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ScienceDirect
International Journal of Pharmaceutics 346 (2008) 57–63
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Controlled dual release of basic fibroblast growth factor and indomethacin from heparin-conjugated polymeric micelle

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Received 2 March 2007; received in revised form 20 April 2007; accepted 4 June 2007
Available online 22 June 2007

Abstract

This work describes the development of heparinized polymeric micelle as a novel injectable carrier for the dual drug delivery that can simultaneously release basic fibroblast growth factor (bFGF) and indomethacin (IMC), which can promote the regeneration of damaged tissue and prevent the inflammatory response after implantation. Tetronic®–PCL–heparin for the preparation of heparinized polymeric micelle was synthesized by introducing PCL as a biodegradable linkage on Tetronic, following the conjugation of heparin. The mean diameter of the formed TCH micelle was around 114 nm and increases in the micelle size after single and dual drug loading were observed. Loading efficiencies of IMC and bFGF were 30.9% and 70.5%, respectively. *In vitro* dual drug release profiles from TCH micelles were investigated. IMC was more slowly released from dual drug-loaded micelle over 3 weeks as compared with single drug-loaded one. bFGF was released over 2 months in a controlled manner. Therefore, the release profile results support that TCH micelle could not only incorporate a hydrophobic drug into the core but also bind with bFGF to heparin that exists on its outer shell. The TCH micelle will have enhanced therapeutic effects on the target site which may be required the multi-function of drugs to use.
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Keywords: Polymeric micelle; Heparin; Dual drug release; Growth factors; Tissue engineering

1. Introduction

Recently, tissue engineering has been attracted to many scientists and surgeons as a therapeutic method to treat patients with having maximum healing effects and minimum inflammatory responses (Vacanti and Langer, 1999; Tabata, 2000, 2001). To advance tissue engineering, they has developed many technologies such as the isolation of specific cells through a small biopsy, culturing them on many scaffolds under precisely controlled culture conditions, delivering the cell-seeded scaffold to the desired site in a patient's body, and so on (Lee et al., 2001; Shin et al., 2003). In particular, successful regeneration of damaged organs or tissues based on tissue engineering often require various growth factors to modulate cellular activities because a large amount of biosignal should be transported from cell to cell and extra-cellular matrix (ECM) (Lee and Mooney, 2001; Ungaro et al. (2005)). The ground of this approach is that growth factors and their specific cell surface receptors consist of a large and complex family of signaling molecules that have been considered to play an important role in a variety of processes of embryonic development and tissue homeostasis (Dailey et al., 2005; Goldfarb, 1996; Martin, 1998; Muenke and Schell, 1995; Naski and Ornitz, 1998; Ornitz and Marie, 2002; Muenke and Wilkie (2000)).

Over the past few decades, several technologies have been developed to deliver growth factors by incorporating them into matrices (Wissink et al., 2001; Obara et al., 2003; Andreopoulos and Persaud, 2006), microparticles (Suetomi et al., 2005; Perets et al., 2003), and so on. However, bioavailability of growth

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0378-5173/$ – see front matter © 2007 Published by Elsevier B.V.
doi:10.1016/j.ijpharm.2007.06.025

factors is generally poor, since they are poorly absorbed and have a short half-life due to the enzymatic degradation and self-aggregation (Edelman et al., 1993). Also, site localization and sustained release of growth factors are very difficult, since unformulated growth factors rapidly diffuse away from the site of injection or transplantation (Kawai et al., 2000). Furthermore, even now there are many problems such as inflammation and side effects on target site, resulting in alternatively transplanting cultivated tissues and treating biomaterials with signaling proteins. Therefore, various therapeutic functions, such as reduction of side effects, growth factor delivery, convenient mode of administration, and anti-inflammatory activity, are required for tissue regeneration.

Polymeric micelles (PMs), supramolecular assemblies of block copolymers, have been receiving intrinsic attention as colloidal carrier systems for delivery of poor absorbable hydrophobic drugs and proteins (Jones and Leroux, 1999; Kakizawa and Kataoka, 2002). The basic mechanism underlying process of micellization in aqueous media is depicted by the core segregation from the outer environment by a palisade-like hydrophilic segment based on hydrophobic driving force of the spontaneous micelle formation (Kwon and Okano, 1996). Generally, PMs consist of the segregated shell embedded in the hydrophilic palisade and the hydrophobic core that pays an important role in container of hydrophobic drugs such as antiphlogistic drug and anticancer drug (Kataoka et al., 2001; Liu et al., 2000; Riess, 2003). At recent years, copolymer designs by many combinations between hydrophilic and hydrophobic polymers were developed to give multi-functionality to PMs.

Heparin has representative antithromgenicity as well as specific binding affinity with some proteins such as growth factors and cytokines, thereby playing an essential role in the regulation of various biological signaling (Cardin and Weintraub, 1989; Capila and Linhardt, 2002). Based on such specific interactions, the chemical tailoring of heparin to amphiphilic copolymers can be utilized as smart functional moiety for the relevant pharmacokinetics and biological maintenance of growth factor delivery.

In the current study, we introduce a novel heparin-conjugated micelle system as an injectable carrier for simultaneous delivery of bFGF as a growth factor and indomethacin (IMC) as a hydrophobic drug. The heparin-conjugated micelle was formed from a Tetronic®–PCL–heparin block copolymer. In the structure of the copolymer, Tetronic® segment allow the micelle to exhibit thermo-sensitivity for injectable application and PCL block gives elevated stability of the micelle due to the formation of semi-crystalline phase and biodegradability. Particularly, heparin conjugation to Tetronic®–PCL copolymer was designed to control the release behavior of bFGF through specific affinity between heparin and bFGF. After the formation of micelle, the micellar properties were characterized and *in vitro* release studies were carried out with single and dual drug-loaded micelles.

2. Materials and methods

2.1. Materials

Tetronic® 1307 was purchased from BASF (BASF, Korea). ε-Caprolactone, stannous octoate (Sn-Oct), succinic anhydride (SA), 4-dimethylaminopyridine (DMAP), triethylamine (TEA), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), and indomethacin (IMC) were provided from Aldrich Chemical Co. Poly(ethylene glycol) was provided from Polyscience Inc. (M_W = 3.4 kDa, pharmaceutical grade). Heparin sodium salt (from porcine intestinal mucosa, 140 units/mg) was supplied from Acros Co. Recombinant human fibroblast growth factor-basic (bFGF) was supplied from CytoLab Ltd. FGF-2 (147) and goat anti-rabbit IgG-FITC was provided from Santacruz Biotechnology Inc. Other chemicals were used as received without further purification process.

2.2. Synthesis and characterization of Tetronic®–PCL–heparin (TCH) copolymer

Tetronic®–PCL (TC) copolymer was synthesized by bulk ring-opening polymerization of ε-caprolactone with Tetronic® 1307 (Fig. 1) as a four-arm initiator and Sn-Oct as a catalyst (Choi et al., 1998). To carboxylate the end groups of TC, TC copolymer, SA, DMAP, and TEA were dissolved in anhydrous dioxane and stirred for 24 h at room temperature (Lee et al., 2001). For the synthesis of TCH (Fig. 1), heparin was conjugated to TC copolymer by using EDC/NHS methods (Chung et al., 2005). The structure of TC and TCH were characterized by FT-IR and ^{1}H NMR. Molecular weight of TC was determined by GPC. The content of conjugated heparin was analyzed by the toluidine blue colorimetric method.

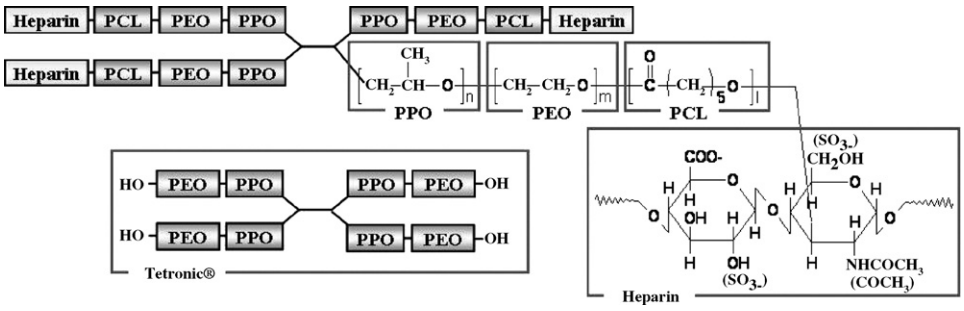

Fig. 1. Chemical structures of Tetronic® and Tetronic®–PCL–heparin (n = 20, m = 62, l = 5).

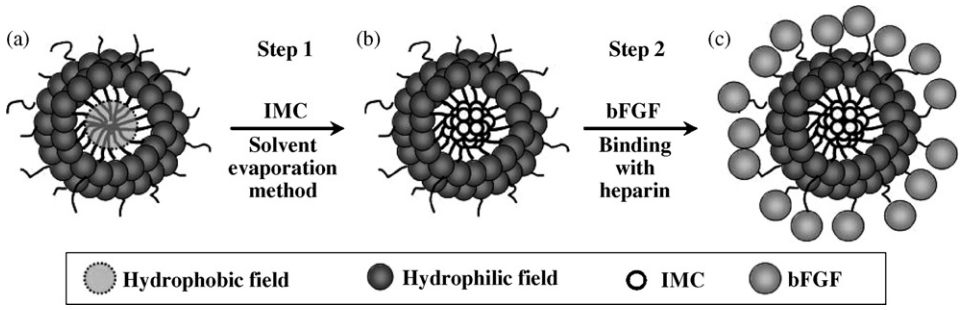

Fig. 2. Schematic representation for preparation procedure of dual drug-loaded Tetronic®–PCL–heparin.

2.3. Preparation of dual drugs-loaded TCH micelles

The TCH micelle containing IMC was prepared by the single emulsion and solvent evaporation method as follows (Fig. 2; (a) → (b)): the TCH copolymer (20 mg/H_2O 1 ml) and IMC (20 mg/methanol 1 ml) were stirred at 37 °C for 24 h for incorporation of IMC into the micelle core. The solution was then incubated overnight in the dry oven at 50 °C, allowing slow evaporation of organic solvent. The residual IMC was completely removed by centrifugation (GS-6R centrifuge (BECKMAN, USA)) at 1000 rpm for 10 min. The separated micelle solution was sonicated by BRANSON 5510 sonicator (Branson ultrasonics, USA) for 30 min, followed by lyophilizing.

The bFGF-loaded TCH micelle was also prepared by the single emulsion and solvent evaporation method (Fig. 2; (a) → (c)). To briefly describe, TCH solution and bFGF (400 ng/ml) were stirred at 37 °C for 24 h, allowing interactions between immobilized heparin and bFGF. The unbound bFGF was removed using salting-out salt methods by adding 10 wt.% PEO 0.1 ml (Ghosh, 2004; Aruna and Lali, 2001). bFGF-loaded micelle solution was sonicated for 30 min and finally lyophilized to give the resultant. The formation of dual drug-loaded TCH micelles was performed through two steps containing above-mentioned methods as described at Fig. 2; (a) → (b) → (c).

2.4. Critical micelle concentration (CMC) and size distribution of TCH micelles

In order to determine the CMC of TCH micelles in distilled water, fluorescence measurement was carried out using pyrene as a probe (Wilhelm et al., 1991). TCH micelle solutions were made with different concentrations at the range of 10^{-6} to 10^{2} mg/ml. Fluorescence excitation spectra were obtained with emission wavelengths of 390 nm as a function of the concentration of TCH micelles with spectrofluorometer (JASCO FP-6500, Japan). Excitation and emission bandwidths were 3.0 and 1.5 nm, respectively.

The mean diameter and size distribution of micelle were measured by dynamic light scattering (DLS) using an argon ion laser system (Brookhaven Instruments BI200SM, UK) set at a wavelength of 633 nm as a laser source. The sample solutions passing through a 0.45 μm filter were transferred into the light scattering cells. The intensity autocorrelation was measured at a scattering angle 90° with a Brookhaven BI-9000AT autocorrelator at 25 °C. The correlation function was accepted when the difference between the measured and the calculated baseline was less than 0.1%. CONTIN algorithms were used in the Laplace inversion of the autocorrelation function to obtain the size distribution of the micelle (Antony et al., 1998).

2.5. FITC labeling on bFGF for confocal observation

bFGF was incubated with FGF-2 (147) as a primary antibody at 37 °C for 60 min. The optimal range of antibody concentration was 0.5–5.0 μg/ml in PBS with 1.5% normal blocking serum. And, the resultant was incubated for 45 min with either biotin-conjugated or fluorochrome-conjugated secondary antibody diluted to 1–5 μg/ml in PBS with 1.5–3% normal blocking serum in a dark chamber.

FITC–bFGF solution (5 ml) and TCH solution (20 mg/ml, 5 ml) were mixed and incubated at 37 °C for 24 h. Prepared samples were observed using a LSM 510 confocal microscope (Carl Zeiss, Germany). This microscope was attached to Zeiss Axiovert 100M Inverted Microscope for Nomarski DIC & Epi-Fluorescence equipped with a 15 mW krypton/argon laser and LP 505 FITC filter sets. The samples were observed with c-apochromat 63.

2.6. In vitro release study

The amount of IMC in the core of TCH micelle was determined with a UV–vis spectrophotometer (JASCO V-750, Japan). To evaluate the loading efficiency (LE) of drugs, freeze-dried micelles were disrupted by an addition of ethanol/THF (1:1, v/v). Then, loading amount of IMC was determined by measuring the UV absorbance at 318 nm. The LE of IMC was calculated by the weight ratio of IMC to pre-weighed IMC-loaded micelles. bFGF quantitative assay was carried out using human FGF basic immunoassay kit (R&D systems) and a visible plate reader (ELISA, Biotrak, UK) by measuring the UV absorbance at the range of 450–540 nm.

In vitro release kinetics of IMC and bFGF from the TCH micelle was performed using previously dried samples (20 mg). The micelle solution (2 wt.%) was placed into a dialysis bag, and then immersed into a large vial containing 49 ml of heated PBS (pH 7.4, 37 °C). At appropriate time intervals, 5 ml of the released medium was collected from each sample and they were replaced by fresh medium.

Table 1
Average diameters of Tetronic® derivative micelles with or without drugs

Drug loading	Diameter[a] (nm)		
	Tetronic®	TC	TCH
No drug	16.9 ± 0.2	25.3 ± 0.5	114.1 ± 0.6
IMC	115.6 ± 0.3	138.6 ± 0.9	211.3 ± 0.7
bFGF	–	54.2 ± 0.2	193.2 ± 0.3
IMC + bFGF	–	152.1 ± 0.3	236.6 ± 0.8

[a] Diameter data was determined by DLS measurements ($n = 3$, mean ± S.D.).

3. Results and discussion

3.1. Structural characterizations and physical properties of the TCH micelle

TC and TCH copolymers were synthesized and characterized according to our previous reports, confirming their chemical structures, molecular weights, and CMCs in accordance with those of the reports (Lee et al., 2007a,b).

The size of micelles for each obtained derivative was observed at various manners of drug loading as presented in Table 1. Micelles showed narrow and unimodal distributions in all samples. In all manners of drug loading, increases in the diameter of micelles were observed as increasing in the length of copolymers. This result indicates that copolymers were assembled to form spherical micelles, which also means the chain length of copolymers determines the diameter of micelles. These elucidations are supported by great increases in the diameter of TCH micelles due to the conjugation of heparin with much higher molecular weight than PCL. Micelles also showed various sizes by varying the loading manner of drugs. Addition of drugs to micelles remarkably increased the diameter of the micelles. As shown in Fig. 2, IMC is incorporated into the hydrophobic core of micelles, whereas bFGF is bound to heparin on the outer surface of micelles. Therefore, it can be inferred that loading of both drugs induced structural expansion of micelles. IMC loading to micelles resulted in significant increases in the diameter of micelles, indicating efficient encapsulation of IMC into the core of micelles. The increased diameter for all micelles was almost same as roughly 100 nm. This result convinces that IMC encapsulation is related with the Tetronic® segment of copolymers. bFGF loading to micelles showed different manner from IMC loading about changes in the diameter of micelles. After loading bFGF, TCH micelle showed drastic increase in diameter, whereas TC micelle revealed little change in diameter. This result is associated with the presence of heparin segment in copolymer. The expansion of TCH micelle resulted from selective interaction of bFGF with heparin, while that of TC micelles seems to be derived from non-specific interactions between bFGF and micelles. In addition, simultaneous loading of two drugs exhibited increased sizes of micelles as compared with loading of each drug.

As shown in Fig. 3, confocal image of TCH micelles revealed round shape and the size distribution with the diameter range of 150–200 nm, which is consistent with DLS data (Table 1) (Lee et al., 2007a,b). This observation indicates that well-dispersed TCH micelles that combined with FITC-labeled bFGF are confirmed from a number of fluorescence spots. This result demonstrates that conjugated heparin of TCH micelle can specifically bind with bFGF and TCH copolymer was assembled to form TCH micelle with expected size and shape.

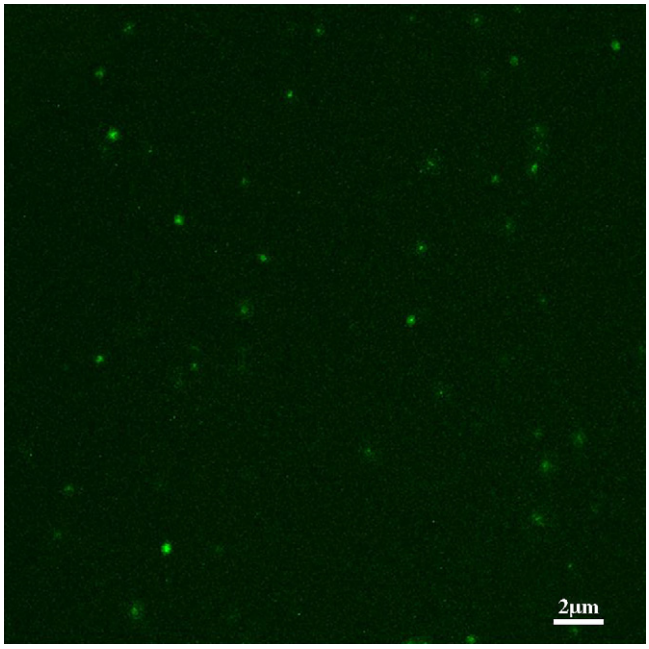

Fig. 3. Confocal image of Tetronic®–PCL–heparin micelle. Panel shows spherical aggregates with less than 200 nm diameter (scale bar = 2 μm).

3.2. Drug loading efficiency of micelles

The loading efficiency (LE) of drugs was investigated in various loading manners of drugs to each micelle as presented in Table 2. The LE of IMC was slightly enhanced when PCL block was conjugated with Tetronic. IMC LEs of TC and TCH were higher than the Tetronic® which did not have the additional hydrophobic moiety. This means that PCL segment also contributed to encapsulate IMC into the micelle. The conjugation of heparin to TC copolymer resulted in almost no changes in both loading manners (single and dual), indicating that heparin do not participate in IMC encapsulation. In the LE of bFGF to micelles, the LE for TCH micelle was very high as compared with that of Tetronic® and TC micelle. This result is described by the presence of heparin into the structure of micelles. As mentioned above, TCH micelle contains heparin that can bind specifically with bFGF in contrary to Tetronic and TC micelle.

Table 2
Drug loading efficiency of Tetronic® derivatives

Drug loading	Loading efficiency[a] (%, $n = 3$, mean ± S.D.)		
	Tetronic®	TC	TCH
IMC (single)	22.2 ± 0.1	30.7 ± 0.1	30.9 ± 0.3
IMC (dual)	–	17.6 ± 2.5	16.6 ± 0.7
bFGF (single)	39.7 ± 8.8	37.5 ± 4.3	70.5 ± 8.2
bFGF (dual)	–	21.6 ± 1.5	54.0 ± 3.8

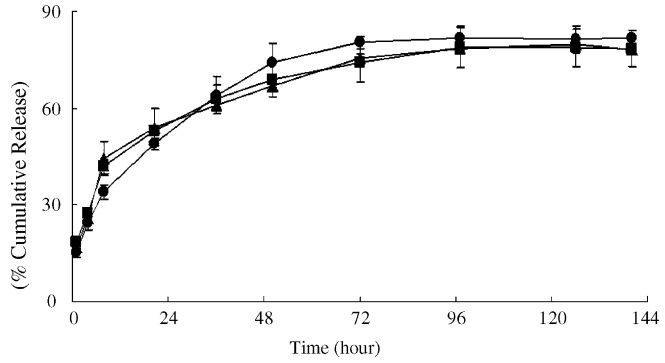

Fig. 4. Cumulative IMC release profiles of Tetronic® derivatives. IMC was released from Tetronic® (●), Tetronic®–PCL (■), and Tetronic®–PCL–heparin (▲) micelles at the concentration of 0.40 mg/ml in PBS solution ($n = 3$, mean ± S.D.).

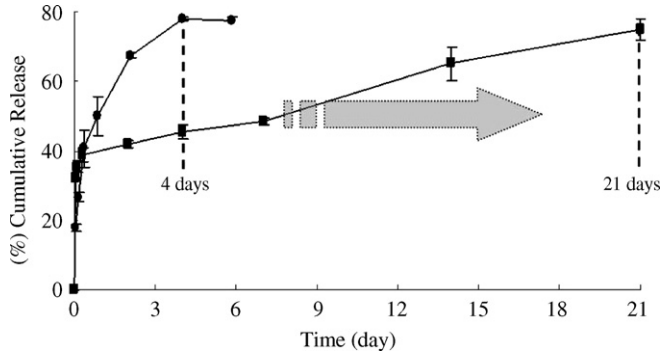

Fig. 5. Cumulative IMC release profiles of TCH micelles. IMC was released from single drug-loaded TCH micelles (●) and dual drug-loaded TCH micelles (■) ($n = 3$, mean ± S.D.).

We can assume that the lower LE of bFGF for Tetronic and TC micelle presumably resulted from not specific binding with heparin but encapsulation by non-specific bindings with micelle structures.

3.3. In vitro release study

Release behaviors of IMC (antiphlogistic drug) and bFGF (growth factor) from micelles were studied under various conditions, which were shown in Figs. 4–7. Fig. 4 shows cumulative

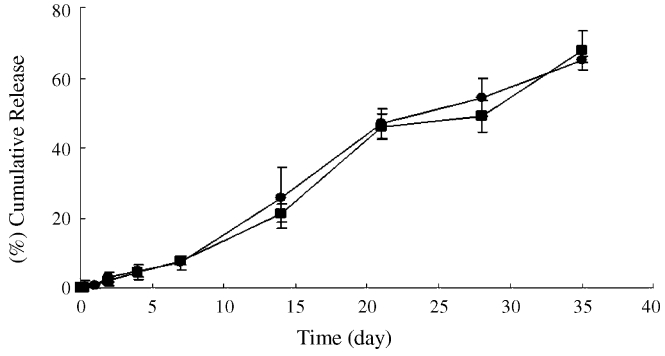

Fig. 6. Cumulative bFGF release profiles of TCH. bFGF was released from single drug (●)-loaded and dual drug (■)-loaded TCH micelles ($n = 3$, mean ± S.D.).

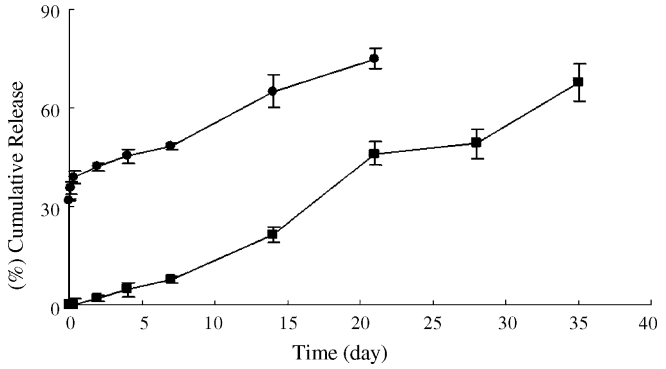

Fig. 7. Cumulative total drug release profiles of dual drug-loaded TCH micelles. IMC (●) and bFGF (■) were released from dual drug-loaded TCH micelles ($n = 3$, mean ± S.D.).

release profile of IMC from each single drug-loaded micelle. IMC was released as same manner from all micelles with showing no initial burst for relatively long release time. Such profiles of IMC describes that loaded IMC was released nearly from the hydrophobic part of Tetronic® segment in all micelles, indicating sustained release behavior and no initial burst of IMC.

Fig. 5 shows difference in release behavior of IMC between single and dual loading of drugs to TCH micelle. While dual drug (IMC and bFGF)-loaded TCH micelle released about 80% of loaded IMC amount for 21 days, single drug (IMC)-loaded TCH micelle released the similar amount of IMC for 4 days. This release profiles indicates that dual drug loading induces significantly sustained release of IMC from TCH micelle. The IMC incorporated into the TCH micelle is probably released by molecular diffusion through the matrix. The diffusion of IMC can be suppressed by varying structure or size of the matrix. As a result, we can demonstrate that the sustained release behavior of IMC in dual drug loading is possibly related with the presence of bFGF on the outer shell of the micelle.

The release behavior of bFGF from TCH micelle was also investigated in both single and dual drug loading as shown in Fig. 6. Contrary to IMC, bFGF did not showed any differences in the release profile between single and dual drug loading, which resulted from the existence of bFGF on the outer surface of TCH micelle. That is, IMC loading dose not affect the release behavior of bFGF from TCH micelle.

Fig. 7 shows the total release profile of dual drugs, IMC and bFGF, from TCH micelles. bFGF showed greatly sustained release profile than IMC for simultaneous release of both drugs, although bFGF and IMC exists on the outer shell and inner core of TCH micelle, respectively. This can be also explained by considering the interaction manner of drugs with TCH micelles. Ionic binding of bFGF to heparin is more favorable to sustained release as compared with hydrophobic interaction of IMC with hydrophobic Tetronic® segment. Although bFGF loading inhibited the release of IMC, the release period of bFGF was longer than that of IMC. In the release profile of IMC, it is observed that initial amount of IMC release was significantly high as comparing with that of bFGF. To elucidate this result, it should be reviewed encapsulation mechanism of drugs. A little amount of loaded IMC is incorporated into the outer matrix of TCH micelle,

though a large amount of the IMC is encapsulated into the core part of the micelle composed of hydrophobic Tetronic® segment. In contrast, almost all of loaded bFGF exist on the outer surface of TCH micelle. As a result, the loaded IMC incorporated in the outer matrix of TCH micelle results in high level of initial release of IMC, whereas the loaded bFGF bound specifically to heparin on the surface of TCH micelle is sustainedly released over the whole release period.

4. Conclusions

The TCH micelle assembled from a heparin-conjugated TC block copolymer (TCH) was developed for drug delivery system, especially dual drug delivery. The TCH copolymer was synthesized by conjugating heparin to the TC copolymer to form a spherical micelle in which heparin chain was exposed on surface and Tetronic® and PCL segments were disposed into inner space. In this micelle, bFGF was bound to heparin on surface and a great portion of IMC was incorporated into hydrophobic core. The TCH micelle containing both IMC and bFGF simultaneously released two drugs with showing different release behaviors over long period of day scale, resulted from different interactions between drugs and segments in TCH micelle. From these results, it is expect that TCH micelle can be utilized to deliver other hydrophobic drugs and proteins, in particular heparin-binding proteins except IMC and bFGF. Ultimately, we suggest that TCH micelle has promising potentials for pharmaceutical and biomedical applications that are required multi-action of drugs to use, such as tissue regeneration and drug delivery system.

Acknowledgments

This work was supported by a grant from the Korea Health 21 R&D Project, Ministry of Health & Welfare (02-PJ3-PG6-EV11-0002), and Nano-Biotechnology Project (Regenomics), Ministry of Science & Technology (B020214), Republic of Korea.

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