ORIGINAL ARTICLE

Structural studies of amphiphilic oligopeptides composed of alternating alanine and ionizable amino-acid residues using CD and ^{13}C CP/MAS NMR spectroscopy

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We report a novel self-assembling system of amphiphilic oligopeptides composed of alternating hydrophobic and ionizable amino-acid residues. Three types of peptides, AD12, AE12 and AK12, were prepared as building blocks for this model system. These peptides formed helical conformations in low-concentration solutions, but these peptides adopted β-sheet structures and precipitated in highly concentrated solutions at a pH that neutralizes the charges on the ionizable side chains. In addition, the mixing of the peptides with positive and negative charges on the side chains of the ionizable amino-acid residues was found to trigger the formation of the β-sheet structure. This system could be used to create new materials based on oligopeptides.

INTRODUCTION

The self-assembly of peptides, proteins or their derivatives has been extensively studied and is a promising approach for the design and construction of novel functional nano-materials. Studying this self-assembly also helps clarify the mechanism of diseases. In particular, artificial amphiphilic peptides with exactly alternating hydrophobic and ionizable amino-acid residues have been investigated as candidate building materials based on peptides. This type of amphiphilic peptide is known to self-assemble into β-sheet structures, which then form nanofibers and hydrogels. This self-assembly occurs because one side becomes hydrophobic and the other becomes hydrophilic due to alternating hydrophobic and hydrophilic amino-acid residues in the β-strand. Weak interactions consisting of hydrophobic interactions and charge interactions work together cooperatively to direct the formation of a larger scale architecture. Zhang *et al*[1] demonstrated that the peptide named EAK16 from the Z-DNA-binding protein Zuotin showed a tendency to self-assemble into a self-supporting gel under physiological pH and salt conditions.[1,2] Since then, many other peptides have been designed to mimic EAK16, and the effects of the sequence and type of hydrophobic residues[3,4] and the effect of a combination of ionizable residues[5,6] on the structural and the physical properties of the self-supporting gels have been studied. These peptide gels have been utilized as biomaterials in constructs such as scaffolds for cell culture *in vivo*[7–9] and *in vitro*.[7,9] In addition, gelating peptides responsive to pH,[10] temperature,[11] ionic strength[12] or UV radiation[13] have been developed to improve the handling of peptide-based gels

using a combination of D-amino acids, proline residues or other amino-acid derivatives.

In this paper, we report a novel self-assembling system of amphiphilic oligopeptides with alternating alanine and ionizable amino-acid residues—Asp (D), Glu (E) and Lys (K). The three types of peptides prepared as building blocks for this system, AD12, AE12 and AK12, contain alanine as the hydrophobic amino-acid residue. These peptides differ from the EAK16 peptide mentioned above in which the ionizable amino-acid residues in each peptide have only positive or negative charge. In addition to the analysis of each peptide, mixtures that combined positive and negative charges on the side chain groups were characterized to establish a new system of peptide self-assembly. These studies should provide useful information for the design and development of new materials based on oligopeptides.

EXPERIMENTAL PROCEDURE

Preparation of peptides and stock solutions

The amphiphilic oligopeptides used in this study were synthesized using a Pioneer solid-phase peptide synthesis system (Applied Biosystems Ltd) with 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. The following peptides were synthesized: AD12: $(AD)_{12}A$, AE12: $(AE)_{12}A$ and AK12: $(AK)_{12}A$. These peptides were cleaved from PS-resins using trifluoroacetic acid solution and were then precipitated using ice-cold diethyl ether. By repeating the dissolution with trifluoroacetic acid and reprecipitation with diethyl ether, the impurities were removed. According to the results of the NMR measurements and the results of previous studies, the purity of the peptides was estimated to be greater than 90%.

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The stock solutions were prepared as follows: each peptide was dissolved in ultrapure water, and the solutions were adjusted to pH 7.0 with hydrochloric acid or ammonia. The concentrations of the peptide solutions were maintained at $1 \, \text{mg ml}^{-1}$ by adding ultrapure water for the circular dichroism (CD) measurements. For the ^{13}C CP/MAS NMR measurements, the stock solutions were prepared by the same procedure as for CD samples except that the final concentration was adjusted to $10 \, \text{mg ml}^{-1}$.

CD measurements

The CD spectra were recorded with a J-820 spectrometer (JASCO co., Tokyo, Japan). All the experiments were performed using a quartz cell with a 1-mm path length over the range of 190–260 nm at ambient temperature.

The samples were prepared by adding 10 volumes of pH-adjusted water, containing hydrochloric acid or ammonia as needed, to the stock solutions immediately before recording the CD spectra. Therefore, the final concentration of each solution was $0.1 \, \text{mg ml}^{-1}$ (refer to each figure caption for the values of concentration in molarity). The pH values were checked after the dilution. For the mixture solutions, first, each stock solution was diluted 10-fold with ultrapure water and then, the diluted solutions were mixed at an one-to-one volume ratio.

^{13}C CP/MAS NMR measurements

^{13}C CP/MAS NMR spectra were obtained on a Varian NMR system spectrometer with an operating frequency of 100.57 MHz for ^{13}C at a magic angle spinning (MAS) rate of 8 kHz. A cross-polarization (CP) contact time of 1 ms was used. The ^{13}C chemical shifts were calibrated indirectly using the methine carbon peak of adamantane observed at 29.47 p.p.m. relative to tetramethylsilane at 0 p.p.m.

To collect solid-state ^{13}C NMR spectra, AD12 and AE12 were reprecipitated by adding hydrochloric acid to the $10 \, \text{mg ml}^{-1}$ stock solutions until the pH values were less than 2. AK12 was reprecipitated by adding ammonia to the stock solution until the pH was greater than 10. Then, the precipitates were collected by centrifugation and freeze dried. To prepare the mixture samples, the stock solutions were mixed at an one-to-one volume ratio. Immediately after mixing, the solutions were freeze dried. For the mixture of AD12 and AK12, different time-course samples were also prepared by letting the mixed solution stand for 1, 3, 8, 24 or 100 h at 4 °C before freeze drying. These mixtures were freeze dried without centrifugation.

RESULTS AND DISCUSSION

Conformational studies of each peptide

The conformation of each peptide (AD12, AE12 and AK12) in aqueous solution at various pHs was investigated by CD, and the results are shown in Figure 1. In case of AD12, shown in Figure 1a, almost all the spectra showed a random coil pattern with negative local maxima at ~ 196 nm. In contrast, for AE12, shown in Figure 1b, the spectra indicated that this peptide underwent a conformational transition between the random coil state and an α-helix structure depending on the pH. The spectrum with two negative local maxima, at 222 and 204 nm, and one positive local maximum, at 196 nm, is typical for peptide solutions in which the peptides have an α-helical conformation. For random coil peptides, the spectrum has a negative local maximum at less than 200 nm. Therefore, the spectra for the low pH region, 2.3, 3.3 and 4.3, indicated that the peptides form α-helices. In contrast, the spectra for the higher pH region (pH values over 5.8) indicated that the peptides were in random coil state. For AK12, shown in Figure 1c, the spectra for the high pHs, 10.1 and 11.6, showed the presence of an α-helical conformation, whereas the spectra for the low pHs (less than 8.6) indicated that the peptides were in a random coil state.

In Figure 1b and c, the pH dependences of the molar ellipticity at 222 nm, $[\theta]_{222}$, are presented as insets. The value of $[\theta]_{222}$ decreases as the amount of α-helix structure increases. It should be noted that the
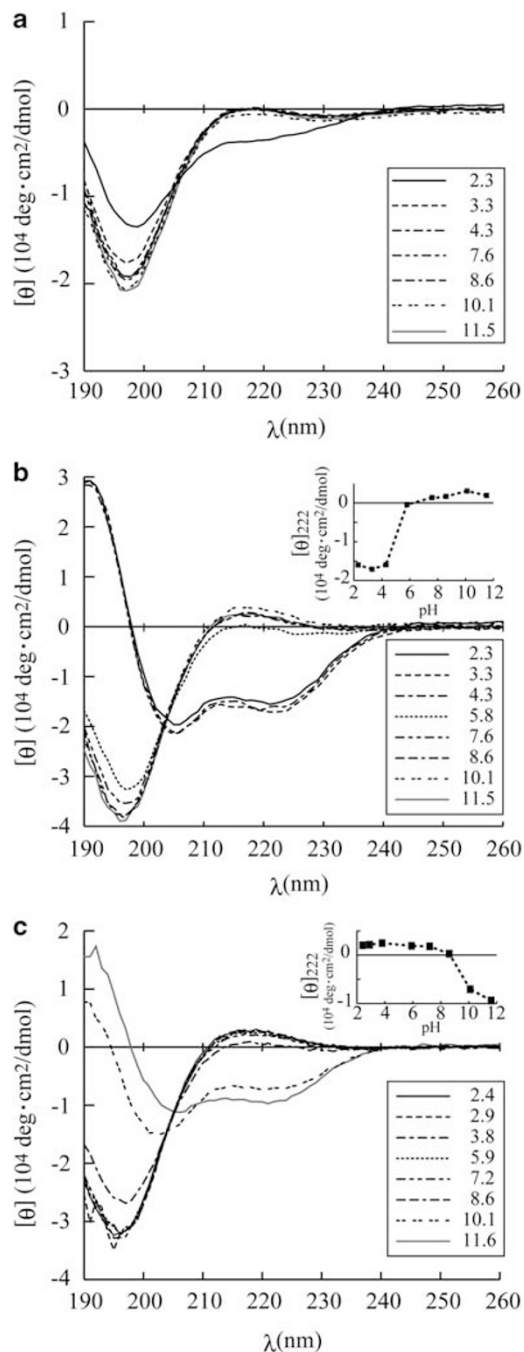

Figure 1 CD spectra of AD12 (**a**), AE12 (**b**) and AK12 (**c**) at various pH values. The concentrations of peptides in molarity are $[\text{AD12}] = 43 \, \mu\text{M}$, $[\text{AE12}] = 40 \, \mu\text{M}$ and $[\text{AK12}] = 40 \, \mu\text{M}$. The insets in (**b**) and (**c**) show the pH dependence of the molar ellipticity at 222 nm ($[\theta]_{222}$), which indicates the presence of α-helical structures.

conformational transitions occurred drastically, and the dependence on pH was completely opposite between AE12 and AK12. Also notable is the fact that the pH regions related to the transition of the peptide conformations were nearly the same as the pK_a values of the side chains, 4.3 for Glu and 10.4 for Lys.

Here, referring to AD12 again, careful analysis of the spectra at pH 2.3 and 3.3 revealed that the intensity of the negative local maxima at 197 nm in these two spectra was lower than that of the negative local maxima for the other solutions at pHs greater than pH 3.3.

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This difference suggests that AD12 also undergoes a pH-dependent conformational change in the vicinity of the pK_a value of the side chain of the aspartic acid residue, 3.9, although the spectral changes were neither drastic nor clear. These results were consistent with the fact that the CD spectrum of poly-Asp in acidic solution did not show a complete helical pattern like that of poly-Glu, even though poly-Asp tended to form an α-helix structure.[14] Considering these results together with the results that AE12 and AK12 formed α-helix structures under the pH conditions at which the charges on the side chains of ionizable amino-acid residues were neutralized, it can be hypothesized that AD12 also adopted a helical conformation in acidic solutions like AE12 did.

These results indicated that the conformations of the peptides changed depending on the pH because the charge states of the side chains on the ionizable amino-acid residues changed. That is, all three types of peptides used in this study are in the random coil state at a neutral pH. However, the conformations change to the helical state immediately when the pH becomes sufficiently low or high to neutralize the side chain charges of the ionizable residues. These conformational transitions were reversible (data not shown).

Figure 2 shows the ^{13}C CP/MAS NMR spectra of the precipitates of the 10 mg ml^{-1} stock solutions of AD12 (A), AE12 (B) and AK12(C). The chemical shift values of the peaks derived from main chain and the Ala Cβ carbons are summarized in Table 1. From the chemical shifts of the main chain carbons, it is clear that all the peptides predominantly formed β-sheet structures.[15] However, in the carbonyl carbon region from 155 to 185 p.p.m., there are some differences in the spectral patterns. The lowest field peaks at ∼176 p.p.m. in the spectra of AD12 and AE12 were assigned to the carboxyl group carbons in the side chains of Asp Cγ and Glu Cδ, respectively.[15] The shoulder peaks on the lower field side of the Ala carbonyl carbon peaks in the spectra of AD12 and AE12 were attributed to the Ala carbonyl carbons in the random coil state. This observation indicates that some of the AD12 and AE12 peptides are in the random coil state, in contrast to AK12.

Turning now to the Cα region, Figure 2a shows a small broad peak on the lower field side of the Asp peak derived from the random coil state, in addition to two sharp peaks at 48.2 and 49.2 p.p.m. derived from the β-sheet structure. These peaks are a good agreement with the assignments for the carbonyl carbon region. In case of AE12, shown in Figure 2b, two distinct Cα peaks, for Ala and Glu, were

observed. Similarly, two Cα peaks, for Ala and Lys, were observed separately for AK12, as shown in Figure 2c. As some of the individual AE12 peptides are in the random coil conformation, as stated above, the separation between the Cα peaks for Ala and Glu is worse than the separation of the corresponding peaks for Ala and Lys of AK12. Some broad peaks attributed to random coil peptides most likely overlapped with the Cα peaks for AE12.

The highest field peaks from 17 to 24 p.p.m. were assigned to Ala Cβ, although the peak for Lys Cγ, at ∼21–23 p.p.m., overlapped with the Ala Cβ peaks in the AK12 spectrum.[15] The shape of the Ala Cβ peak is known to be related not only to the conformation of the peptide but also to the intermolecular arrangement of the peptides.[16–18] Regarding the Ala Cβ signal in the spectrum for AE12, the broad peak at 18.2 p.p.m. was assigned to the random coil peptides, and the other peaks at lower field were all derived from the β-sheet structures. The lowest peak at 23.8 p.p.m. was assigned to the parallel β-sheet structure, and the other two peaks, one at 21.4 p.p.m. and the other a shoulder peak on the higher side of the peak at 21.4 p.p.m., were assigned to the anti-parallel β-sheet structures. This observation indicates that AE12 formed both parallel and anti-parallel β-sheet structures. Moreover, the entire shapes of the peaks for Ala Cβ are quite similar to those of poly-Ala.[16] This similarity indicates that the Ala Cβ carbons are in the same environment as those in poly-Ala. In other words, this result indicates that the β-sheet structure is formed by hydrophobic interactions between the methyl groups in the Ala residues. Regarding AD12, there was no peak at ca. 24 p.p.m. assigned to the parallel β-sheet structure. Therefore, AD12 formed mainly anti-parallel β-sheet structures. In the case of AK12, two peaks were assigned to the parallel β-sheet structure at 23.9 p.p.m. and to the anti-parallel β-sheet at 21.3 p.p.m., although the Lys Cγ peak overlapped in-between the peaks at 21.3 and 23.9 p.p.m. These spectral characteristics demonstrate that AK12 formed both parallel and anti-parallel β-sheet structures, as did AE12. The small broad peak at ∼18 p.p.m. indicates that AK12 was also present in the random coil state. However, the amount of random coil AK12 is considerably less than the amounts of AD12 and AE12 in the random coil conformation.

Conformational changes in the peptide mixtures

There are many reports describing self-assembling amphiphilic oligopeptides that have alternating hydrophobic and ionizable amino-acid residues with both positive and negative charges, as
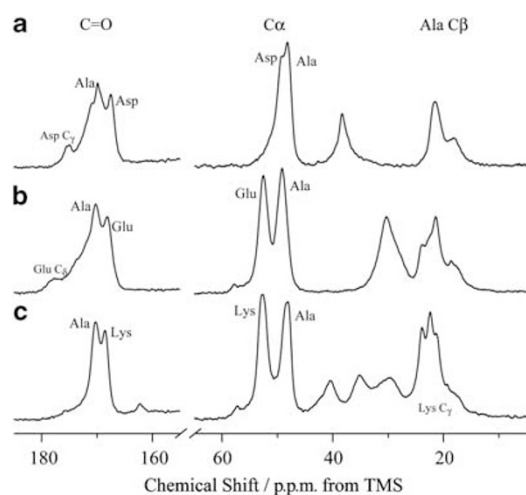

Figure 2 ^{13}C CP/MAS NMR spectra of the precipitates of AD12 (**a**), AE12 (**b**) and AK12 (**c**).

Table 1 ^{13}C CP/MAS NMR chemical shifts for AD12, AE12 and AK12

		C=O	Cα	Cβ
AD12	Ala	169.8	48.2	18.2
				21.5
	Asp	167.5	49.4	
AE12	Ala	170.2	49.2	18.2
				21.4
				23.8
	Glu	168.1	52.6	
AK12	Ala	170.3	48.2	21.3
				22.5[a]
				23.9[a]
	Lys	168.5	52.7	

[a]It would be overlapped by the Lys Cγ peak.

mentioned in the introduction. These peptides self-assemble into β-sheet structures and form gel-like materials. This gelation is thought to be the result of assembly via a combination of hydrophobic interactions and ionic forces, based on the hydrophobic–hydrophilic arrangement and the charges of the self-complementary peptides. When mixing a carboxylic peptide with an amide peptide, the charges of the peptides cancel out, allowing the peptides to self-assemble into β-sheet structures. Such mixing is possible with our peptides.

Time-course CD spectra of the peptide mixtures are shown in Figure 3. As shown in Figure 3a, the conformations of the peptides in the mixture of AD12 and AK12 changed gradually over time from random coils to β-sheets. The typical CD spectral pattern indicative of a β-sheet structure is characterized by two extreme values: a positive local maximum at $\sim$195–200 nm and a negative local maximum at 217 nm. The increase in the absolute value of the negative local maximum at 217 nm reflects an increase in the amount of β-sheet peptides. The inset in Figure 3a, which shows the change in $[\theta]_{217}$ over time, illustrates that the amount of β-sheet peptide increased exponentially in the early stage and then came to an equilibrium over 24 h later.

A gradual conformational change was also observed in the ^{13}C CP/MAS NMR measurements, as shown in Figure 4a. These samples were prepared by freeze drying after mixing the 10-mg ml^{-1} stock solutions and letting them stand for 0–100 h. Regarding the carbonyl carbon region, the strong main peak at 173 p.p.m. appears in

the spectrum for the sample freeze dried immediately after mixing (top marked 0 h) with a weak shoulder peak at 170 p.p.m. This main peak decreases with time, and the intensity of the shoulder peak increases in a complementary manner. The shoulder peak at 170 p.p.m. becomes the main peak more than 8 h later, and the shoulder peak eventually splits into two peaks at 169 and 170 p.p.m. (bottom marked 100 h). These two peaks were assigned to the carbonyl carbons in the β-sheet structure, at 169 p.p.m. for the Asp and Lys residues and at 170 p.p.m. for the Ala residue. These chemical shift values in the final spectra show good agreement with those of each peptide shown in Figure 2 and Table 1. The rest of the broad peaks at 173 to 176 p.p.m. were assigned to the carbonyl carbons in the random coil state and the Cγ carbon of the Asp residues. The small peaks at 159.5 p.p.m. in the spectra from 0 to 24 h can be assigned to the carbamate group formed by the amide group at the end of the side chain in the Lys residues.[19]

For the highest field peaks assigned to the Ala Cβ carbons, the first main peak appeared at 17 p.p.m. This peak was derived from the random coil state (top marked 0 h). The peak intensity decreases with time, and the peak at 21.9 p.p.m. appeared in a complementary manner. The increase in the peak intensity at 21.9 p.p.m. is indicative of the formation of and increase in the amount of anti-parallel β-sheet structures, although the Lys Cγ peak also overlapped.

The Cα carbon region is complex, and it is more difficult to assign signals in this region than in the carbonyl carbon and Ala Cβ regions. However, it is possible to assign the signals by considering the
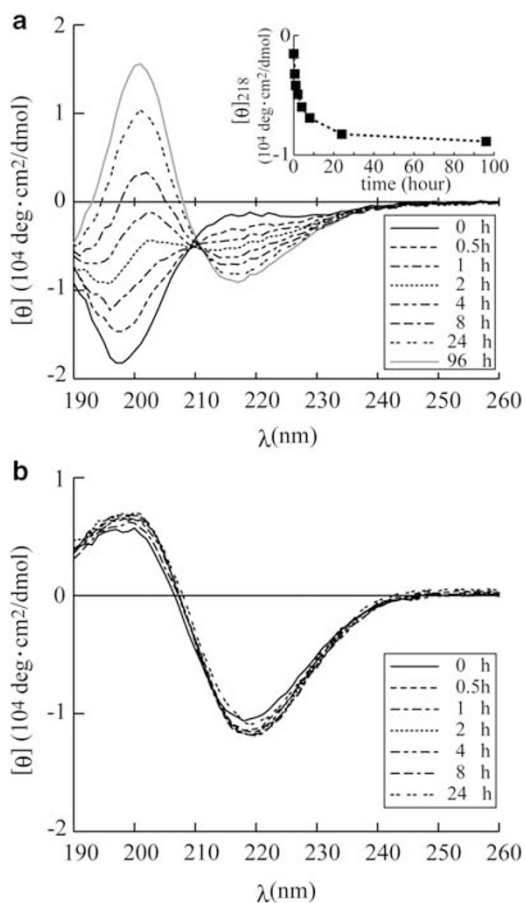

Figure 3 CD spectra of the mixtures AD12 and AK12 (**a**) and AE12 and AK12 (**b**). The inset in (**a**) shows the time-course of the molar ellipticity at 217 nm ($[\theta]_{217}$), which indicates the presence of β-sheet structures.
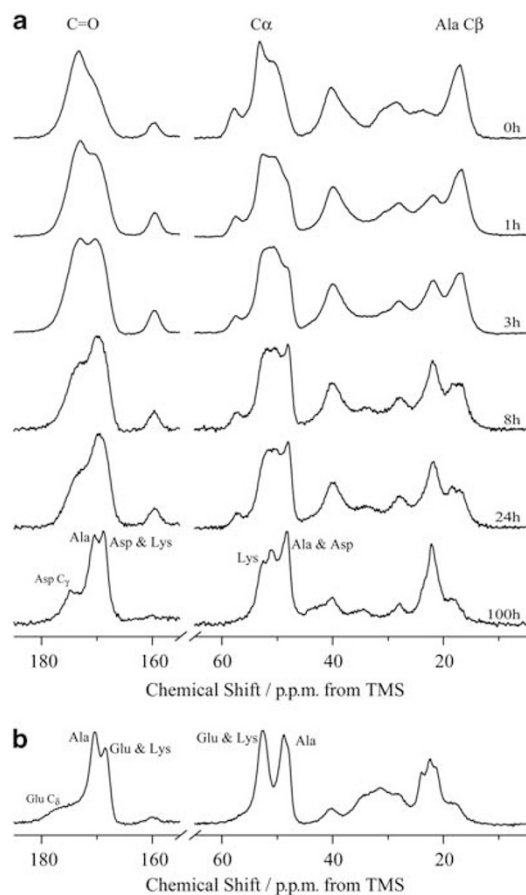

Figure 4 ^{13}C CP/MAS NMR spectra of the mixtures of AD12 and AK12 (**a**) and AE12 and AK12 (**b**). Explanatory notes in part (**a**) show the elapse time before freezing after mixing.

assignments of the carbonyl and Cβ carbon regions. Starting first with the peaks in the spectrum for 100 h later, there are three peaks, one each at 48.3, 51.1 and 52.6 p.p.m. The highest field peak at 48.3 p.p.m. was assigned to the Ala and Asp Cα peaks in the β-sheet structure because of the chemical shift values and the fact that the assignments of the carbonyl and Ala Cβ carbons indicated that the mixture formed β-sheet structures. The lowest field peak at 52.6 p.p.m. was assigned to the Lys Cα in the β-sheet structure using the same reason as for the highest field peak. The chemical shift values, 48.3 and 52.6 p.p.m., conformed to those of each peptide in Figure 2. A middle peak at 51.1 p.p.m. can be assigned to the random coil state because the peaks in the carbonyl and Ala Cβ carbon regions indicate that the mixture included random coil peptides. The highest field peak at 48.3 p.p.m. appeared starting at 1 h later as a shoulder peak, and the size of this peak increased with time. This result indicates that the amount of β-sheet structure increased with time, a conclusion that coincides with the analyses of the carbonyl carbon and Ala Cβ regions. The lowest peak at 52.6 p.p.m. assigned to the Lys Cα in the β-sheet structure seemed to be observed from the beginning. However, the chemical shift value of the peak in the 0 h spectrum was slightly different from that in the 100 h spectrum, at 53.2 p.p.m. This chemical shift value is close to that of the Ala and Asp Cα carbons in an α-helix.[15] In addition, another peak was observed at 57.5 p.p.m. in the 0–24 h spectra, which can be assigned to the Lys Cα carbons in the α-helix.[15] Therefore, it was concluded that the structure of the mixture of AD12 and AK12 changed over time from a random coil state including a partial helical conformation to an anti-parallel β-sheet structure including some random coil peptides.

In contrast, the mixture of AE12 and AK12 formed a β-sheet structure immediately after mixing. In the CD spectra from 30 min to 8 h later, shown in Figure 3b, no spectral change was observed, although a slight increase in the absolute value of $[\theta]_{217}$ was found compared with the value immediately after mixing. In the spectrum for 24 h later, the absolute value of $[\theta]_{217}$ decreased again. This decrease was caused by the aggregation and precipitation of peptides, as reflected by the fact that both the positive and negative maximum values in the spectrum decreased, and the spectrum did not pass through the isosbestic point. This clear difference in the behavior of the conformational change for the mixture of AD12 and AK12 was assumed to be due to the difference in the side chain length between Glu and Asp.

The ^{13}C CP/MAS NMR spectrum shown in Figure 4b, corresponding to the mixture of AE12 and AK12 that was freeze dried immediately after mixing, agrees with the results of the CD analyses. This mixture formed a precipitate immediately after mixing. This precipitate formation is another difference from the case of the AD12 and AK12 mixture, which formed stacked peptides gradually over time. In the carbonyl carbon region, there were two sharp peaks at 168.5 and 170.4 p.p.m. These peaks can be assigned to the carbonyl carbons in the β-sheet structure, at 170.4 p.p.m. for Ala and at 168.5 p.p.m. for Glu and Lys. The small broad peak at the lower field may be derived from the carbonyl carbon in the random coil state and the carboxylic carbon in the side chain of the Glu residues.

The Cα region also shows two peaks. The higher field peak at 48.8 p.p.m. can be assigned to the Ala Cα carbon, and the lower field peak at 52.7 p.p.m. is assigned to the Glu and Lys Cα carbons. In the case of the highest field region assigned to the Ala Cβ carbon, which also includes the Lys Cγ carbon peak, there are three distinct peaks other than the Lys Cγ peak at 22.4 p.p.m. The smallest broad peak was assigned to the random coil peptide, and the other two sharp

peaks at 21.4 and 23.9 p.p.m. were assigned to the anti-parallel and parallel β-sheet structures, respectively. Thus, the mixture of AE12 and AK12 formed mainly β-sheet structures, and in addition, the β-sheet structure included both parallel and anti-parallel arrangements according to the results of the solid-state NMR spectroscopy.

These peptides were in the random coil state at neutral pH, as shown in Figure 1. In addition, each peptide stock solution did not aggregate on its own, even more than 1 week after preparation. The ^{13}C CP/MAS NMR spectra of freeze-dried samples prepared from high-concentration stock solutions were measured to confirm the expectation. All of the peptides, AD12, AE12 and AK12, were in the random coil state, as expected (data not shown). Therefore, the formation of β-sheet structures and precipitation in these mixtures was considered to be caused by the mixing procedure. After the mixing, there are both positive and negative ions in the system. Thus, the side-chain charges cancel out, allowing the intermolecular interactions necessary for the formation of β-sheet structures. In other words, the β-sheet structures were formed as the result of electrostatic interactions between two types of peptides, AD12 and AK12 or AE12 and AK12.

CONCLUSIONS

Amphiphilic oligopeptides with alternating alanines and ionizable amino-acid residues, AD12, AE12 and AK12, underwent conformational transitions between the random coil state and a helical conformation depending on the pH in low-concentration aqueous solution. However, the ^{13}C CP/MAS NMR spectra indicated that these peptides assembled into β-sheet structures in high-concentration solutions at the pH at which the charges on side chains of the ionizable amino-acid residues are neutralized. That is, the conformation of these peptides depends not only on the pH but also on the peptide concentration. Furthermore, the CD and ^{13}C CP/MAS NMR data indicated that the mixing of peptides with charges that can neutralize them can trigger the self-assembly of these peptides. This self-assembling peptide system will be a good candidate for the development of materials based on peptides because of its easy preparation. The differences in the time required to form β-sheet structures between systems (AD12 and AK12 or AE12 and AK12) suggest that the gelation speed can be controlled by changing the combination of peptides used.

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