Colloid Polym Sci (2008) 286:777–786
DOI 10.1007/s00396-008-1836-8

ORIGINAL CONTRIBUTION

Aggregates formation and pH response of mixed dodecyl-terminated copolypeptides containing tryptophan

Hideki Sugimoto · Eiji Nakanishi · Yumi Mizuno · Takashi Yasumura · Katsuhiro Inomata

Received: 4 October 2007 / Revised: 18 December 2007 / Accepted: 24 December 2007 / Published online: 24 January 2008

Abstract In this study, two kinds of amphiphilic copolypeptide, C_{12}-poly(*N*-hydroxyethyl L-glutamine-*co*-L-tryptophan) (C_{12}-EGT-T) and C_{12}-poly(L-glutamic acid-*co*-L-tryptophan) (C_{12}-GAT-T) were prepared. By using the mixture of these amphiphilic copolypeptides, pH response of aggregate formation, critical aggregate concentration, the sizes of the aggregates and sustained-release behavior of model substances were investigated. The mixture of the amphiphilic copolypeptides formed aggregates in aqueous medium and showed the ability to uptake model substances such as pyrene and 5-fluorouracil into their hydrophobic moiety. Moreover, pH response was observed in the sustained-release behavior of model substances and the pH region where these properties changed was from pH 7.4 to pH 4.5 in the mixture of C_{12}-EGT-T/C_{12}-GAT-T at 50/50 (mol/mol). These results could be explained by dissociation of trifluoroacetic-acid-treated tryptophan residues in the hydrophilic moieties and destabilization of the aggregates by electrostatic repulsion.

Keywords Amphiphilic copolypeptide · Aggregates formation · pH response

Introduction

Amphiphilic polymer have attracted considerable attention due to the capability to self assemble in aqueous solution [1–6]. Multitudes of amphiphilic polymers have been synthesized and their aggregative properties were investigated. Amphiphilic polymer aggregation systems have very interesting properties and the morphologies could be controlled by stimuli (temperature, pH, and so on), so the polymers have numerous underlying value in application areas such as microcapsules, drug delivery system (DDS), and stabilization of emulsion [7–11]. DDS is a particularly interesting field because such systems are not only able to deliver the medicine to the organ which needs it but can improve therapeutic effects and reduce adverse effects [12–15]. Kataoka and coworkers [16–18] have developed polymeric micelle carrier system using biodegradable polymer including poly(ethylene oxide) and polypeptides.

Synthetic polypeptides composed of the same basic units as those of proteins are expected to be excellent functional materials. Polypeptides also exhibit several conformations such as α-helices, β-sheets, and random coils, depending on pH and the kind of amino acid used, and are expected to serve as intelligent materials for DDS and other applications [19, 20]. However, applied DDS studies taking advantage of the dissociation or the conformation changes of polypeptides with change in pH have rarely been reported.

In our previous study, it was found that L-tryptophan (Trp), having a bulky indole ring and well known as the most hydrophobic of natural amino acids, treated with trifluoroacetic acid (TFA) exhibited pH-reversible color changes from red (below pH 4.0) to yellow (above pH 5.5) and that the color changes were caused by dissociation of the indole ring of Trp [21]. Moreover, poly(*N*-hydroxyethyl L-glutamine)-*graft*-poly(L-Trp) and poly(*N*-hydroxyethyl L-glutamine)-*block*-poly(L-Trp) containing TFA-treated poly(Trp) as hydrophobic component showed a pH response in critical aggregation concentration, the size of aggregates, release behavior of model substances [22, 23]. However, the pH response region was shifted unfortunately to lower pH

H. Sugimoto · E. Nakanishi (✉) · Y. Mizuno · T. Yasumura · K. Inomata
Graduate School of Engineering, Nagoya Institute of Technology, Gokiso-cho, Showa-ku,
Nagoya, Aichi 466-8555, Japan
e-mail: nakanishi.eiji@nitech.ac.jp

(between pH 5.0 and pH 2.0) than that of TFA-treated L-Trp monomer by robust aggregation of hydrophobic poly(Trp) moieties.

The aim of the present study was to prepare pH response copolypeptide carrier in which the aggregated structure changes in the neutral pH region.

In this study, two types of dodecyl-terminated copolypeptide, C_{12}-poly(*N*-hydroxyethyl L-glutamine-*co*-L-tryptophan) (C_{12}-EGT-T) and C_{12}-poly(L-glutamic acid-*co*-L-tryptophan) (C_{12}-GAT-T), were prepared by aminolysis or saponification of starting copolypeptide, C_{12}-poly(γ-benzyl L-glutamate-co-Trp) (C_{12}-GT), with 2-amino-1-ethanol or 0.1N NaOH *aq*. These amphiphilic copolypeptides were then treated with TFA to obtain the pH response amphiphilic copolypeptides C_{12}-EGT-T and C_{12}-GAT-T. The aggregation behavior, model substance uptake ability, and pH dependence of sustained-release behavior of mixture of C_{12}-EGT-T and C_{12}-GAT-T were evaluated. These dodecyl-terminated copolypeptides treated with TFA were expected to show the pH response in aggregate formation and release of model substance at pH region which is similar to Trp monomer treated with TFA by introducing the tryptophan residues into the hydrophilic part.

Experimental

Materials

Synthesis of starting dodecyl-terminated copolypeptide, C_{12}-poly(γ-benzyl L-glutamate-co-Trp)

Starting copolypeptide consisting of hydrophobic dodecyl (C_{12}) alkyl chain, γ-benzyl L-glutamate and L-Trp, C_{12}-poly (γ-benzyl L-glutamate-*co*-Trp) (C_{12}-GT), was obtained by polymerization of γ-benzyl L-glutamate (γ-BLG) and L-Trp *N*-carboxyanhydride (NCA) with *n*-dodecylamine (C_{12}-NH_2). γ-BLG and Trp NCA were obtained according to the method described in previous papers [22, 23]. Each NCA was recrystallized from ethyl acetate with petroleum ether. The γ-BLG NCA (80mol%) and Trp NCA (20mol%) were dissolved in 1/1 (*v*/*v*) mixture of dioxane and dichloromethane (conc. 10wt.%) and then C_{12}-NH_2 as an initiator was added at NCA-to-C_{12}-NH_2 molar ratio of 20 to obtain C_{12}-GT. The C_{12}-GT was precipitated in an excess of cold methanol and dried in vacuo. All solvents used in synthesis of C_{12}-GT were distilled twice.

Preparation of C_{12}-poly(N-hydroxyethyl L-glutamine-co-L-tryptophan)

The amphiphilic copolypeptide, C_{12}-EGT-T, was prepared by aminolysis of C_{12}-GT with 2-amino-1-ethanol at 50 °C for 5days. The poly(*N*-hydroxyethyl L-glutamine-*co*-L-tryptophan) (EGT) part is water soluble and it seems that EGT does not affect the aggregate behavior because the conformation of EGT in water is random coil [24]. Therefore, EGT was used as a hydrophilic component in amphiphilic copolypeptide. The amphiphilic copolypeptide was obtained by dialysis of the reaction mixture (molecular cutoff 3,500) and lyophilized. Debenzylation of C_{12}-GT was confirmed by the disappearance of absorption due to ester groups at 1,720cm^{-1} in Fourier transform infrared (FTIR) spectrum.

Preparation of C_{12}-poly(L-glutamic acid-co-L-tryptophan)

The amphiphilic copolypeptide, C_{12}-GAT-T, was prepared by saponification of C_{12}-GT with 0.1N NaOH *aq* at r.t. The poly(L-glutamic acid-*co*-L-tryptophan) (GAT) part is also water soluble. The amphiphilic copolypeptide was obtained by dialysis of the reaction mixture (molecular cut off, 3,500) and lyophilized. Debenzylation of C_{12}-GT was confirmed by the disappearance of absorption due to ester groups at 1,720cm^{-1} in FTIR spectrum.

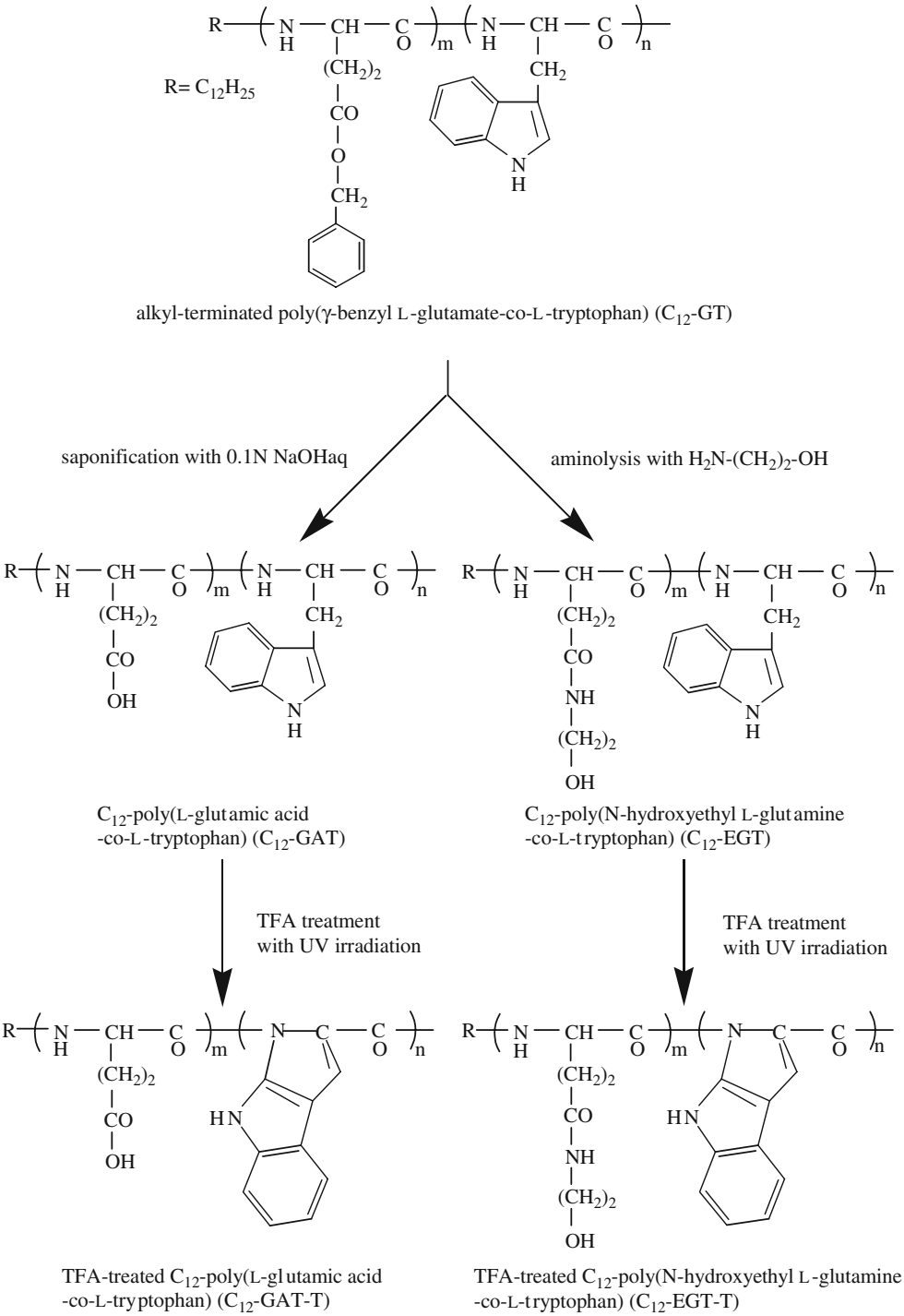
Preparation of pH response dodecyl-terminated copolypeptides with TFA

The amphiphilic copolypeptides prepared above were dissolved in TFA with irradiating ultraviolet (UV; wavelength 365nm, intensity 300μW/cm^2) for 3days [21–23]. The two kinds of amphiphilic copolypeptides treated with TFA, C_{12}-EGT-T and C_{12}-GAT-T, were obtained by dialysis of the reaction mixture and lyophilized. Table 1 shows compositions of C_{12}-EGT-T and C_{12}-GAT-T evaluated by ^{1}H-nuclear magnetic resonance (NMR) in dimethyl sulfoxide (DMSO)-d_6. The compositions were calculated with integral curve at 7ppm (for Trp residue), 2.2–2.6ppm (for β,γ methylene in glutamic residue), and 1.2ppm (for methyl in dodecyl group). A schematic diagram of preparation of amphiphilic copolypeptide treated with TFA is shown in Scheme 1.

Table 1 The compositions of amphiphilic copolypeptides evaluated by ^{1}H-NMR in DMSO-d_6

Sample code	Molecular weight			Trp cont (mol%)
	PHEG	PGA	Trp	
C_{12}-EGT	3268	–	750	17.9
C_{12}-EGT-T	2924	–	699	18.4
C_{12}-GAT	–	2838	791	20.8
C_{12}-GAT-T	–	2838	779	20.6

Scheme 1 Preparation scheme of pH response amphiphilic copolypeptides



Preparation of dodecyl-terminated copolypeptides aggregates including model substances

The mixed amphiphilic copolypeptides aggregates including model substances, pyrene (Py) and 5-fluorouracil (FU), into hydrophobic core were prepared by dissolving of each amphiphilic copolypeptide in the aqueous solution of model substance at given amount, and then the ultrasonic wave was irradiated for the solution. The amphiphilic copolypeptides–model substance mixed solution was transferred into cellulose tube (molecular cutoff 3,500) to remove excess amount of model substance and dialyzed in distilled water until the model substance was not detected in the outside solution. The uptake amount of model substance was determined with a preprepared analytical curve. Fluorescence intensity of 384nm (excitation wavelength 336nm) was employed for Py. In the

case of 5-FU, the absorption intensity at 260nm was measured with UV–visible (Vis) spectrophotometer.

Reagents and solvents

All reagents and solvents were purchased from Peptide Institute Inc. (amino acids) and Nacalai Tesque, Inc. and were used without further purification except for the solvents used in copolypeptide synthesis. Py as a nonionic model drug and 5-FU as an anticancer drug were used in this study. 5-FU is known as conventional antimetabolite [25] and produces adverse effects such as dehydration, inflammation of the intestines, and so on. Moreover, solubility of 5-FU for water is very low. Therefore, administration of 5-FU by DDS seems to be a very significant method [26–28].

Measurements

Methods

The composition of amphiphilic copolypeptide was estimated by ^{1}H-NMR (BRUKER Model AVANCE200 spectrometer) measurements. DMSO-d_6 was used for the measurement. To confirm debenzylation in aminolysis and saponification reaction, FTIR (Nicolet Instruments Model AVATAR 320S FTIR spectrophotometer) measurements were carried out. FTIR spectra were measured by the KBr method in the region of 4,000–400cm^{-1}. To evaluate pH response of copolypeptide treated with TFA, UV–Vis absorption spectra were measured by a HITACHI Model U-3310 spectrophotometer with a quartz cell having a path length of 1.0cm. The pH was adjusted by 1.0N HCl *aq* and 1.0N NaOH *aq*. Fluorescence spectroscopy was performed in a SHIMADZU Model RF-5300FC spectrophotofluorometer to evaluate the critical aggregate concentration (CAC) and aggregate stability of amphiphilic copolypeptides. 1-Anilino-8-naphtalene sulfonic acid magnesium salt (ANS) and Py were used as fluorescent probe. The excitation wavelength of 350 and 336nm were employed for the measurement. To elucidate the conformation of amphiphilic copolypeptide, circular dichroism (CD) measurements were carried out with a JASCO Model J-725 CD/ORD spectrometer with a quartz cell having a path length of 0.1 and 1.0cm. Dynamic light scattering (DLS) measurement was carried out with a cylindrical cell having a diameter of 12mm in order to estimate the size of the aggregates formed by amphiphilic copolypeptides. He–Ne laser (632.8nm) was used as the light source for the measurement. Atomic force microscope (AFM) analysis was performed with NanoScope® IIIa from Veeco Instruments in tapping mode to observe the morphology of the aggregates. The sample was prepared by drying the aqueous solution including the aggregates on silicon wafer at r.t. for 5h and then at 100 °C for 5min [29].

Evaluation of aggregates stability of dodecyl-terminated copolypeptides mixture

The fluorescence intensity of Py which is not included in aggregates is strongly affected and quenched by existence of Cu^{2+} ion [30, 31]. The stability of mixed amphiphilic copolypeptides aggregates was evaluated by monitoring of fluorescence intensity of Py when Cu^{2+} ion was added to amphiphilic copolypeptides–Py solution at desired pH. The ratio of quenching of fluorescence was calculated by following equation,

$$\text{The ratio of quenching} = I_q/I_0$$

where, I_q is fluorescence intensity of Py with Cu^{2+} ion and I_0 is fluorescence intensity of Py without Cu^{2+} ion. The mixed amphiphilic copolypeptides and Py concentrations in the measurements were 1.5×10^{-4}mol/l and 1.2×10^{-7}mol/l, respectively.

Evaluation of release behavior from mixed dodecyl-terminated copolypeptides aggregates with 5-FU

The solution of mixed amphiphilic copolypeptides aggregates including 5-FU was poured into a dialysis tube (molecular weight cutoff 3,500) [32]. The tube was immersed into desired pH solution and the released amount of model drug of external solution was measured with time by spectroscopic analytical method. The absorption intensity at 260nm was measured with UV–Vis spectrophotometer. The released percentage was calculated by the following equation:

$$\text{Released percentage}\ (\%) = C_r/C_0 \times 100$$

where, C_r is released amount of model substance and C_0 is initial uptake amount of model substance.

Results and discussion

pH response of dodecyl-terminated copolypeptides

Figure 1 shows pH dependence of Vis spectra of C_{12}-EGT-T, C_{12}-GAT-T, and the mixture. The amphiphilic copolypeptide treated with TFA, C_{12}-EGT-T and C_{12}-GAT-T, showed variations in Vis spectra in the region from pH 3.0 to pH 5.0 and from pH 5.5 to pH 7.0, respectively, and the effect which introduced Trp residues treated with TFA into the hydrophilic part in amphiphilic

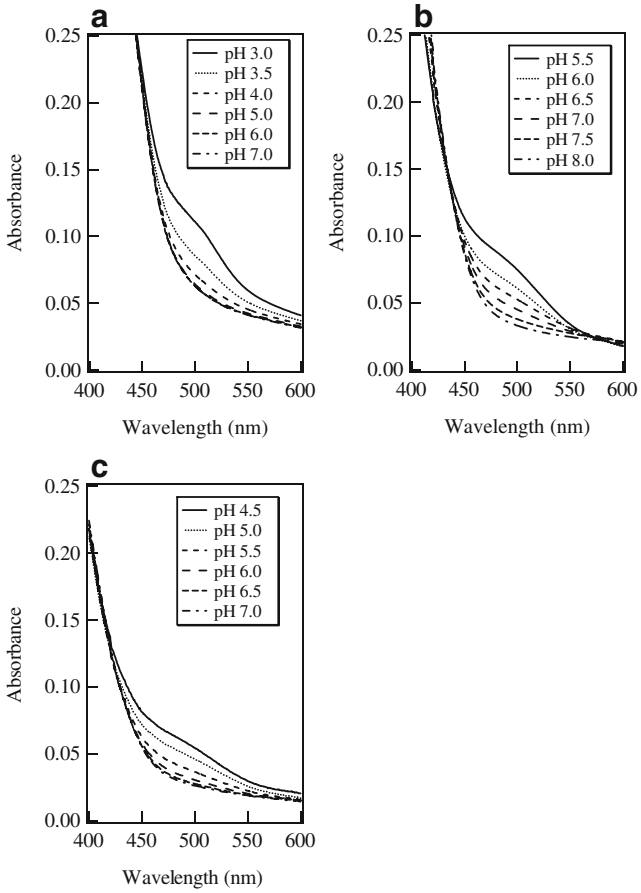


Fig. 1 Vis spectra of deodecyl-terminated copolypeptide at various pH, **a** C_{12}-EGT-T, **b** C_{12}-GAT-T and **c** mixture. Copolypeptide conc.= 5.0×10^{-3} mol/l

copolypeptide was able to be confirmed. On the other hand, pH dependence of Vis spectrum was not observed in untreated C_{12}-EGT and C_{12}-GAT. The indole ring of untreated Trp has weak basicity and it cannot dissociate because the lone pair of nitrogen is employed to hold the aromaticity. On the other hand, the ten π-electrons needed to form an aromatic ring exist in TFA-treated Trp (see the structure of Trp residues after TFA treatment in Scheme 1), except for the lone pair of nitrogen, so that TFA-treated Trp has a strong basicity compared with normal Trp and is regarded as aromatic amine. In a previous study, a TFA-treated random copolypeptide, poly(*N*-hydroxyethyl L-glutamine-*co*-L-Trp), showed variation in the Vis spectrum in the region from pH 4.0 to pH 5.5, and nonaqueous titration revealed that the color change was caused by dissociation of the indole ring of Trp treated with TFA [21]. It is considered that TFA-treated Trp residues in the amphiphilic copolypeptide dissociated at lower pH region because the TFA-treated amphiphilic copolypeptide showed similar color change and pH response by TFA treatment as the TFA-treated random copolypeptide. The difference in dissociation pH of TFA-treated Trp between C_{12}-EGT-T and C_{12}-GAT-T seems attributable to the difference in hydration state and accelerate of protonation of the indole ring in TFA-treated Trp by existence of surrounding carboxylic acids of glutamic acid residues [33]. Table 2 summarized pH response regions of mixed amphiphilic copolypeptides by the same UV–Vis method. In all compositions, the mixed amphiphilic copolypeptides indicated intermediate pH response region between C_{12}-EGT-T and C_{12}-GAT-T. Moreover, in Fig. 1c, two-step change or increment of absorbance below pH 4.5 was not observed. From this result, C_{12}-EGT-T and C_{12}-GAT-T seem to have formed the aggregates in two components with the common hydrophobic domain. The pH response region of mixture was affected by copolypeptides composition, that is, the concentration of carboxylic acids of glutamic acid residues in mixture, and pH response region of the 50/50mol mixture was from pH 7.0 to pH 4.5. This pH response region is similar to the pH change as the lysosome endocytoses material in the cell [34], and the mixed copolypeptide aggregates seems suitable for DDS. In the next sections or later, CAC, size of aggregates, and release behavior of the mixture of C_{12}-EGT-T and C_{12}-GAT-T at 50/50 (mol/mol) were evaluated.

Secondary structure of dodecyl-terminated copolypeptides

Conformation of amphiphilic copolypeptides in aqueous solution was evaluated by CD measurement. Figure 2 indicates CD spectra of C_{12}-EGT and C_{12}-GAT at various pH. CD spectra of C_{12}-EGT confirm that random coil was dominant at measurement pH region. Same result was obtained for TFA-treated C_{12}-EGT-T and the conformation was random coil. C_{12}-GAT showed pH

Table 2 The pH response regions of amphiphilic copolypeptide mixtures in UV–Vis measurement

Molar ratio (C_{12}-EGT-T:C_{12}-GAT-T)	pH response region
100:0	5.0–3.0
90:10	formed precipitates
80:20	formed precipitates
70:30	6.5–4.5
60:40	6.5–4.5
50:50	7.0–4.5
40:60	7.0–4.5
30:70	7.0–4.5
20:80	7.5–5.0
10:90	7.5–5.0
0:100	8.0–5.5

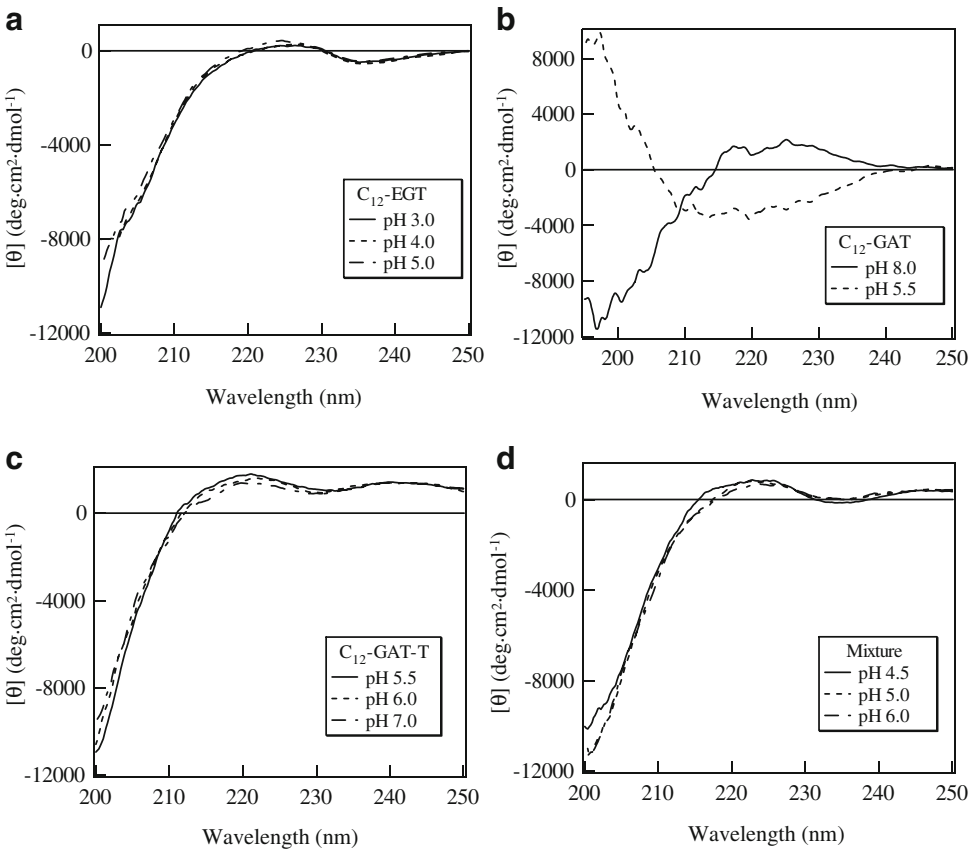


Fig. 2 CD spectra of dodecyl-terminated copolypeptides at various pH, **a** C_{12}-EGT, **b** C_{12}-GAT, **c** C_{12}-GAT-T and **d** Mixture. Copolypeptide conc.=3.0×10^{-3} mol/l

dependence of the conformation (see Fig. 2b) and formed α-helix slightly at pH 5.5 which corresponds to pKa of carboxylic acids of glutamic acid residues. On the other hand, the C_{12}-GAT-T and mixed dodecyl-terminated copolypeptide did not form α-helix below pKa of carboxylic acids of glutamic acid residues and their conformation were still random coil (see Fig. 2c,d). These results in Vis and CD measurements (shift of dissociation pH of TFA-treated Trp and delay of α-helix formation) seem to suggest that TFA-treated Trp residues and carboxylic acids in glutamic acid residues may have induced the dissociation of the each other.

Evaluation of critical aggregates concentration of mixed dodecyl-terminated copolypeptides

The CACs of mixed amphiphilic copolypeptides were evaluated by fluorescence measurements with ANS. In the fluorescence spectrum of ANS, fluorescence maximum wavelength (λmax) shifts to shorter wavelength (blue shift) when solvent polarity around this probe molecule was decreased. Figure 3 shows the variation of λmax for ANS as a function of mixed amphiphilic copolypeptides concentration at pH 7.4. The blue shift of λmax from approximately 525nm to approximately 470nm was observed with increasing mixed copolypeptides concentration. This blue shift of λmax reflects the formation of amphiphilic copolypeptide aggregates and uptake of ANS into their hydrophobic cores. The inflection point in the curve was qualified as CAC. The CACs for mixed amphiphilic copolypeptides obtained from the fluorescence measurements were listed in Table 3 with the CACs of each TFA-treated and untreated dodecyl-terminated copolypeptides.

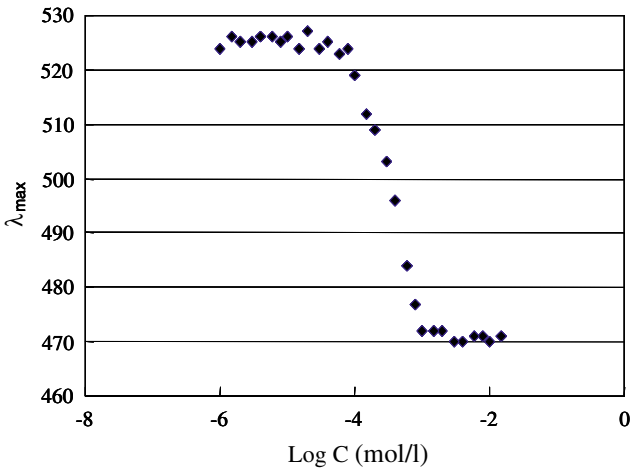


Fig. 3 Variation of fluorescence maximum wavelength (λmax) for ANS as a function of mixed amphiphilic copolypeptides (C_{12}-EGT-T/C_{12}-GAT-T=50/50) concentration. [ANS]=1.6×10^{-6} mol/l

Table 3 The CACs of each amphiphilic copolypeptide

Sample code	pH	CAC (mol/l)	Dissociating groups
C_{12}-EGT	5.0	3.5×10^{-4}	Non
	3.0	3.0×10^{-4}	Non
C_{12}-EGT-T	5.0	1.0×10^{-4}	Non
	3.0	6.0×10^{-4}	TFA-treated Trp
C_{12}-GAT	8.0	2.4×10^{-4}	–COOH
	5.5	2.0×10^{-4}	–COOH
C_{12}-GAT-T	8.0	0.9×10^{-4}	–COOH
	5.5	3.6×10^{-4}	–COOH, TFA-treated Trp
Mixture	7.4	1.1×10^{-4}	–COOH
C_{12}-EGT-T/C_{12}-GAT-T=50/50	4.5	7.5×10^{-4}	–COOH, TFA-treated Trp

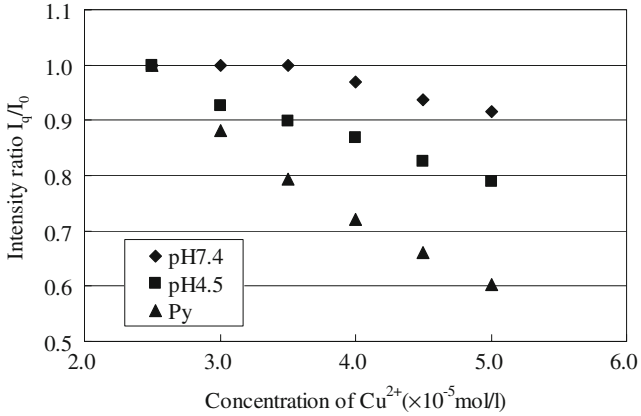


Fig. 4 Variation of fluorescence intensity ratio (I_q/I_0) for Py against Cu^{2+} concentration. The mixed amphiphilic copolypeptides (C_{12}-EGT-T/C_{12}-GAT-T=50/50) and Py concentrations in the measurements were 1.5×10^{-4} and 1.2×10^{-7} mol/l, respectively

The CACs of untreated dodecyl-terminated copolypeptides were not changed drastically at measured pH. While, mixed amphiphilic copolypeptides exhibited different CAC with pH, and the CACs became intermediate value or almost equal to those of independent TFA-treated dodecyl-terminated copolypeptides and the mixture indicated only one CAC value. If C_{12}-EGT-T and C_{12}-GAT-T form aggregates independently, two critical micelle concentration (CMC) values or higher CMC value than that of each component will be observed. This result seems to support that C_{12}-EGT-T and C_{12}-GAT-T formed the aggregates in two components with the common hydrophobic domain. Moreover, it is considered that the difference of CAC originates from the dissociation of TFA-treated Trp moieties and the aggregates of mixed TFA-treated amphiphilic copolypeptides were destabilized by electrostatic repulsion, hydration of Trp residues, and lowering of hydrophobic coagulation power of the aggregates at lower pH region.

Evaluation of aggregates stability of dodecyl-terminated copolypeptides mixture

The stability of mixed amphiphilic copolypeptides aggregates against pH was estimated by quenching of fluorescence intensity of Py with Cu^{2+}. Figure 4 shows the variation of fluorescence intensity ratio (I_q/I_0) against Cu^{2+} concentration. In the Py solution without the mixed amphiphilic copolypeptide, the fluorescence of Py was strongly quenched by the addition of Cu^{2+} ion and the decrease of the fluorescence intensity became slow with increase of Cu^{2+} ion concentration. While in the case of mixed TFA-treated amphiphilic copolypeptides aggregates, large difference in quenching of fluorescence of Py against pH was observed and the fluorescence of Py was greatly quenched at pH 4.5 in comparison with the result at pH 7.4. This phenomenon indicates that the aggregate-formed amphiphilic copolypeptides become unstable because TFA-treated Trp dissociates at pH 4.5.

Evaluation of the size of mixed amphiphilic copolypeptides aggregates

To evaluate the size of mixed amphiphilic copolypeptides aggregates, DLS measurements were performed and the results are summarized in Table 4 with the results of each TFA-treated and untreated amphiphilic copolypeptide. The DLS measurements revealed that amphiphilic copolypeptides, C_{12}-EGT and C_{12}-GAT, formed aggregates with approximately 80 and 100nm in Rh(θ→0), respectively. The pH dependence of aggregate size was observed for mixed TFA-treated amphiphilic copolypeptides as same as independent C_{12}-EGT-T and C_{12}-GAT-T. The variation of Rh(θ→0) were *approximately* 65nm at pH 7.4 and *approximately* 50nm at pH 4.5. This changes in Rh(θ→0) of aggregates caused by changing pH originated in

Table 4 The sizes of amphiphilic copolypeptide aggregates evaluated by DLS measurement

Sample code	pH	Rh (θ→0)	Size distribution
C_{12}-EGT	5.0	78.8	0.069
	3.0	80.3	0.067
C_{12}-EGT-T	5.0	74.3	0.090
	3.0	51.7	0.490
C_{12}-GAT	8.0	107.5	0.033
	5.5	110.8	0.034
C_{12}-GAT-T	8.0	122.7	0.120
	5.5	90.9	0.890
Mixture	7.4	64.5	0.069
C_{12}-EGT-T/C_{12}-GAT-T=50/50	4.5	48.2	1.000

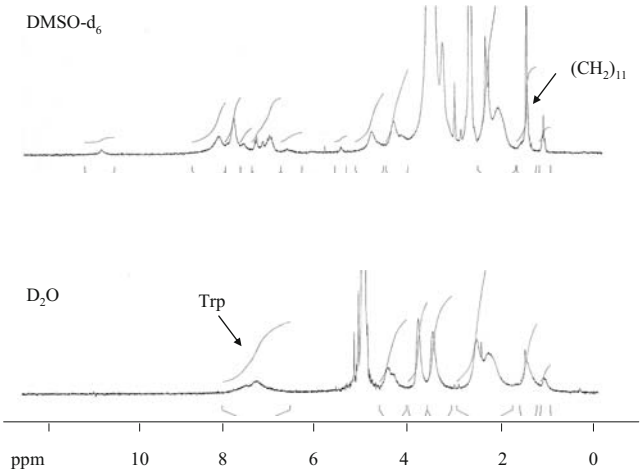


Fig. 5 ^{1}H-NMR spectra of C_{12}-EGT-T/C_{12}-GAT-T mixture in DMSO-d_6 and D_2O

dissociation and electrostatic repulsion of TFA-treated Trp moieties. If the mixture of C_{12}-EGT and C_{12}-GAT form simple core–shell type micelle, the Rh of spherical micelle formed by these amphiphilic copolypeptide can be calculated from 3.4nm (for C_{12}-GAT only) to 4.0nm (for C_{12}-EGT only) [35]. Therefore, it is not possible to explain the size of aggregates formed by these amphiphilic copolypeptides in terms of a simple core–shell structure. Figure 5 indicates ^{1}H-NMR spectra of C_{12}-EGT-T–C_{12}-GAT-T mixture in DMSO-d_6 and D_2O. As can be seen from the Fig. 5, it was confirmed that not only signal of alkyl chain (around 1–2ppm) but also signal of Trp residues (around 7–8ppm) became broad peak in D_2O. The large increase in aggregate size indicates formation of secondary aggregates, originating from weak aggregation of Trp residues in hydrophilic part and the weak interaction does not affect the dissociation of Trp residues treated with TFA. Conversely, the aggregate size decreases at lower pH region as decrease of the average number of associated molecules decreases because of the disruption of the hydrophobic domains caused by dissociation of Trp. The model proposed to account for the change in aggregate size is presented in Fig. 6. The AFM images of TFA-treated amphiphilic copolypeptide aggregates in dry state are shown in Fig. 7. Though the presence of aggregates with approximately 60nm was confirmed in AFM images, the aggregates had a wide-size distribution; aggregates of *approximately* 30–80-nm diameter were also observed.

Release behavior of model substance from amphiphilic copolypeptide aggregates

The uptake abilities of the aggregates were estimated using 5-FU by measuring the amount of 5-FU dissolved into the hydrophobic region of the mixed copolypeptides aggregates. Uptake amounts and loading efficiencies of 5-FU for mixed amphiphilic copolypeptides aggregates is summarized in Table 5 with values of each TFA-treated and untreated amphiphilic copolypeptide. The loading amount (wt.%) of each model substance was calculated from the weight of 5-FU loaded into aggregates divided by the weight of the loaded aggregates. The loading efficiency (%) was evaluated by dividing the weight of 5-FU loaded into aggregates by the initial weight of 5-FU. The difference of primary structure did not influence the uptake amount and loading efficiency of 5-FU. However, the TFA treatment greatly influenced the uptake amount and efficiency, and these values for TFA-treated amphiphilic copolypeptides became higher than those of untreated amphiphilic copolypeptides. The increase of uptake amount and efficiency seems to originate from increase of hydrophobicity of aggregates by the change of Trp residues with TFA treatment to tricyclic structure [19–21], as well as the difference in CACs (see Table 3). The uptake amount and loading efficiency of

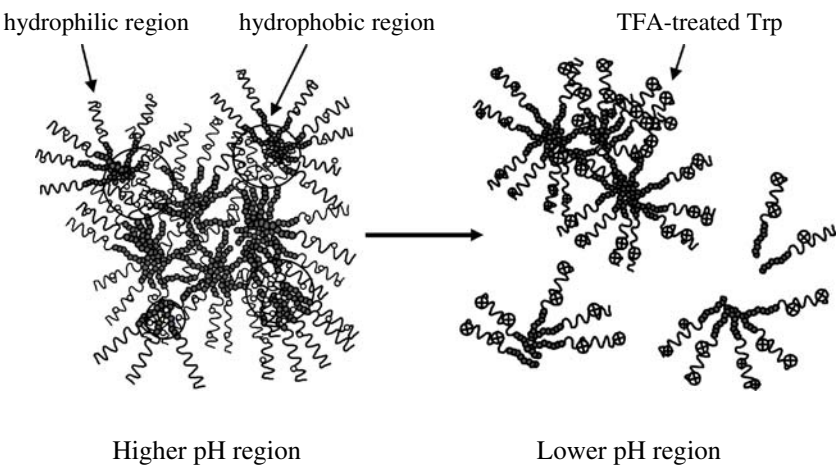


Fig. 6 Presumed model of aggregate size change in pH response amphiphilic copolypeptide aggregates

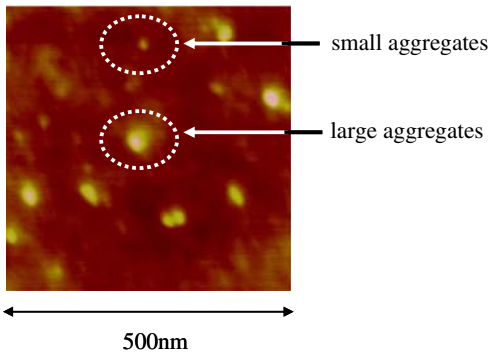


Fig. 7 AFM images of mixed amphiphilic copolypeptide aggregates (C_{12}-EGT-T/C_{12}-GAT-T=50/50) on silicone wafer (dry state)

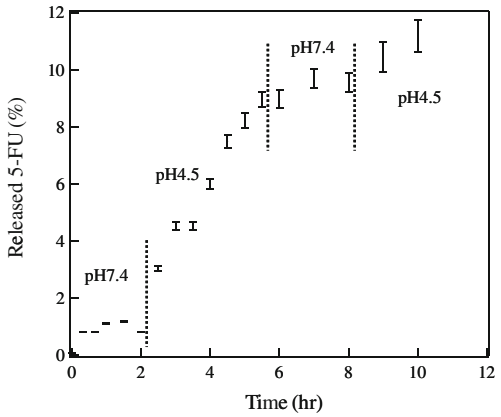


Fig. 8 Release profiles of 5-FU from mixed amphiphilic copolypeptides aggregate (C_{12}-EGT-T/C_{12}-GAT-T=50/50)

mixed amphiphilic copolypeptides aggregates was similar to those of independent TFA-treated amphiphilic copolypeptides. Figure 8 shows the pH dependence of release behavior of 5-FU from mixed amphiphilic copolypeptides aggregate. The amount of 5-FU released was normalized with the total amount of 5-FU incorporated into each aggregate. As can be seen from Fig. 8, the release rate of 5-FU was accelerated by changing the pH from 7.4 to 4.5 and started to decrease when the pH increased to 7.4 again. At pH 7.4, the release of 5-FU was negligible. This release increased with decreasing pH, and continuous release was observed at pH 4.5. Thus, although 5-FU was retained in the hydrophobic regions of the aggregates at pH 7.4, they were released at pH 4.5, since the hydrophobic interaction holding the aggregates together were weakened by dissociation of TFA-treated Trp.

Table 5 The loading amounts and loading efficiencies of 5-FU for each amphiphilic copolypeptide aggregate

Sample code	Loading amount[a] (wt.%)	Loading efficiency[b] (wt.%)
C_{12}-EGT	1.2	7.9
C_{12}-EGT-T	2.0	19.2
C_{12}-GAT	1.3	8.8
C_{12}-GAT-T	2.4	20.1
Mixture (C_{12}-EGT-T/C_{12}-GAT-T=50/50)	2.5	18.8

Polymer concentration=3×10^{-3} M; 5-FU concentration=1.5×10^{-3} M; loading pH: pH 5.0 (series of C12-EGT), pH 8.0 (series of C12-GAT), pH 7.4 (mixture); solvent: distilled water.

[a] Loading amount (wt.%)=weight of 5-FU loaded into aggregates/weight of loaded aggregates

[b] Loading efficiency (wt.%)=weight of 5-FU loaded into aggregates/initial feed weight of 5-FU

Conclusions

In this study, aggregates of mixed amphiphilic copolypeptides were prepared, and their pH response and release behavior were investigated. The major conclusions of this investigation were as follows:

(1) The dodecyl-terminated copolypeptides treated TFA showed the pH response at pH region which is similar to Trp monomer treated with TFA by introducing the tryptophan residues into the hydrophilic part.

(2) The pH response region of TFA-treated amphiphilic copolypeptides could be shifted to near natural pH region by mixing of each TFA-treated amphiphilic copolypeptide.

(3) The mixed TFA-treated amphiphilic copolypeptides, C_{12}-EGT-T/C_{12}-GAT-T, in aqueous solution formed aggregates containing hydrophobic region and these aggregates could assimilate model substances into the hydrophobic region.

(4) The mixture of TFA-treated amphiphilic copolypeptides showed pH dependence of aggregate size: dissociation of TFA-treated Trp residues resulted in a change in average aggregate diameter from *approximately* 65 nm at pH 7.4 to *approximately* 50 nm at pH 4.5.

(5) In studies of release behavior of 5-FU from mixed amphiphilic copolypeptides aggregates, release rates of 5-FU was found to be accelerated by decreasing the pH 7.4 to 4.5, and release rate was depressed when the solution pH rose to 7.4 again.

Thus, novel pH-responsive aggregates were prepared by using amphiphilic copolypeptides treated with TFA. In the future, more detailed examination and DDS applications will be carried out.

References

1. Kataoka K, Harada A, Nagasaki Y (2001) Adv Drug Deliv Rev 47:113
2. Zhulina EB, Borisov OV (2002) Macromolecules 35:9191
3. Verdonck B, Gohy JF, Khousakoun E, Jérôme R, Prez FD (2005) Polymer 46:9899
4. Lokitz BS, Convertine AJ, Ezell RG, Heidenreich A, Li Y, McCormick CL (2006) Macromolecules 39:8594
5. Kim BS, Mather PT (2006) Polymer 47:6202
6. Ge Z, Xie D, Chen D, Jiang X, Zang Y, Liu H, Liu S (2007) Macromolecules 40:3538
7. Murthy KS, Ma QG, Clark CG, Remsen EE, Wooly KL (2001) J Chem Soc Chem Commun 2001:773
8. Lee ES, Shin HJ, Na K, Bae YH (2003) J Control Rel 90:363
9. Hayashi H, Iijima M, Kataoka K, Nagaaki Y (2004) Macromolecules 37:5389
10. Verdonck B, Du PF, Goethals EJ (2003) Macromol Chem Phys 204:2090
11. Garnier S, Laschewsky A (2006) Langmuir 22:4044
12. Yokoyama M (1998) J Jpn Soc Biomater 16:276
13. Maeda H, Seymour LW, Miyamoto Y (1992) Bioconjugate Chem 3:351
14. Ogawa Y (2000) Drug Deliv Syst 15:429
15. Thunemann AF, Beyermann J, Kukula H (2000) Macromolecules 33:5906
16. Kumagai M, Imai Y, Nakamura T, Yamasaki Y, Sekino M, Ueno S, Hanaoka K, Kikuchi K, Nagano T, Kaneko E, Shimokado K, Kataoka K (2007) Colloids Surf B Biointerfaces 56:174
17. Yuan X, Harada A, Yamasaki Y, Kataoka K (2005) Langmuir 21:2668
18. Itaka K, Yamauchi K, Harada A, Nakamura K, Kawaguchi H, Kataoka K (2003) Biomaterials 24:4495
19. Kim KS, Kim TK, Graham NB (1999) Polym J 31:813
20. Nakanishi E (1999) Kobunshi 48:407
21. Sugimoto H, Nakanishi E, Hibi S (1998) Polymer 39:5739
22. Sugimoto H, Nakanishi E, Hanai T, Yasumura T, Inomata K (2004) Polym Int 53:972
23. Sugimoto H, Nakanishi E, Yamauchi F, Yasumura T, Inomata K (2005) Polymer 46:10800
24. Nakanishi E, Sugiyama E, Shimizu Y, Hibi S, Maeda M, Hayashi T (1991) Polym J 23:983
25. Calabresi P, Parks RE Jr (1985) Antimetabolites. In: Goodman LS, Raal TW, Murad F (eds) Pharmacological basis of therapeutics. McMillan, New York, pp 1268–1276
26. Seymour LW, Duncan R, Duffy J, Ng SY, Heller J (1994) J Control Rel 31:201
27. Nichifor M, Schacht EH, Seymour LW (1996) J Control Rel 39:79
28. Giammona G, Pitarresi G, Craparo EF, Cavallaro G, Buscemi S (2001) Colloid Polym Sci 279:771
29. Xu JP, Ji J, Chen WD, Shen JC (2005) Macromol BioSci 5:164
30. Cheng CH, Hsia HC, Shyan CC (1999) Polym J 31:535
31. Cao P, Munk P, Ramireddy C, Tuzar Z, Webber SE (1991) Macromolecules 24:6300
32. La SB, Okano T, Kataoka K (1996) J Pharm Sci 85:85
33. Sugimoto H, Nakanishi E, Kamiya M, Hibi S (1999) Polym Bull 42:395
34. Ohya Y, Ouchi T (2001) Off J Jpn Soc Drug Deliv Syst 16:143
35. Brant DA, Flory PJ (1965) J Am Chem Soc 87:2791