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ATRP를 이용한 Lysine 말단기를 가진 펩타이드-고분자 하이브리드 합성

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Solid Phase Synthesis of Lysine-exposed Peptide-Polymer Hybrids by Atom Transfer Radical Polymerization

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초록: 펩타이드-고분자 하이브리드 소재(PPs)들은 선택적 용매에서 나노구조 형성을 위한 잠재적 구성 요소로서 많은 연구분야에 이용되고 있다. PPs는 잘 정의된 펩타이드-고분자로 이루어진 바이오콘주게이트의 손쉬운 제조방법과 다양한 응용분야에서 이들의 고유활성도에 대한 연구는 중요한 이슈이다. 본 연구에서는 atom transfer radical polymerization(ATRP)와 고체상 펩타이드 합성법을 이용하여 펩타이드-고분자 하이브리드 소재를 제조하였다. PYGK (proline-tyrosine-glycine-lysine) 펩타이드를 제조하기 위하여 일반적인 고체상 펩타이드 합성법을 이용하였다. PYGK 펩타이드는 섬유소용해(fibrinolysis) 과정에서 플라스미노젠과 반응하는 PFGK(proline-phenylalanine-glycine-lysine)와 유사한 펩타이드이다. 펩타이드와 펩타이드 개시제는 MALDI-TOF와 ^{1}H NMR을 이용하여 분석하였다. 펩타이드-고분자인 pSt-PYGK는 GPC, IR, ^{1}H NMR 분석법, 그리고 TLC를 이용하여 분석하였다. 구형 마이셀 집합체는 TEM과 SEM으로 측정하였다. 본 합성방법은 고유결합 활성도를 가진 잘 정의된 펩타이드-고분자 하이브리드 소재를 합성할 수 있는 기회를 제공한다.

Abstract: Recently, the peptide(or protein)-polymer hybrid materials (PPs) were sought in many research areas as potential building blocks for assembling nanostructures in selective solvents. In PPs, the facile routes of preparing well-defined peptide-polymer bio-conjugates and their specific activities in various applications are important issues. Our strategy to prepare the peptide-polymer hybrid materials was to combine atom transfer radical polymerization (ATRP) method with solid phase peptide synthesis. The standard solid phase peptide synthesis method was employed to prepare the PYGK (proline-tyrosine-glycine-lysine) peptide. PYGK is an analogue peptide, PFGK (proline-phenylalanine-glycine-lysine), which interacted with plasminogen in fibrinolysis. The peptide and the peptide-initiator were characterized with MALDI-TOF mass spectrometry and ^{1}H NMR spectrometer. The peptide-polymer, pSt-PYGK was characterized by GPC, IR, ^{1}H NMR spectrometer and TLC. Spherical micellar aggregates were determined by TEM and SEM. Current synthesis methodology suggested opportunities to create the well-defined peptide-polymer hybrid materials with specific binding activity.

Keywords: atom transfer radical polymerization, plasminogen, coagulation, polystyrene, polymer-protein hybrid.

Introduction

The peptide-polymer hybrid materials (PPs) were sought in many research areas as potential building blocks for assembling nanostructures in selective solvents.[1-3] In PPs, the facile routes of preparing well-defined peptide-polymer bioconjugates and their specific activities in various applications are important issues.[4] The hybrid between polymers and peptides have received many attentions in recent years as a new class of biomaterials for various applications, which could self-assemble into nano-structures like micelles, spheres, rods, vesicles, and tubes.[5-7] In particular, amphiphilic block copolymers consisting of covalently connected hydrophobic and hydrophilic blocks offer many advantages as effective drug delivery systems. They can form micelles by self-assembling in water due

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to the different solubility of each block in selective solvents. A hydrophobic core, being able to incorporate various hydrophobic drugs, is surrounded by a hydrophilic corona, capable of water solubility and biocompatibility of the micelles.[8,9]

Many examples of hybrid block copolymers composed of a nano-biological segment, proteins, and one or two homopolypeptide sequences were reported.[10-12] Recently, the interest of utilizing the peptide-polymer hybrid materials (PPs) increased as building blocks, which could be assembled into nano-structures in selective solvents.[13-16]

Recent advances in polymer synthesis, particularly in the field of controlled/living radical polymerization (CRP) techniques, such as atom transfer radical polymerization (ATRP), nitroxide-mediated polymerization (NMP), and reversible addition-fragmentation chain transfer (RAFT), stimulated the development of novel well-defined polymer-based bioconjugates.[4,17,18] In particular, atom transfer radical polymerization (ATRP) was proved to be a one of the most widely investigated techniques among all the living polymerization techniques to synthesize such peptide-polymer bioconjugates.[3] Indeed, ATRP could be a facile technique for preparing well-defined polymers with narrow molecular weight distribution, predictable chain length, controlled microstructure, defined chain-ends, and controlled architecture.[19] Moreover, the chemistry of ATRP is tolerant of many functional groups, thereby permitting the controlled synthesis of a broad range of polymers. The ATRPs of various monomers from different particles were studied extensively for surface modification. Many studies have been reported on the CRP techniques were employed as a new synthetic route to PPs. Ten *et al*. reported the solid phase supported synthesis routes to obtain the oligopeptide-based RAFT agents.[20] The peptide-polymer hybrid materials were synthesized by the ATRP from resin-supported peptides by Washburn *et al*.,[21] and the peptide-polymer bioconjugate block copolymers through the nitroxide-mediated radical polymerization (NMRP) from a solid support was shown by Wooley group.[12]

In this study, we described our strategy to prepare the peptide-polymer hybrid materials by combining ATRP method with solid phase peptide synthesis. The standard solid phase peptide synthesis method was used to prepare the peptide. The peptide sequence in this work is PYGK (proline-tyrosine-glycine-lysine). PYGK was an analog of known peptide, PFGK (proline-phenylalanine-glycine-lysine), which interacted with plasminogen in blood coagulation and fibrinolysis. The synthesized peptide was functionalized with the initiator for ATRP method. The peptide-based initiator was used for the polymerization of styrene from solid support. The peptide and the peptide-initiator were characterized with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry and hydrogen nuclear magnetic resonance (^{1}H NMR) spectrometer. With pSt-PYGK, the micellar aggregates were induced and binding assay of PYGK of pSt-PYGK with plasminogen was studied. The peptide-polymer, pSt-PYGK was characterized by gel permeation chromatography (GPC), infrared (IR), hydrogen nuclear magnetic resonance (^{1}H NMR) spectrometer and thin-layer chromatography (TLC) and spherical micelles were determined by transmission electron microscope (TEM) and scanning electron microscope (SEM). In addition, the effects of polystyrene (pSt), PYGK, and pSt-PYGK in coagulation and fibrinolysis were studied.

Experimental

Materials. Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), 1-hydroxybenzotriazole hydrate (HOBt), fmoc-Lys (BOC)-Wang resin, fmoc-Gly-OH, fmoc-Tyr (tBu)-OH, and fmoc-Pro-OH were purchased from Novabiochem, *N,N*-dimethylformamide (99.9+%), 1,3-diisopropylcarbodiimide (DIPCDI, 99%), trifluoroacetic acid (TFA, 99+%), piperidine (99.5+%), potassium cyanide (97%), pyridine (99.9+%), dichloromethane (DCM, 99.9%), ninhydrin, *N,N,N′,N″,N″*-penetamethyldiethylenetriamine (PMDETA, 97%), 2-bromopropionyl bromide (98%), and phenol (99+%) were purchased from Aldrich and used without further purification. Anisole (Junsei Chemical, 99.8%), triethylamine (TEA, Junsei Chemical, 99.0%), deuterium oxide (D_2O, Cambridge Isotope Laboratories, Inc.), methanol-*d* (CD_3OD, Cambridge Isotope Laboratories, Inc.), chloroform-D ($CDCl_3$, Cambridge Isotope Laboratories, Inc.), tetrahydrofuran (THF, J.T. Baker, for use in GPC), ethyl ether (anhydrous, J.T. Baker), and methanol (Hayman, 99.9%) were also used as received. Copper (I) bromide (98%) was purchased from Aldrich, purified by stirring acetic acid overnight, filtered to collect the solid, and washed with methanol and ethyl ether. Styrene (KENTO Chemical Co., INC.) was used after distilled from calcium hydrate. Calcium chloride, thrombin, tissue-plasminogen activator (t-PA), Tris buffer, phosphate buffer, clear Nunc microtiter plate were purchased from Sigma. Plasmin chromogenic substrate, Isovaleryl-Phe-Lys-pNA · HCl (PL1), was from Bachem Inc. Pooled normal plasma was prepared from 5 healthy subjects whose laboratory results were within normal ranges. PT-Fibrinogen

HS and SynthASil (Instrumental Laboratory, Lexington, MA) were used for prothrombin time (PT) and activated partial thromboplastin time (aPTT), respectively.

Instruments. ^{1}H NMR (500 MHz) spectra were recorded as solutions on a Varian Inova 500 spectrometer with the solvent signal as standard (or TMS signal as standard). Molecular weights (M_n) and molecular weight distributions (M_w/M_n) were determined using gel permeation chromatography (GPC) calibrated with polystyrene standards and equipped with Agilent 1100 pump, RID detector and PSS SDV (5 μm, 10^5, 10^3, 10^2 Å 8.0×300.0 mm) column. IR spectra were obtained on a JASCO FT-IR 460 PLUS using KBr pallets. Mass spectra were obtained from PE Voyage-DE PRO and Voyager with 337 nm Nitrogen Laser and Linear mode Flight tube. Transmission electron microscopy (TEM) images were obtained on a H-7600 (HITACHI, LTD) instrument at 100 kV. Scanning electron microscopy (SEM) images were obtained on a S-4100 (HITACHI, LTD). Thin layer chromatography (TLC) was performed with 25 TLC aluminium sheets 20×20 cm silica gel 20F.

PYGK Peptide Synthesis. Peptide synthesis was carried out on fmoc-Lys (BOC)-functionalized Wang resin by a standard solid phase method. Fmoc-Lys (BOC)-resin (0.108 mmol, 0.163 g) was added to a 10 mL reaction vessel and was dispersed in DMF allowed to equilibrate for 1 h to swell the bead. After washing the resin with DMF 3 times, the resin was washed 4 times with DMF and 7 mL of 20% piperidine in DMF was added to remove the protecting group 4 times. Deprotected resin was washed with DMF 6 times. In relation to the concentration of the pre-coupled amino acid on the solid support, dissolved fmoc-Gly-OH (5X), PyBOP (4.9X), and HOBt (5X) in DMF to the 5 equivalents of the amino acid, HOBt and the 4.9 equivalents of PyBOP was dissolved in a minimum amount of DMF (3 mL). Then 10 equivalents of DIPCDI were added to the mixture. The mixture was allowed to equilibrate for 10 min to completely activate the carboxylic acid group by shaking. The activated amino acid was then added to the reactor, which contained 163 mg of resin and reacted with shaking for 8 h at RT. Upon completion the beads were washed 4 times with DMF. The deprotection and coupling steps were repeated until the synthesis of peptide sequence, PYGK was completed. Every step was checked with Kaiser test. Full conversion of each coupling cycle was verified with Kaiser test. The liberation of PYGK was accomplished by 3 h treatment with the 1 mL of cleavage mixture (95% TFA, 2.5% DIPCDI, and 2.5% H_2O) two times. The oligopeptide was isolated from collected TFA solution by diethyl ether precipitation, centrifugation.

Synthesis of PYGK-Br. PYGK-Wang-resin (0.09 mmol) was added to a 10 mL vessel, added 2 mL of DMF to swell out the resin, and 2-bromopropionyl bromide (2.00 mL, 0.86 mmol) and TEA (0.07 mL, 0.86 mmol) solution in DMF was added. The mixture was shaken overnight, and then the resin-bound initiator was washed with DMF and dried. The liberation of PYGK-Br was accomplished by 3 h treatment with the 1 mL of cleavage mixture (95% TFA, 2.5% DIPCDI, and 2.5% H_2O) two times. The PYGK-Br was isolated from collected TFA solution by diethyl ether precipitation, centrifugation.

Synthesis of PYGK-pSt. Styrene (2.00 mL, 1.73×10^{-2} mmol), anisole (2.00 mL) and PMDETA (0.05 mL, 0.22 mmol) were added to a N_2 purged Schlenk flask. After three freeze-pump-thaw cycles, Cu(I)Br (30.98 mg, 0.22 mmol) was added to the flask, followed by two freeze-pump-thaw cycles. After 30 min stirring, the resin-bound peptide initiator (24.84 mg, 4.32×10^{-2} mmol) was added to the flask. The reaction mixture was heated in an oil bath at 90 °C. After 5 h, the polymerization was stopped by exposing the reaction mixture to air and cooling down the flask to the room temperature. The reaction mixture was washed with THF and methanol, repeatedly. The liberation of PYGK-pSt was accomplished by 3 h treatment with the 3 mL of cleavage mixture (95% TFA, 2.5% DIPCDI, and 2.5% H_2O) two times. The solution was incubated 2 times by shaking for 3 h and the resin was washed with TFA for the last time to remove the remained peptide. Then, THF was added to dissolve the polymer end functionalized peptide. The solution was collected and precipitated with methanol.

Cleavage Reaction. PYGK Cleavage: the beads were placed into a 5 mL vessel, and washed with 3 times of 100% DMF, DCM, and anhydrous methanol. And then the beads were dried under vacuum for 4 h in a chamber. The peptide was cleaved with a 1 mL of 95% TFA solution (TFA: DIPCDI: water = 95: 2.5: 2.5). The solution was incubated by shaking for 3 h and repeated 2 more times. After collecting the TFA solution, PYGK was precipitated by adding cold ethyl ether drop by drop to the TFA solution, and they were obtained by centrifugation.

pSt-PYGK Cleavage: the beads were placed into a 10 mL vessel, and washed 5 times with THF to remove polystyrene without the peptide. After washed 5 times with methanol, 3 mL of 95% TFA solution (TFA: DIPCDI: water = 95: 2.5: 2.5) was added to cleave off the pSt-PYGK from the resin. The solution was incubated 2 times by shaking for 3 h and the resin

was washed with TFA for the last time to remove the remained peptide, which didn't convert to the polymer. Then, THF was added to dissolve the polymer end functionalized peptide. The solution was collected and precipitated with methanol.

Preparation of Micellar Aggregates. The micellar aggregates of PYGK-pSt were prepared by adding water in THF solution of the polymer. A polymer concentration of 0.25 mg/mL was used and 15% (v/v) of water added dropwise to the solution with stirring to induce micellization. For comparison, the polystyrene dissolved in THF was prepared with same method. The solution was dropped on silicon wafer or carbon coated grid for SEM or TEM analysis, respectively.

Results and Discussion

To introduce PYGK peptides sequence into synthetic polymers, combination of solid phase peptide synthesis and atom transfer radical polymerization were employed as shown in Figure 1. The synthetic scheme for peptide-polymer hybrid was similar to works previously reported by others.[4]

Characterization of the Peptide and the Peptide-initiator. The mass of PYGK and PYGK-initiator were measured using MALDI-TOF mass spectrometry. Samples were prepared from a solution of 40% (vol/vol) acetonitrile in 0.1% (vol/vol) trifluoroacetic acid. All samples were prepared by spotting and performed using the most commonly used matrix for peptide analysis, α-cyano-4-hydroxycinnamic acid (α-CHCA). The mass of the peptide, PYGK (M: 463 m/z) synthesized by solid phase peptide synthesis, was confirmed by one major peak at 464 m/z (M+H). In the analysis of PYGK-initiator, the emergence of the peak at 598/600 m/z (M+H) corresponding to the proton-cationized molecule peak of PYGK-initiator also indicated that PYGK was converted to PYGK-initiator. The ^{1}H NMR spectrum of PYGK was shown in Figure 2 and also provides information on the structure of the peptide obtained by 95% TFA treatment after synthesis with solid phase method. The peaks were assigned to the structure of PYGK as shown in Figure 2. In Figure 3, the peak from the initiator at 1.80 ppm was appeared, but the integration value was not corresponding to the number of the proton (-$CHCH_3Br$). This result indicates that not all PYGK converted to PYGK-initiator.

Characterization of Peptide-Polymer Hybrid Materials. Gel permeation chromatography with THF as eluent, using RI detector was used to measure the molecular weight of pSt-PYGK. Figure 4 shows the chromatogram of the peptide end functionalized polystyrene. The number-average molecular weight was about 24000 g/mol and the polydispersity index (PDI) was 1.27, indicating that the polymerization of pSt-

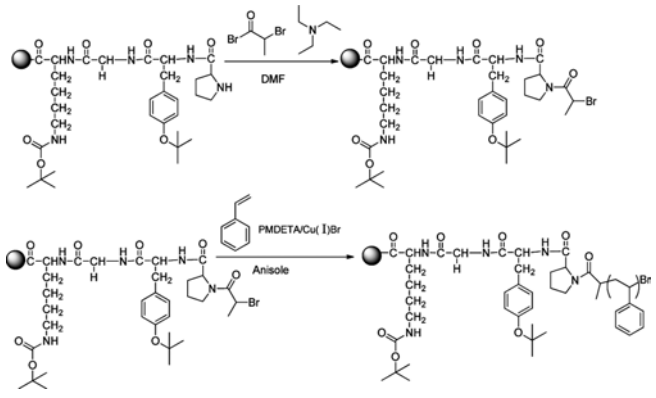

Figure 1. Solid phase synthesis of the peptide-polymer (pSt-PYGK) hybrid materials.

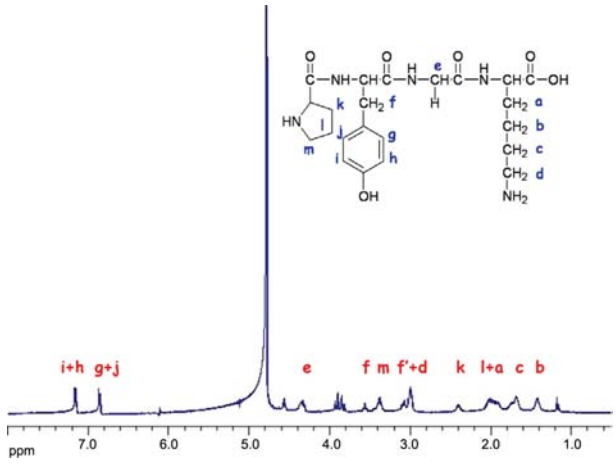

Figure 2. ^{1}H NMR spectrum of PYGK.

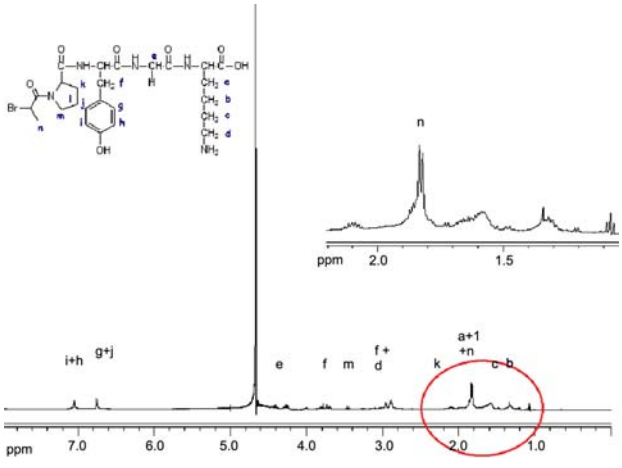

Figure 3. ^{1}H NMR spectrum of PYGK-initiator.

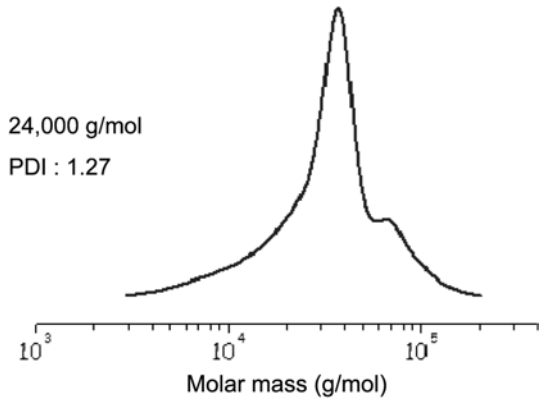

Figure 4. Gel permeation chromatogram of pSt-PYGK.

PYGK proceeded in a controlled way. The GPC trace of pSt-PYGK exhibited a high-molecular weight shoulder. The molecular weight for the small peak in GPC chromatogram was about twice as high as that of the major peak. The small peak might be due to the coupling product of growing polystyrene. As polystyrene chains are growing, the coupling between chains tethered on one resin can occur. Because of the coupling, the polymer with molecular weight twice higher than that of major product can be obtained, resulting in increase of the PDI.

Polystyrene containing the peptide was characterized with FTIR to compare pSt-PYGK to polystyrene without the peptide. Figure 5 shows the IR spectra of polystyrene (a) and pSt-PYGK (b). In Figure 5(a), the major polystyrene peaks (=C-H *sp2* stretch over 3000 cm^{-1}, -C-H *sp3* stretch below 3000 cm^{-1}, mono subst. 2000~1667 cm^{-1}, mono subst. oop 756 and 698 cm^{-1}, aromatic C=C 1600~1475 cm^{-1}) were confirmed, and in Figure 5(b), not only the major polystyrene peaks but the amide peak of the peptide (amide I 1700 cm^{-1} and amide II 1650 cm^{-1}) was observed. The structure of pSt-PYGK was also analyzed by ^{1}H NMR spectroscopy. First, ^{1}H NMR spectrum of pSt-PYGK (M_n~24000 g/mol) was obtained, but the signal from peptide at the α-polymer chain end was difficult to find presumably due to the high degree of polymerization of polystyrene (M_n~24000 g/mol).

Therefore, the lower molecular weight pSt-PYGK (M_n~3000 g/mol)) was prepared (monomer: initiator: catalyst = 400: 1: 5) by adding 20% phenyl ethyl bromide as a molar ratio, which yielded low molecular weight pSt-PYGK. The ^{1}H NMR spectrum of pSt-PYGK was obtained in two different solvents; $CDCl_3$ (Figure 6(a)) and the mixture of $CDCl_3$ and CD_3OD ($CDCl_3$: CD_3OD = 2:1) (Figure 6(b)). These results indicate that pSt-PYGK may aggregate in $CDCl_3$. This strong tendency of pSt-PYGK to form aggregates restricts the NMR spectroscopic resolutions. To improve the resolution of pSt-PYGK, several solutions were considered; (1) alteration solitary organic solvents (2) mixtures of organic solvents like $CDCl_3$, CD_3OD, and DMSO-d_6, (3) mixtures of $CDCl_3$ with protic solvents (4) Influence of transversal relaxation time on signal intensity.[22,23] In this work, (3) was used to improve the resolution. All of the peptide peaks could be observed between 2 and 5 ppm in $CDCl_3$/CD_3OD mixture supporting the successful formation of peptide-polystyrene hybrid structures. But an improvement of spectral resolution is still required to optimize of disaggregation.

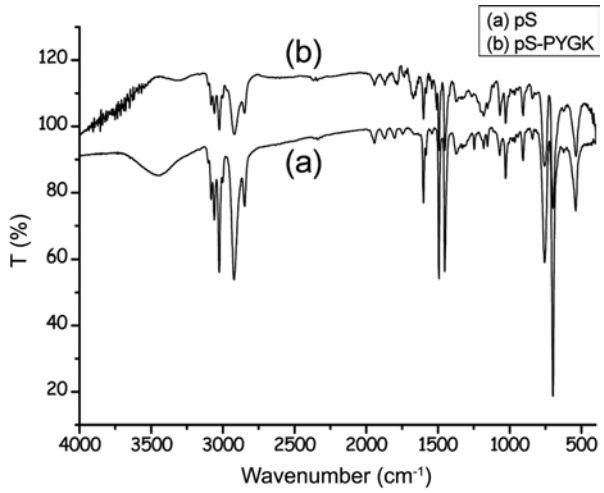

Figure 5. FTIR spectra of polystyrene (a); pSt-PYGK (b).

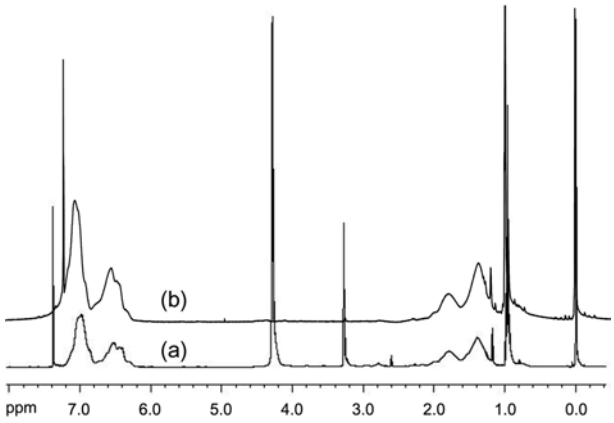

Figure 6. ^{1}H NMR spectra of pSt-PYGK (M_n~3000 g/mol) from $CDCl_3$ (a); $CDCl_3$/CD_3OD (b).

The hybrid structures of peptides and polystyrene was further confirmed by thin-layer chromatography analysis using commercial silica plates. Methyl chloride (MC), *n*-hexane, methanol mixture was used as eluent. For the comparison, polystyrene (M_n~20000 g/mol and PDI~1.09) was analyzed

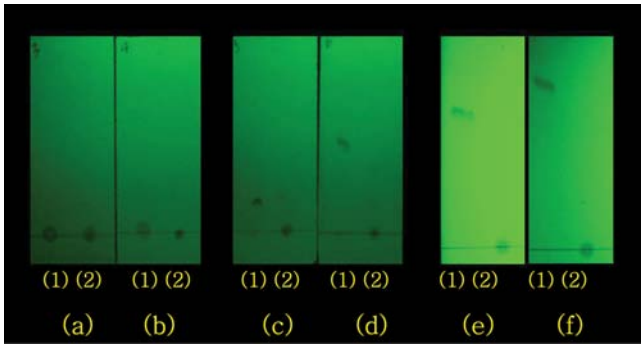

Figure 7. TLC chromatogram of polystyrene (1) and pSt-PYGK (2): (a) MC:*n*-hexane (1:2); (b) MC:*n*-hexane:MeOH (1:2:0.02); (c) MC:*n*-hexane (1:1); (d) MC:*n*-hexane (1:1:0.01); (e) MC:*n*-hexane (1.5:1); (f) MC:*n*-hexane (1.5:1:0.02).

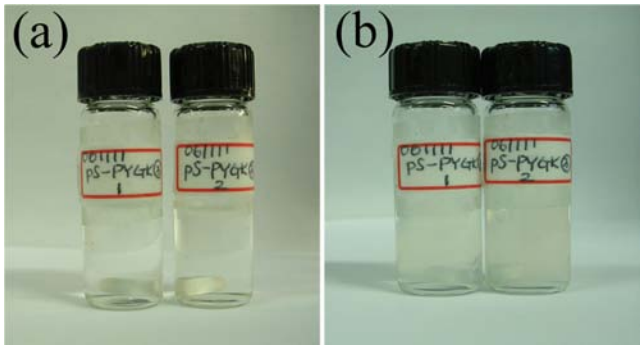

Figure 8. The photographs of pSt-PYGK dissolved in THF (1: 0.25 mg/mL, 2: 0.5 mg/mL) (a) and after adding 15 wt% (v/v) of water in THF (b).

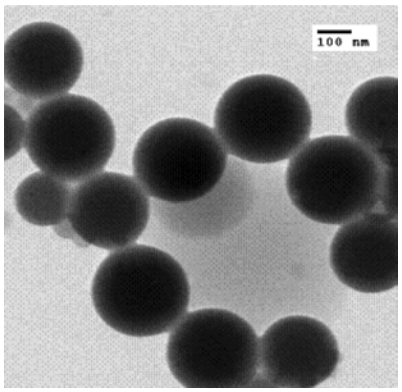

Figure 9. TEM image of the micelles prepared from pSt-PYGK at 0.25 mg/mL of the initial concentration.

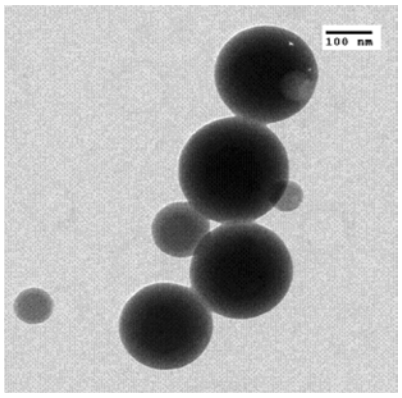

Figure 10. TEM image of the micelles prepared from pSt-PYGK at 0.5 mg/mL of the initial concentration.

with TLC. Polystyrene and pSt-PYGK were functionalized with bromine because they were prepared by ATRP. Despite similar structure, there was a difference in elution behavior between two polymers. As shown in Figure 7, it was confirmed that the elution behavior of pSt-PYGK was markedly different from polystyrene samples because of the end functional group. pSt-PYGK had a carboxylic acid group at another functional site from the peptide. Therefore, as the increase of the solvent polarity, the R_f values of polystyrene clearly increased on silica plates, but there was no difference and movement in the case of pSt-PYGK, due to the carboxylic acid group as a functional group. This carboxylic acid group causes the strong interaction on silica plates, so this effect of absorption for carboxylic acid terminated polystyrene was observed.[24] To increase the R_f values of pSt-PYGK, the molecular weight of the solvent should be increased (over 37000 g/mol) to sufficiently overcome the interaction energy.[24,25]

Characterization of the Polymer Micelles. Micellar aggregates were prepared with two different initial concentrations of pSt-PYGK in THF. Figure 8 shows the micelle formation. Figure 8(a) is the pSt-PYGK dissolved in THF (1: the initial concentration of pSt-PYGK is 0.25 mg/mL, 2: the initial concentration of pSt-PYGK is 0.5 mg/mL) and they were clear solutions. After adding 15 wt% (v/v) of water dropwise to each solution with a micropipette under stirring to induce polymer micellization, the turbidity of the solution was increased (Figure 8(b)). Figure 9 and Figure 10 show representative TEM images of the micellar aggregates (TEM images of micelles prepared with low (0.25 mg/mL) (Figure 9) and high (0.5 mg/mL) (Figure 10) concentrations. The size of the micellar aggregates at high concentration was relatively larger. The particle size was about ~ 200 nm for lower molecular weight of pSt-PYGK and about ~ 250 nm for higher molecular weight of pSt-PYGK. As a control, the polystyrene without the peptide dissolved in THF and added 15 wt% (v/v) of water. It was also measured with TEM. In Figure 11, no well-defined structures were observed. TEM results demonstrated that polystyrene

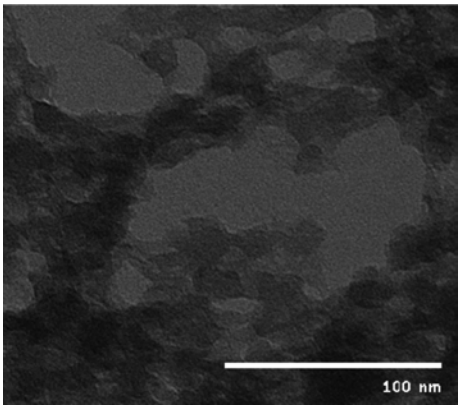

Figure 11. TEM image of the controlled micellization with polystyrene.

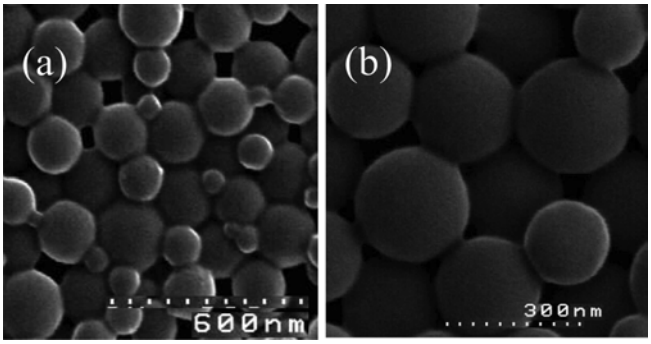

Figure 12. SEM images of the micelles prepared from pSt-PYGK; the initial concentration is (a) 0.25 mg/mL; (b) 0.5 mg/mL.

synthesized by solid phase synthesis method from the resin with the peptide and the initial concentration of polymer had influence on the micelle size.

The structure of polymeric micelles induced from pSt-PYGK was characterized by SEM. Figure 12 shows the SEM images obtained from the two solutions (Figure 12(a): 0.25 mg/mL and Figure 12(b): 0.5 mg/mL). Similar size and morphology of micelles with the images from TEM were observed on SEM.

Conclusions

The synthesis of PYGK-pSt was conducted on a solid support by solid phase peptide synthesis and ATRP methods. These peptide-polymer hybrid materials were characterized by ^{1}H NMR, FTIR, GPC, and TLC. The spherical micelle aggregates from pSt-PYGK were induced and determined by TEM and SEM. This synthetic methodology provides many opportunities to create the well-defined peptide-polymer hybrid materials with specific binding activity.

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