

Novel Macromolecular Self-organization of Poly(ethylene glycol)-*block*-poly(L-histidine): pH-induced Formation of Core-shell Nanoparticles in Aqueous Media

Joon Sig Choi, Eun Jung Lee, Sung-Jun Park, Hie-Joon Kim, and Jong Sang Park*

School of Chemistry & Molecular Engineering, Seoul National University, San 56-1,
Shillim-dong, Kwanak-ku, Seoul 151-742, Korea
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Block copolymers composed of hydrophilic groups and hydrophobic segments can sometimes self-assemble in a selective solvent forming a supramolecular structure. Many studies have been concentrated on block copolymers that form polymeric micelles in an organic solvent.^{1,2} Recently, a few studies have focused on block copolymers forming micelles upon dissolution into water.³⁻⁵ Copolymers with hydrophilic blocks such as poly(ethylene glycol) (PEG) and hydrophobic blocks composed of a side-group protected biodegradable poly(amino acid) chain have potential utility as drug delivery systems.⁶ Hydrophobic drugs can be physically loaded within polymeric micelles or chemically conjugated to poly(amino acid) with subsequent polymeric micelle formation.⁷

There are a few examples of water-soluble block copolymers that can self-assemble in a pH-dependent manner.^{8,9} We were interested in poly(L-histidine), which is a biodegradable polypeptide¹⁰ and thought to possess an amphoteric nature due to the imidazole group of histidine.¹¹ So, we have designed the poly(ethylene glycol)-*block*-poly(L-histidine) (PEG-*b*-PLH) block copolymer and characterized its pH-dependent supramolecular self-assembly by ¹H NMR, size exclusion chromatography, and dynamic light scattering.

We performed the stepwise repeated liquid phase synthesis method rather than ring-opening polymerization to prepare the block copolymer for the following two reasons. First, this method does not require a hazardous reagent such as triphosgene that is widely used in the ring-opening polymerization method. Second, in the case of ring-opening polymerization, it is hard to control the number of repeating units, but it is possible to exactly control the poly(L-histidine) residues by the liquid phase method.

Methoxy poly(ethylene glycol) (mPEG)-amine (Sigma, St. Louis, MO)¹² was used as the polymeric supporter, and poly(L-Histidine) was prepared by repeated liquid phase peptide synthesis using fluoren-9-ylmethoxycarbonyl (Fmoc) chemistry.¹³ mPEG-amine was stirred with 6 equivalents of *N*-hydroxybenzotriazole (HOBt), 2-(1H-Benzotriazole-1-yl)-1,3,3-tetramethyluronium hexafluorophosphate (HBTU), *N*- α -Fmoc-*N*-in-trityl-L-histidine (AnaSpec, Inc., San Jose, CA) and *N,N*-diisopropylethylamine (DIPEA), respectively

in anhydrous *N,N*-dimethylformamide (DMF). After the coupling reaction reached completion, the mixture was precipitated with a 10-fold excess of cold ether and further washed two times with ether. 30% piperidine was used for deprotection of the Fmoc group of histidine. The reaction mixture was precipitated and washed with cold ether as described above. From the reaction mixture, only mPEG-coupled products were precipitated in cold ether and other byproducts were removed by washing with excess ether. In order to remove small traces of excess reagents, the precipitate was recrystallized in pure ethanol. The precipitates were dried *in vacuo* and prepared for further coupling reaction. The coupling and deprotection reactions were repeated 18 times.¹⁴ The progress of each reaction was monitored by ninhydrin test until completed. After treating 90% trifluoroacetic acid to remove the trityl groups of poly(L-histidine), the final product was precipitated in ether and washed with excess ether. The aqueous copolymer solution was dialyzed for 1 day against water using a Spectra/Por dialysis membrane (molecular weight cut-off = 6000-8000, Spectrum, Los Angeles, CA) and collected by freeze-drying (overall yield 37%). This linear block copolymer was identified by ¹H NMR and the matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (PerSeptive Biosystems, Inc., Framingham, MA).¹⁵ The M_w and M_n values of this copolymer were 8384 and 8313, respectively ($M_w/M_n = 1.01$).

As the pH changed, it was expected that the degree of protonation and solubility of the PLH segment of this block copolymer might change. This was indeed the case as observed in the ¹H NMR spectra. Proton peaks of the imidazole groups were observed to shift upfield with increasing pD (pD was adjusted by NaOD and DCl.) (Table 1). Moreover, the signals of the PLH segment drastically decreased at

Table 1. ¹H NMR at different pD conditions (δ in ppm)

	pD		
	2.8	5.6	8.0
<i>a</i> -H	3.4	3.4	3.4
<i>b</i> -H	3.7	3.7	3.7
<i>c</i> -H	4.6	4.6	4.5
<i>d</i> -H	3.1	3.1	2.9
<i>e</i> -H	7.2	7.0	6.8
<i>f</i> -H	8.6	8.1	7.6

*To whom correspondence should be addressed: e-mail: pfjspark@plaza.snu.ac.kr; Tel: +82-2-880-6660; Fax: +82-2-889-1568

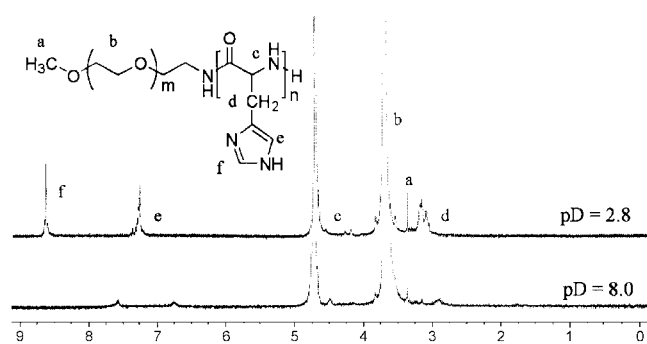


Figure 1. ^1H NMR spectra of mPEG-*b*-PLH at pD = 2.8 and 8.0.

pD 8.0 where the imidazole groups were completely deprotonated (Figure 1). The ratio of integration value for *d*-H of PLH to PEG at pD 8.0 ($r = 0.0090$) decreased by about 86% in comparison with that at pD 2.8 ($r = 0.066$).¹⁶ As the PLH homopolymer is soluble in water at $\text{pH} \leq 3$,¹⁷ the PLH segments in this block copolymer probably self-assembled one another forming polymeric micelles at higher pH, which may cause proton signal shielding or peak broadening of the PLH block.^{18,20}

To further assess the pH-induced self-organization phenomena, size-exclusion chromatography measurements were performed at room temperature under some different pH conditions (pH was adjusted with TFA). The liquid chromatography apparatus was equipped with an HPLC pump and UV detector (200 nm). The column was a SB-805HQ (molecular weight range = 500-4,000,000). The polymer was dissolved in the eluent solution to a concentration of 1.0 mg/mL and the flow rate was 0.6 mL/min. As expected, only one peak with the retention time of 18.8 min was observed at pH 2.9, the level that is below the threshold for the PLH block to aggregate. As the pH of the eluent solution increased to 4.0, two peaks larger in size were observed (13.6 and 16.1 min, respectively). Even though we do not know exactly the reason for the results, it was presumed that further aggregation among the block copolymers might have occurred.

The size of the polymeric aggregates was determined by dynamic light scattering (Brookhaven BI-DS Instruments) at a scattering angle of 90° . The aqueous PEG-*b*-PLH solution constructed nanoparticles at pH 5.4 (sodium acetate buffer) and 7.4 (phosphate buffered saline) with two subpopulations.²¹ These reported phenomena are consistent with the previously described results concerning the amphiphilic block copolymer, poly(ethylene oxide)-*block*-poly(β -benzyl L-aspartate).²² The larger aggregates are thought to be such as al-

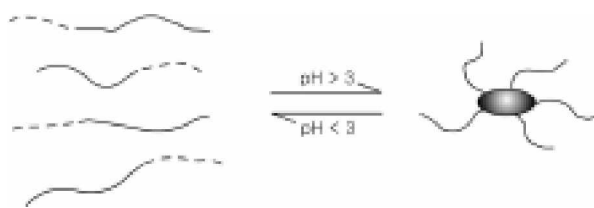


Figure 2. Schematic representation of the pH-dependent supra-molecular assembly of mPEG-*b*-PLH in an aqueous solution. Solid line is PEG chain and dotted line is poly(L-histidine).

ready observed in the size exclusion chromatography.

In conclusion, it was confirmed that the PEG-*b*-PLH copolymer could self-assemble in aqueous media in a pH-dependent manner. This pH-sensitive copolymer formed nano-sized particles above pH 3. As illustrated in Figure 2, it is presumed that PEG blocks form the outer shell of nanoparticles and the PLH segments construct the aggregated inner core.

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- $M_w = 5757$, $M_n = 5697$, $M_w/M_n = 1.01$, which is determined by MALDI-TOF mass spectrometry.
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- ^1H NMR (D_2O) δ 8.6, 7.2 (s, 2H, imidazole group), 4.6 (m, 1H, α -H), 3.6 (s, $-\text{CH}_2\text{CH}_2\text{O}-$), 3.3 (s, 3H, $\text{CH}_3\text{O}-$), 3.1 (br m, 2H, β -H). The M_w and M_n values of the product based on its structural formula were calculated to be 8224 and 8164, respectively.
- The calculated ratio based on the structure is 0.069.
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- The averaged diameter of each two population was 25.7, 183 (pH 5.4) and 26.9, 273 nm (pH 7.4).
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