Stably-dispersed and Surface-functional Bionanoparticles Prepared by Self-assembling Amphipathic Polymers of Hydrophilic Poly(γ-glutamic acid) Bearing Hydrophobic Amino Acids

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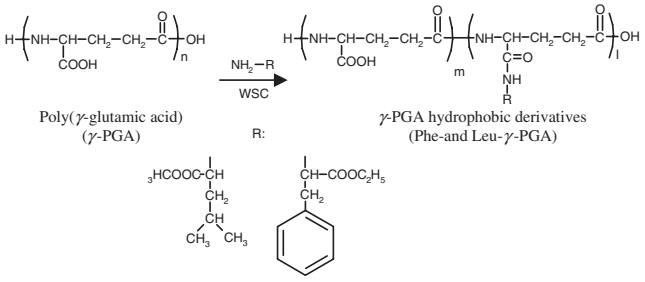
(Received November 27, 2003; CL-031153)

Surface-functional bionanoparticles were prepared by self-assembling amphipathic poly(γ-glutamic acid) derivatives with a hydrophilic backbone and hydrophobic phenylalanine and leucine side groups. Poly(γ-glutamic acid) bearing phenylalanine nanospheres exhibited excellent water-dispersibility, surface functionality and appropriate size (200 nm) for medical use. Additive PEG conjugation assisted nanoparticle formation of leucine-grafted poly(γ-glutamic acid).

Amphipathic polymers have received great interest in recent years because they can form self-assembled nano-organisms, i.e., micelles, vesicles, spheres, tubes, and fibers in organic solvent/water systems and aqueous solution. Block copolymer micelles and particles have been studied to serve as drug or gene delivery carriers.[1] In the past 20 years, we have studied the synthesis, characteristics and applications of core-corona self-assembling graft copolymer nanospheres composed of hydrophobic polystyrene and hydrophilic macromonomers.[2] Core-corona nanospheres have functional corona layers where drugs, peptides and viruses are conjugated. Core-corona nanospheres act as controlled release carriers and nanosphere-based vaccines.[3] However, the biodegradability and biocompatibility are required for medical use which polystyrene-based nanospheres cannot achieve. Although several biodegradable nanoparticles were recently reported,[4] they possess low dispersion-stability in soap-free aqueous conditions, low size-controllability, or low chemical reactivity on the surface. In drug carrier or nanosphere-based vaccines, soap is undesirable and more than a 100 nm size along with surface-reactivity is required for protein immobilization on the particle surface. In the present study, we tried to prepare bionanoparticles (more than a 100 nm size) with high water-dispersibility and surface reactivity, using poly(γ-glutamic acid) (γ-PGA). γ-PGA is produced by microorganisms which have a high molecular weight, biodegradability and biocompatibility.[5] We developed a modified method of γ-PGA to produce drug delivery carriers and tissue engineering materials.[6] Here we synthesized amphipathic γ-PGA derivatives bearing hydrophobic L-phenylalanine ethyl and L-leucine methyl esters.

Scheme 1 shows the synthesis of γ-PGA bearing L-phenylalanine ethyl esters (Phe–γ-PGA) and γ-PGA bearing L-leucine methyl esters (Leu–γ-PGA). Synthesis procedure is shown below: 4.7 unit mmol γ-PGA (number-average molecular weight, Mn, of 3.1×10^5, polydispersity of 1.2, donated by Meiji Seika Kaisya, Ltd.) was dissolved in 0.3 N–$NaHCO_3$ aqueous solution (50 mL), and an adequate amount of WSC, phenylalanine ethyl esters and leucine methyl esters (ACROS ORGANICS Co., New Jersey, USA) were added, and stirred for 30 min at 4 °C. After several hours, the solution became translucent and was maintained at room temperature for an additional 24 h. Low-molecular-weight chemicals were removed by dialysis using a Spectra/Pore membrane (cut-off molecular weight of 50000 Da) for 3 days. γ-PGA derivatives were obtained by freeze-drying for 3 days.



Scheme 1. Chemical structures of γ-PGA and γ-PGA hydrophobic derivatives.

Table 1. Preparation of Phe–γ-PGA and Leu–γ-PGA

	γ-PGA	HAA[a]	WSC	Yield	C.D.[b]
	unit mmol	mmol	mmol	%	%
Phe–γ-PGA-9	4.7	4.7	0.47	55	8.6
Phe–γ-PGA-20	4.7	4.7	2.3	51	20
Phe–γ-PGA-38	4.7	4.7	3.5	55	38
Phe–γ-PGA-42	4.7	4.7	7.0	53	42
Phe–γ-PGA-58	4.7	4.7	9.3	66	58
Leu–γ-PGA-25	4.7	12	12	43	25
Leu–γ-PGA-30	4.7	14	14	52	30

[a] HAA is hydrophobic amino acids (Phe and Leu).

[b] C.D.: Convertion degrees were determined by ^{1}H-NMR (400 MHz) using the integrals of the methylene peaks of γ-PGA and the phenyl group peaks of Phenylalanine. In the case of leucine, the methyl groups were estimated.

Reaction conditions are shown in Table 1. The degree of γ-PGA derivative conversion was determined by ^{1}H NMR. Phe–γ-PGA and Leu–γ-PGA substitutions were confirmed with IR spectra. Hydrophobic amino acid substitution ratios were controlled by altering the amount of WSC and hydrophobic amino acids. γ-PGA is known to dissolve in alkaline solutions, THF and DMF. On the other hand, in the case of Phe–γ-PGA-9, -20 and -38, samples were dissolved in water or DMF, DMSO and alkaline solutions. However, Phe–γ-PGA-42 and -58 were dissolved in chloroform, THF and DMSO.

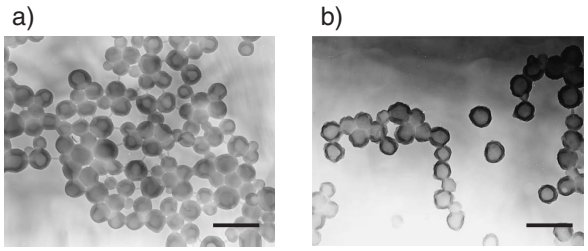


Figure 1. TEM micrographs of a): Phe–γ-PGA-58 and b): PEG-grafted Leu–γ-PGA-30. Scale bars are 500 nm.

10 mg of Phe–γ-PGAs and Leu–γ-PGAs were self-assembled by solvent exchange method (from 1 mL DMSO to 1 mL distilled water), which is normally used to form polymeric micelles consisting of diblock copolymers, to yield a clouded solution.[7] A drop of the solution was cast onto collodion-coated Cu-mesh for transmission electron microscopy (TEM) observation, followed by drying in vacuo and carbon-spattering. TEM images showed that Phe–γ-PGA-9, -20 and -38 showed coexistent among fiber-like and small-size aggregations. The small-size aggregations were not spherical and their sizes were polydisperse (<50–60 nm). Leu–γ-PGAs showed submicro–micro order aggregations. In contrast, Phe–γ-PGA-42 and -58 formed self-assembled nanospheres (Figure 1a). Diameter of Phe–γ-PGA-58 nanospheres approximated 200 nm, estimated by TEM (average of 50 particles). Phe–γ-PGA-58 nanospheres were stably dispersed in distilled water for a week. Furthermore, freeze-dried samples could be re-dispersed in aqueous solution, and reproduced almost the same particle size. The specific self-assembly behavior of γ-PGA derivatives having high phenylalanine content in aqueous solutions may be due to multiple phenyl groups stacking as shown in Figure 2. Leu–γ-PGA aggregation may be caused by loose packing of intermolecular hydrophobic interaction. These results suggest that the stacking effect of phenyl groups is important for self-assembly behavior of γ-PGA derivatives.

To prepare nanospheres from Leu–γ-PGA, amino-terminated PEG (PEG–NH_2: $M_w = 2000$, NOF Co., Tokyo, JAPAN) was additionally conjugated to Leu–γ-PGA-30 as follows. Leu–γ-PGA-30 and PEG–NH_2 were dissolved in distilled water, and then WSC was added and stirred for 24 h at room temperature, and dialyzed over 3 days. The degree of PEG conjugation was about 0.4%, estimated by ^{1}H NMR. PEG-grafted Leu–γ-PGA-30 self-assembled by solvent exchange method (from 1 mL DMSO to 1 mL distilled water). Figure 1b demonstrates the successful formation of PEG-grafted Leu–γ-PGA-30 nanoparticles in aqueous solution (diameter: about 225 nm). Nanoparticles were also stable in distilled water. These results indicated that PEG-grafted chains could enhance the hydrophilicity and inhibit the loose packing of intermolecular hydrophobic interaction of polymer chains. Both bionanoparticles contained an abundant amount of carboxylic groups, which was confirmed by infrared studies.

In conclusion, we demonstrated an effective method for producing bionanoparticles derived from biologically occurring molecules; γ-PGA and phenylalanine ethyl ester. Bionanoparticles had excellent water-dispersibility, 200 nm diameters and surface chemical functionality of carboxyl groups. Additional PEG conjugation supports nanoparticle formation of Leu–γ-PGA. These bionanoparticles should be conjugated to drugs, peptides and viruses onto the surfaces as biodegradable and biocompatible carriers in delivery systems and nanoparticle-based vaccinations.

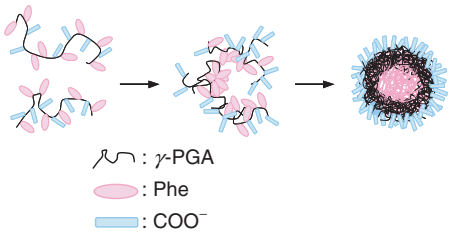


Figure 2. Schematic illustration of self-assembling Phe–γ-PGA-58.

This work was supported by Core Research for Evolutional Science and Technology (CREST) from Japan Science and Technology Agency (JST).

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Published on the web (Advance View) March 6, 2004; DOI 10.1246/cl.2004.398