Heparinized Bioactive Polymers for Biomedical Applications

Ki Dong Park,[*,1]*, Dong Hyun Go, Jin Woo Bae, Kyung Soo Jee*

[1]Department of Molecular Science and Technology, Suwon 443-749, Korea
kdp@ajou.ac.kr

Introduction

The incorporation of heparin to biomaterials has been widely studied to improve the biocompatibility (blood and cell) of biomaterials surfaces. Heparin is known to modulate the biological activity of heparin-binding proteins like serine protease, growth factors and cytokines. Heparinized surfaces are known to be effective to curtail the surface-induced thrombosis which is mainly due to the inhibition of fibrin formation on the surfaces. In addition, the optional binding of the growth factor to extracellular matrix-associated or cell-surface heparins results in a fine control of the bioavailability of the proteins and cells. Amphiphilic Tetronic® is tetra-functional block copolymer derived from the sequential addition of propylene oxide and ethylene oxide to core. Micelles formed from amphiphilic block copolymers have recently attracted significant attention in diverse fields of medicine and biology. An aqueous solution of amphiphilic copolymer shows thermoreversible behavior – a free flowing sol at room temperature but becomes a gel at body temperature. Moreover, a prominent feature of these copolymers, specifically related to drug delivery applications, is the ability to self-assemble in aqueous solutions into multimolecular aggregates having spherical or rod-like morphologies.

In author's laboratory, various kinds of heparinized polymers including heparinized thermosensitive polymers (Tetronic®-PLA(PCL)-heparin copolymers) and star-shaped PLA-Heparin copolymers have been developed as a novel blood/cell compatible biomaterial. Their physico-chemical properties and biological activities have been investigated.

Experimental

Injectable Tetronic®-PLA(PCL)-heparin copolymers. Tetronic®-PLA (TL) copolymer was synthesized by a bulk ring-opening polymerization of L-lactide with Tetronic® and stannous octoate as a catalyst at 110℃ for 45 hrs. The product was precipitated in diethyl ether and further purified through dissolving in chloroform and precipitation in diethyl ether. The residual solvent was eliminated in vacuo at room temperature for over 60 hrs. Then, TL was coupled with heparin by 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and N-hydroxysuccinimide (NHS) in MES buffer at room temperature for 24 hrs. The product was dialyzed against distilled water (MWCO : 15,000) for 3 days and then lyophilized to result in the product (Figure 1). The structure of block copolymer composed of Tetronic®-PLA-heparin conjugate (TLH) was confirmed by FT-IR, ^{1}H-NMR, GPC. Tetronic®-PCL-heparin conjugate (TCH) was synthesized and characterized by same procedure using ε-caprolactone. The sol-gel transition behaviors of aqueous polymer solution were investigated by vial tilting method and the contents of bound heparin were measured by toluidine blue assay. The anticoagulant activity was evaluated by activated partial thromboplastin time (APTT) test *in vitro*. Biofunctionality of TLH hydrogel was investigated by bFGF binding assay. TCH polymeric micelle containing Indomethacin (IMC) and bFGF were prepared by solvent evaporation method. TCH micelle (20mg/dH_2O 1mL) and IMC (20mg) were dissolved in ethanol (1mL) in a glass vial. Afterwards, the solution was stirred at 37℃ for 24 hrs. IMC loaded TCH micelle and bFGF (400ng/mL) was stirred at 37℃ for 24 hrs, allowing sufficient interactions between immobilized heparin and bFGF. In order to determine the critical micelle concentration (CMC) of copolymeric nano-micelles in distilled water, fluorescence measurements were carried out using pyrene as a probe. The size of nano-micelles and their distribution were measured by DLS and TEM.

Star shaped PLA-heparin copolymers. Star-shaped PLA (sPLA) were synthesized by bulk ring-opening polymerization of L-lactide with pentaerythritol as a four-arm initiator and stannous octoate as a catalyst at 130℃, 6hr. And then, heparin was immobilized to sPLA backbone using carbonyldiimidazole (CDI) (Figure 2). Star-shaped PLA-heparin (sPLA-Hep) conjugate has been characterized by FT-IR, colorimetric assay and static contact angle measurement. In addition, the blood compatibility was evaluated by platelet adhesion, protein adsorption and activated partial thromboplastin time (APTT) test in vitro. bFGF and fibronectin was pre-adsorbed on sPLA-Hep surface with different concentration. hMSCs were cultured on different samples (sPLA±heparin, sPLA-Hep±bFGF and/or fibronectin) to evaluate the cell adhesion and proliferation. The proliferation rate of hMSCs was measured by WST-1 test and the cellular adhesion and spreading was quantified by image analyzing software. Cytoskeletons are stained by immunocytochemistry to show the interaction of heparinized surfaces and cytoskeletons. The long-term stabilization of heparin-bound proteins was confirmed by immunoprecipitation method.

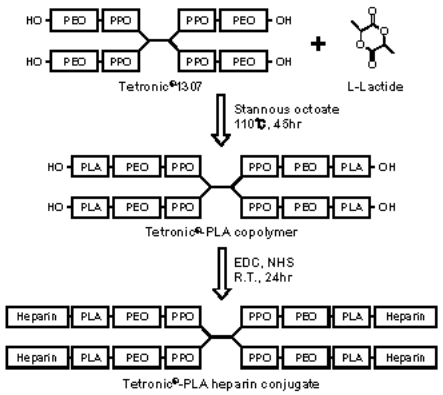

Figure 1. Synthetic scheme of TLH

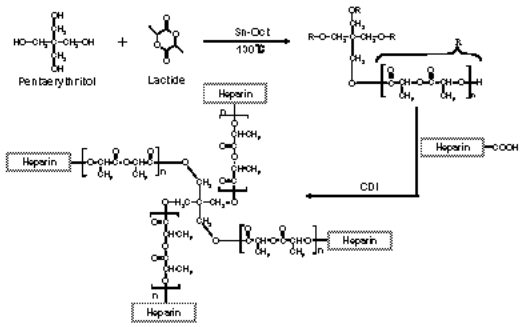

Figure 2. Synthetic scheme of sPLA-Hep

Results and discussion

Injectable Tetronic®-PLA(PCL)-heparin copolymers. TLH and TCH obviously demonstrated the presence of PEO, PPO, PLA, PCL and heparin blocks at FT-IR and ^{1}H-NMR spectra. TLH(TCH) demonstrated sol-to-gel reversible behaviors as shown in Figure 3. The contents of bound heparin of TLH are measured to be 0.61 $\mu g/\mu g$. The anticoagulant activity of TLH is shown to be 67.2% as compared to free heparin. TLH hydrogel demonstrated a higher affinity with bFGF than other samples. Furthermore, TLH hydrogel conjugate showed significant sustained release behaviors over 100hr, while the control showed a rapid bFGF release of more than 90% (Figure 4).

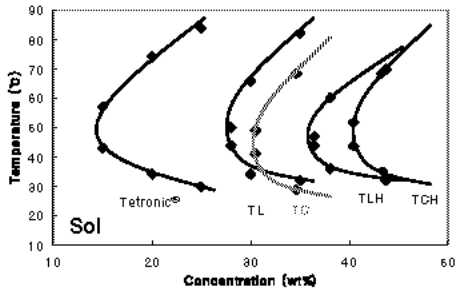

Figure 3. Phase diagram of TLH(TCH) hydrogel

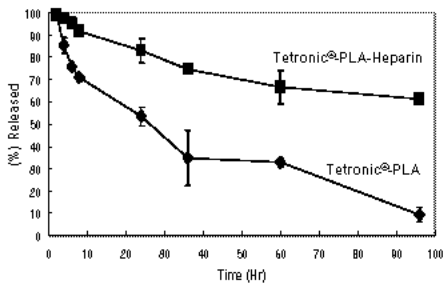

Figure 4. *in vitro* bFGF release profiles of TLH hydrogel

In the IMC DLE study of TC and TCH (30.7%, 30.9%), drug loading efficiency is higher than Tetronic® (22.2%), which did not have the additional hydrophobic moiety. Immobilization of heparin to TC led to increased binding of bFGF more than two times (70.5 %). In the dual drug delivery system, Release of IMC, which was incorporated into a micelle core, was delayed by bFGF enveloping a shell of micelles. IMC release out of the micelle core was sustained over 3 weeks. In the case of bFGF release, it showed a sustained release over two months (Figure 5).

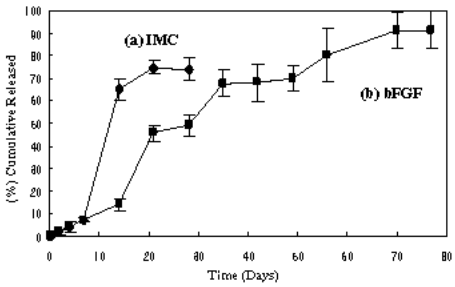

Figure 5. Dual controlled release of TCH micelles

Star shaped PLA-heparin copolymers. FT-IR spectrum of sPLA-Hep indicated that peaks appeared at 1267cm^{-1}, 1299cm^{-1}, 1456cm^{-1} could be assigned to C-S bonding. This peaks indicated that heparin was bound to sPLA. sPLA-Hep coated surface showed the lower static contact angle (higher hydrophilicity) as compared to sPLA surface. The sPLA-Hep significantly prolonged the clotting time (APTT) as compared to sPLA surface, which might be due to the bioactivity of the bound heparins. Bioactivity of immobilized heparin is shown to be 38% as compared to free heparin. *In vitro* platelet adhesion and protein adsorption study revealed that lower platelet and protein adhered on sPLA-Hep surface than sPLA surface. (Table 1)

Table 1. Platelet adhesion and protein adsorption of sPLA and sPLA-Hep

	Platelet adhesion (%)	Protein adsorption (μg/cm^2)
sPLA	50.42±2.30	1.25±0.13
sPLA-Hep	28.57±3.74	0.84±0.14

The hMSCs adhesion and proliferation was increased under the existence of heparin on PLA surface. Cellular activity was increased by bFGF binding on sPLA-Hep surface (Figure 6). The cell spreading area was increased by long-term binding of fibronectin treated sPLA-Hep surface (Figure 7). Highest hMSCs proliferation rate was shown in sPLA-Hep surface treated with both bFGF and fibronectin.

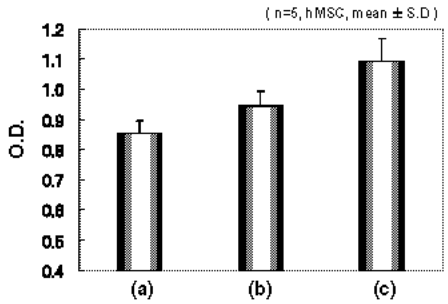

Figure 6. *in vitro* hMSC culture (with bFGF)
(a) sPLA, (b) sPLA-Hep,
(c) sPLA-Hep + bFGF 10ng/ml

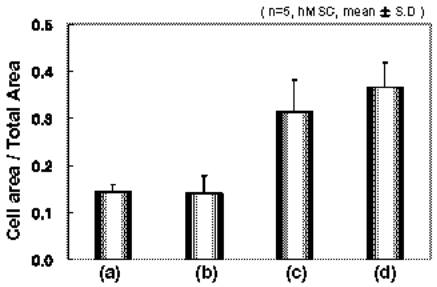

Figure 7. *in vitro* hMSC culture (with fibronectin)
(a) sPLA, (b) sPLA-Hep,
(c) sPLA + fibronectin 5μg/ml,
(d) sPLA-Hep + fibronectin 5μg/ml

Conclusions

Novel heparinized polymers including thermosensitive TLH(TCH) and sPLA-heparin have been developed for the application in the blood contacting surfaces and cell compatible biomaterials. In addition, obtained results attest the usefulness of heparinized polymers as delivery carriers of heparin–binding proteins like growth factors, especially in tissue engineering and protein delivery system.

Acknowledgement

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References

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