and graft copolymer. The carbonyl absorption band of carboxylic acid in C6S of graft copolymer became weak, and the new characteristic amide I band appeared at 1639 cm^{-1}, whereas C6S exhibited the characteristic absorption at 1608 cm^{-1}, which is asymmetric carbonyl stretching vibration. The result suggested that the amide bonds between carboxylic groups of C6S and amine ones of MATP were formed.

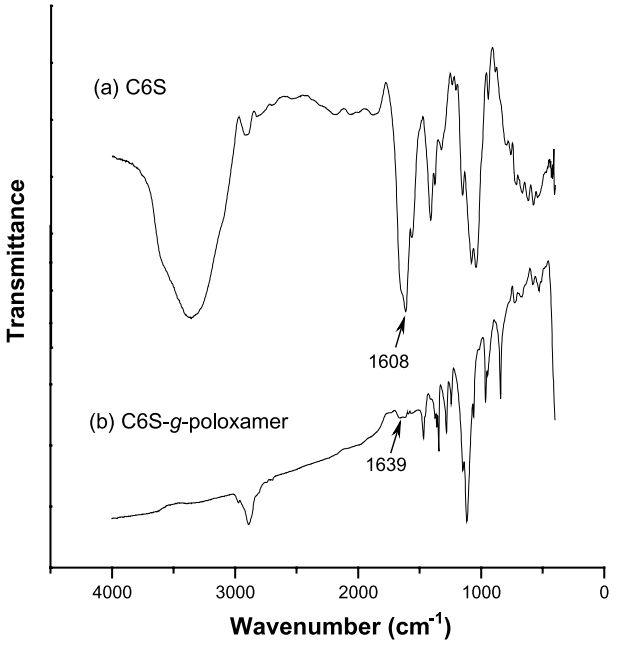

FIGURE 4 **FTIR spectra of Chondroitin 6-Sulfate (C6S) (a) and Poloxamer grafted onto Chondroitin 6-Sulfate (C6S-*g*-Poloxamer) (b).**

Gelation Temperature

The gelation temperature of graft copolymers is shown in Fig. 5. The change of gelation temperature was dependent on the content of C6S in the graft copolymer and the concentration of the graft copolymer solution. The more the content of C6S and ciprofloxacin, the higher was the gelation temperature. This result suggested that hydrophilic properties of C6S and ciprofloxacin interfered with the hydrophobic interaction of Poloxamer. In addition, sol–gel transition of graft copolymer did not occur if C6S was over 10 wt % in the copolymer owing to the increased hydrophilic property of C6S. Furthermore, gelation temperature of C6S-*g*-Poloxamer at 20 wt % concentration was higher than that of HA-*g*-Poloxamer because of the more hydrophilic property of C6S than HA, in condition that the grafted content of C6S or HA in each copolymer was almost equivalent (Cho et al., 2003).

In Vitro Release Studies

The cumulative percent of ciprofloxacin released from the copolymer gel as a function of time is shown in Fig. 6. For the drug containing Poloxamer as shown in the (a) curve of Fig. 6, almost all ciprofloxacin was released after 4 h, indicating that the drug-loaded

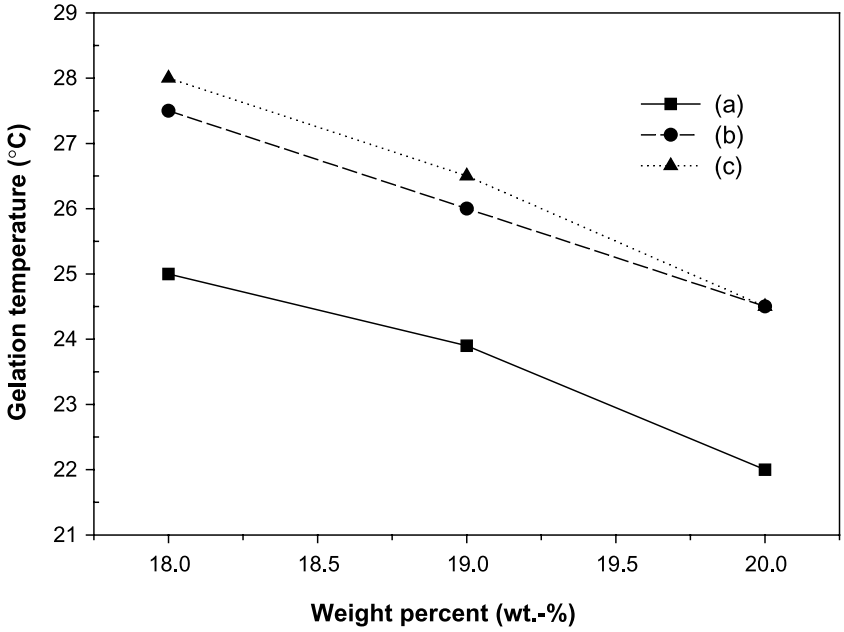

FIGURE 5 **The gelation temperature of Poloxamer (a), Chondroitin Sulfate-*g*-Poloxamer (Chondroitin Sulfate, 0.517 wt %) (b), and Chondroitin Sulfate-*g*-Poloxamer (Chondroitin Sulfate, 2.42 wt %) (c) by vertical method. Bars represent mean ± standard deviations for n = 3.**

Poloxamer is in the sol-state within 4 h. On the other hand, for the ciprofloxacin-loaded graft copolymer (C6S, 2.42 wt %), the drug was released about 80% after 12 h as shown in the (c) curve of Fig. 6. The results suggested that the release of ciprofloxacin from the graft copolymer was sustained, compared with Poloxamer itself, and decreased with increasing the content of C6S in the copolymer due to the mucoadhesive property (Hurst et al., 1987; Saettone et al., 1994; Yamamoto et al., 2004) and high viscosity of C6S (η_{rel}: C6S; 2.77 and Poloxamer: 1.58). Although in case the content of C6S in graft

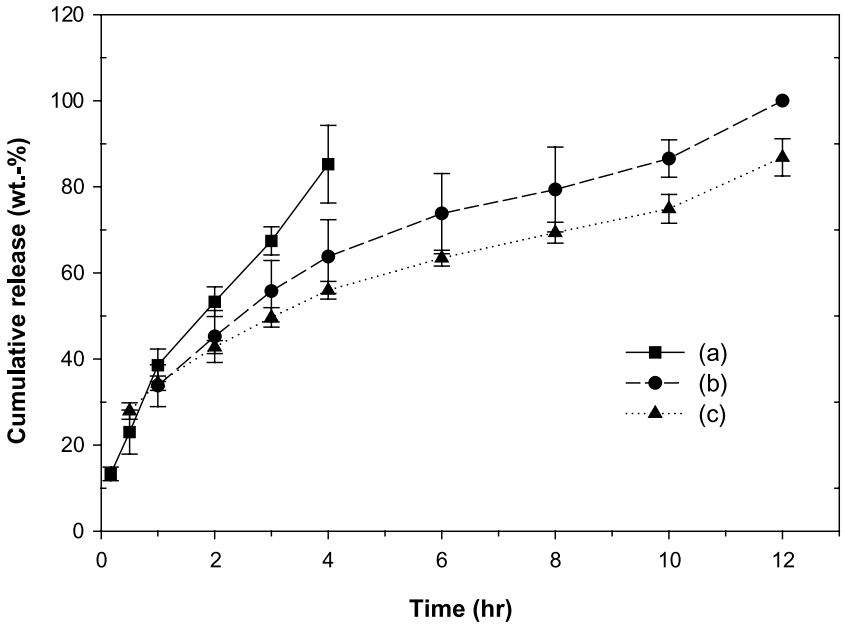

FIGURE 6 **Release of Ciprofloxacin from Poloxamer (a), Chondroitin Sulfate-*g*-Poloxamer (Chondroitin Sulfate, 0.517 wt %) (b), and Chondroitin Sulfate-*g*-Poloxamer (Chondroitin Sulfate, 2.42 wt %) (c) with 1.75 wt % Ciprofloxacin in vitro. Bars represent mean ± standard deviations for n = 3.**

copolymer increased in more than limited amount (10 wt %), a reversible sol–gel transition was not undergone. This result indicated that a relative increase of hydrophilicity of C6S prevents the hydrophobic interaction of polypropylene oxide in Poloxamer, leading to no gelation of graft copolymer. However, the drug release from C6S alone in vitro could not be performed because of the nonoccurrence of sol–gel transition of C6S itself.

Attachment of Human Lens Cells (B3)

Figures 7a, b, and c show the phase-contrast micrographs of human lens cells (B3) adhered to PS dish, Poloxamer-, and C6S-*g*-Poloxamer-coated surfaces after 0 h, 1 day, and 2 days, respectively. The micrographs showed that more cells were adhered to C6S-*g*-Poloxamer-coated surface than Poloxamer-coated one after 1 day and cells were still adhered to C6S-*g*-Poloxamer-coated surface after 2 days, although almost cells were detached from Poloxamer-coated one (data not shown). Cells adhered to C6S-*g*-Poloxamer-coated surface were observed as spherical shapes and transformed ones with colony formation after 1 day and 2 days, respectively, whereas the adhered cells to PS dish were observed as spreading shapes after 2 days. It is thought that the morphological differences of adhered cells between C6S-*g*-Poloxamer-coated surface and Poloxamer-coated surface may relate to specific

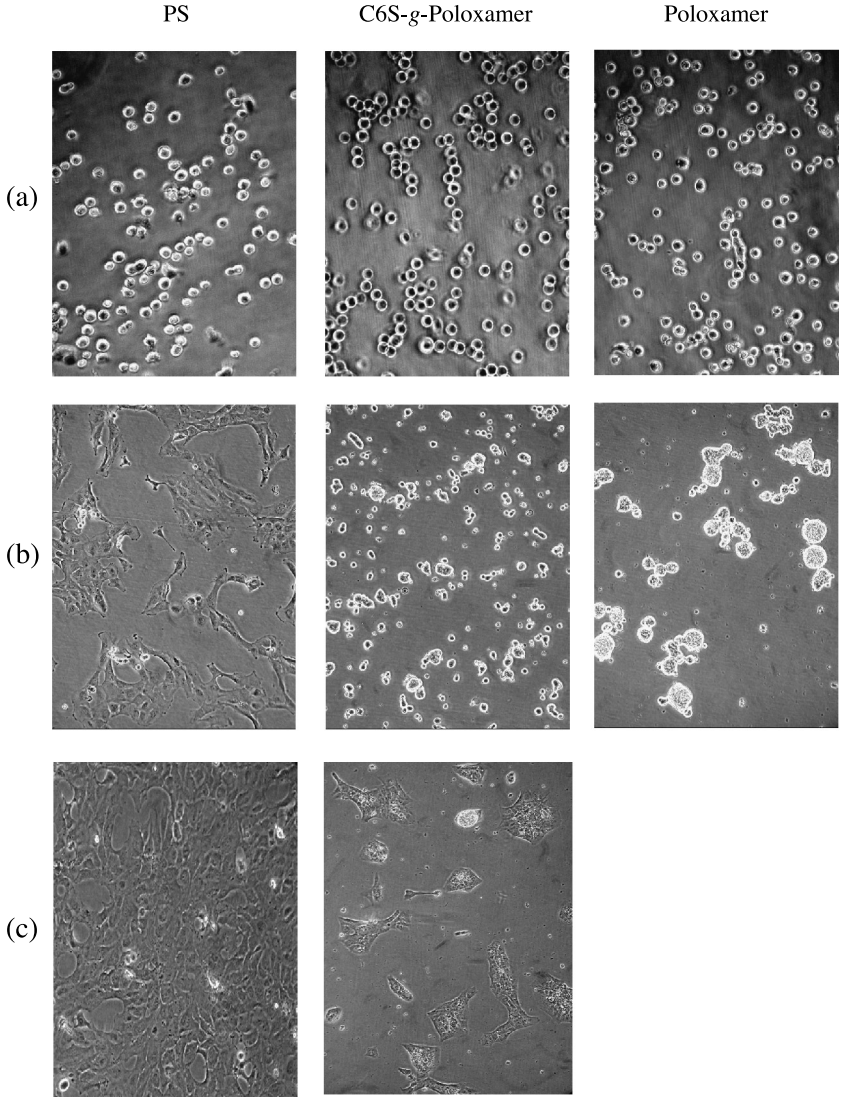

FIGURE 7 **Phase-contrast microphotographs of human lens cells (B3) adhered to Poloxamer-coated surface and C6S-*g*-Poloxamer-coated surface: (a) 0 h, (b) 1 day, and (c) 2 days.**

interaction between C6S and human lens cells (B3), although the exact mechanism should be clarified in future.

CONCLUSIONS

The gelation temperature of C6S-*g*-Poloxamer copolymer was dependent on the concentration of the graft copolymer and the content of C6S. Ciprofloxacin release behavior in vitro, as well as the adhesion and morphology of Human lens cells (B3), was affected by the introduction of C6S into Poloxamer. The results of this study indicate that the bioadhesive and thermally gelling of these graft copolymers will be expected to be an excellent drug carrier for the prolonged delivery to surface of the eye.

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REFERENCES

Cafaggi, S., Leardi, R., Parodi, B., Caviglioli, G., Russo, E., & Bignardi, G. (2005). Preparation and evaluation of a chitosan salt–Poloxamer 407 based matrix for buccal drug delivery. *Journal of Controlled Release*, *102*, 159–169.

Cho, K. Y., Chung, T. W., Kim, B. C., Kim, M. K., Lee, J. H., Wee, W. R., & Cho, C. S. (2003). Release of ciprofloxacin from Poloxamer-graft-hyaluronic acid hydrogels in vitro. *International Journal of Pharmaceutics*, *260*, 83–91.

Chung, T. W., Yang, J., Akaike, T., Cho, K. Y., Nah, J. W., Kim, S. I., & Cho, C. S. (2002). Preparation of alginate/galactosylated chitosan scaffold for hepatocyte attachment. *Biomaterials*, *23*, 2827–2834.

Davies, Y., Lewis, D., Fullwood, N. J., Nieduszynski, I. A., Marcyniuk, B. M., Albon, J., & Tullo, A. (1999). Proteoglycans on normal and migrating human corneal endothelium. *Experimental Eye Research*, *68*, 303–311.

Desai, S. D. (1998). In vitro evaluation of pluronic F127-based controlled-release ocular delivery systems for pilocarpine. *Journal of Pharmaceutical Sciences*, *87*, 226–230.

Edsman, K., Carlfors, J., & Petersson, R. (1998). Rheological evaluation of Poloxamer as an in situ gel for ophthalmic use. *European Journal of Pharmaceutical Sciences*, *6*, 105–112.

Faassen, A. E., Mooradian, D. L., Tranquillo, R. T., Dickinson, R. B., Letourneau, P. C., Oegema, T. R., & McCarthy, J. B. (1993). Cell surface CD44-related chondroitin sulfate proteoglycan is required for transforming growth factor-β-stimulated mouse melanoma cell motility and invasive behavior on type I collagen. *Journal of Cell Science*, *105*, 501–511.

Hsiue, G. H., Hsu, S. H., Yang, C. C., Lee, S. H., & Yang, I. K. (2002). Preparation of controlled release ophthalmic drops, for glaucoma therapy using thermosensitive poly-*N*-isopropylacrylamide. *Biomaterials*, *23*, 457–462.

Hurst, R. E., Rhodes, S. W., Adamson, P. B., Parsons, C. L., & Roy, J. B. (1987). Functional and structural characteristics of the glycosaminoglycans of the bladder luminal surface. *Journal of Urology*, *138*, 433–437.

Katakam, M., Ravis, W. R., & Banga, A. K. (1997). Controlled release of human growth hormone in rats following parenteral administration of Poloxamer gels. *Journal of Controlled Release*, *49*, 21–26.

Kirker, K. R., Luo, Y., Nielson, J. H., Shelby, J., & Prestwich, G. D. (2002). Glycosaminoglycan hydrogel films as bio-interactive dressings for wound healing. *Biomaterials*, *23*, 3661–3671.

Lee, V. H. (1990). New directions in the optimization of ocular drug delivery. *Journal of Ocular Pharmacology*, *6*, 157–164.

Lin, H. R., & Sung, K. C. (2000). Carbopol/pluronic phase change solutions for ophthalmic drug delivery. *Journal of Controlled Release*, *69*, 379–388.

MaCarthy, K. J., Accavitti, M. A., & Couchman, J. R. (1989). Immunological characterization of a basement membrane-specific chondroitin sulfate protegoglycan. *Journal of Cell Biology*, *109*, 3187–3198.

Miller, S. C., & Donovan, M. D. (1982). Effect of Poloxamer 407 gel on the miotic activity of pilocarpine nitrate in rabbits. *International Journal of Pharmaceutics*, *12*, 147–152.

Miyazaki, S., Suisha, F., Kawasaki, N., Shirakawa, M., Yamatoya, K., & Attwood, D. (1998). Thermally reversible xyloglucan gels as vehicles for rectal drug delivery. *Journal of Controlled Release*, *56*, 75–83.

Moghimi, S. M., & Hunter, A. C. (2000). Poloxamers and poloxamines in nanoparticle engineering and experimental medicine. *TIBTECH*, *18*, 412–420.

Rediske, A. M., Koenig, A. L., Barekzi, N., Ameen, L. C., Slunt, J. B., & Grainger, D. W. (2002). Polyclonal human antibodies reduce bacterial attachment to soft contact lens and corneal cell surfaces. *Biomaterials*, *23*, 4565–4572.

Ruoslahti, E., & Yamaguchi, Y. (1991). Proteoglycans as modulators of growth factor activities. *Cell*, *64*, 867–869.

Saettone, M. F., Monti, D., Torracca, M. T., & Cheton, P. (1994). Muco-adhesive ophthalmic vehicles: evaluation of polymeric low-viscosity formulations. *Journal of Ocular Pharmacology*, *10*, 83–92.

Sechoy, O., Tissie, G., Sebastian, C., Maurin, F., Driot, J. Y., & Trinquand, C. (2000). A new long acting ophthalmic formulation of carteolol containing alginic acid. *International Journal of Pharmaceutics*, *207*, 109–116.

Spancake, C. W., Mitra, A. K., & Kildsig, D. O. (1991). Kinetics of aspirin hydrolysis in agueous solutions and gels of poloxamines (Tetronic 1508). Influence of microenvironment. *International Journal of Pharmaceutics*, *75*, 231–239.

Vadnere, M., Amidon, G., Lindenbaum, S., & Haslam, J. L. (1984). Thermodynamic studies on the gel–sol transition of some pluronic polyols. *International Journal of Pharmaceutics*, *22*, 207–218.

Wight, T. N., Kinsella, M. G., & Qwarnstrom, E. E. (1992). The role of proteoglycans in cell adhesion migration and proliferation. *Current Opinion in Biology*, *4*, 793–801.

Yamamoto, A., Yamada, K., Muramatsu, H., Nishinaka, A., Okumura, S., Okada, N., Fujita, T., & Muranishi, S. (2004). Control of pulmonary absorption of water-soluble compounds by various viscous vehicles. *International Journal of Pharmaceutics*, *282*, 141–149.