Colloid Polym Sci (2002) 280: 1157–1161
DOI 10.1007/s00396-002-0742-8

SHORT COMMUNICATION

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Aggregational behavior of *N*-butyloxycarbonyl-L-glutamic acid oligomeric benzyl esters (exact residue numbers 4, 6 and 8) in benzene. SAXS studies

Received: 14 March 2002
Accepted: 8 May 2002

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Abstract *N*-Butyloxycarbonyl(BOC)-L-glutamic acid oligomeric benzyl esters with exact residue numbers (BOCN_pZ, $N_p = 4$, 6 and 8) have been synthesized by a stepwise procedure in a liquid phase. The SAXS intensity spectra of the BOCN_pZ–benzene systems have been analyzed on the basis of the rod-like aggregate model, in which the β-sheet monomers are one-dimensionally stacked antiparallel to each other. The extracted parameters (the number-averaged aggregation number, the monomer–monomer bond energies corresponding to hydrogen bonding energies, and the number-averaged molecular weights) for these aggregates have been compared with those for the aggregates formed by *N*-acetyl-L-glutamic acid oligomeric benzyl esters (AN_pZ, $N_p = 4$, 6 and 8) in benzene (PCCP, 2001, 3, 3140–3149). The results indicate that it is more difficult to form aggregates in the BOCN_pZ systems than in the AN_pZ systems. This difference is due to the bulky BOC group, which hinders the formation of aggregates.

Introduction

A tremendous number of studies on poly-γ-benzyl-L-glutamate (PBLG) have been carried out by various methods for more than 40 years. However, physicochemical studies on the oligopeptides with exact residue number are relatively few compared with those for PBLGs with average residue numbers (that is, degree of polymerization).

In our previous studies [1, 2], for *N*-acetyl- and *N*-octanoyl-L-glutamic acid oligomeric benzyl esters with exact residue numbers ($N_p = 2$, 3, 4, 5, 6, 8, 10, 12 and 14), the microstructures of the aggregates formed by these oligopeptides in dioxane and benzene have been examined by use of IR, SANS and SAXS spectra. The results may be summarized as follows. The IR spectral data for the oligomers with $N_p \geqq 4$ provided ample evidence that the predominant antiparallel β-sheet structure is stabilized above the critical aggregation concentration, while other conformers, including β-turns, may coexist below the critical aggregation concentration. IR results for the shorter oligomers ($N_p < 4$) have indicated that specific conformers (β-turns), which are different from the β-sheet structures, may be preferentially stabilized upon aggregation. On the basis of these IR data, the model of the rod-like aggregate, in which the β-sheet monomers are one-dimensionally stacked antiparallel to each other, has been presented for analysis of their SANS and SAXS intensity spectra. The best fit SANS and SAXS intensity spectra have provided the monomer–monomer bond energy (αkT), corresponding to the hydrogen bonding energies, the number-averaged aggregation numbers, and the number-averaged molecular weights for these oligopeptide aggregates, which can be regarded as supramolecular aggregates.

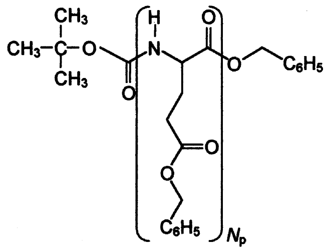
In this present study, the SAXS spectra of *N*-butyloxycarbonyl-L-glutamic acid oligomeric benzyl esters with exact residue number ($N_p = 4$, 6 and 8) have been analyzed by use of a rod-like aggregation model and have been compared with parameters extracted from the SANS and SAXS spectral analyses of *N*-acetyl-L-glutamic acid oligomeric benzyl esters (AN_pZ, $N_p = 4$–8) [1].

Experimental

Materials

N-Butyloxycarbonyl(BOC)-L-glutamic acid oligomeric benzyl esters ($BOCN_pZ$, N_p = residue number) with exact residue numbers were prepared from *N-tert*-butyloxycarbonyl-L-glutamic acid γ-dibenzyl ester and L-glutamic acid α,γ-dibenzyl ester toluene *p*-sulfonate (the Peptide Institute, Inc., Osaka, Japan) in dichloromethane in the presence of trimethylamine, by the stepwise procedure described previously [3].

BOCN_pZ



The oligomers thus prepared were recrystallized in dichloromethane–petroleum ether. The samples were identified by elemental analysis and the agreement between the calculated and observed values for each atom of these samples was within 0.3%.

Confirmation of the residue number (N_p) was made by ^1H NMR: the number of NH signals, their relative intensities and the relative peak areas of BOC CH_3 ^1H signal and the benzyl CH_2 signals were used. The residue numbers (N_p) of the $BOCN_pZ$ series used for the present study were 4, 6 and 8 (abbreviations: BOC4Z, BOC6Z and BOC8Z, respectively).

Benzene was distilled in the presence of CaH_2, and was used for preparation of the sample solutions after confirming the absence of water from the absence of an IR band at 3500 cm^{-1}.

X-Ray powder diffraction pattern and infrared absorption measurements

X-Ray powder diffraction patterns were obtained by use of an RAD-RC diffractometer with counter-monochromator (CuL_α, 50 kV, 110 mA). Infrared absorption spectra were recorded on a Perkin-Elmer 1600 Fourier-transform infrared (FTIR) spectrometer (4000–400 cm^{-1}) with the sample dispersed in KBr discs for the solid states and with the sample–benzene solution sandwiched between two CaF_2-plate windows (spacer 0.015–0.1 mm).

Small angle X-ray scattering measurements

Small angle X-ray scattering (SAXS) measurements were carried out with a quartz cell of 1 mm path length and incident X-ray wavelength of 1.49 Å using the SAXS spectrometer for enzymes, installed at the BL10C (or BL15A) line of the 2.5 GeV storage ring in the Photon Factory at the High Energy Accelerator Research Organization, Tsukuba, Japan.

The magnitude of the momentum transfer (Q) is given by Eq. (1),

$$Q = \frac{4\pi}{\lambda}\sin\left(\frac{\theta}{2}\right) \tag{1}$$

where θ is the scattering angle and λ is the incident wavelength.

Theoretical background for SAXS analysis

In the one-dimensional rod-like aggregation model [1, 4], we may define the monomer–monomer bond energy (in this present study, hydrogen-bonding energy) in the aggregate, relative to isolated monomers in solution, as equal to αkT (k = Boltzman constant and T = absolute temperature, K).

The density distribution function (D) of molecules in aggregates, which consist of i molecules, is expressed by the following equation,

$$D_i = i\left[1 - \left(1\Big/\sqrt{f\mathrm{e}^{\alpha}}\right)\right]^i \mathrm{e}^{-\alpha}/f \tag{2}$$

where f is the molal fraction of the solute. This equation is restricted to diluted systems in which the inter-aggregate interactions can be ignored.

Since D_i takes the maximum i value ($i_{\max}$) at $\partial D_i/\partial i = 0$, $i_{\max}$ is expressed as:

$$i_{\max} = \sqrt{f\mathrm{e}^{\alpha}} = N_n \tag{3}$$

$i_{\max}$ is equal to the number-average aggregation number (N_n). Thus, the αkT can be calculated by the following equation.

$$\alpha kT = [\ln(N_n^2/f)]kT \tag{4}$$

The dependence of the X-ray-scattering intensity $I(Q)$ on the magnitude of a scattering vector can be expressed as a function of both the particle structural factor $P(Q)$ and size- and orientation-weighted interparticle-structural factor $S'(Q)$, as follows,

$$I(Q) = I_0 P(Q) S'(Q) \tag{5}$$

where I_0 is the extrapolated zero-angle scattering intensity, which is independent of the Q value. When interactions between aggregates are neglected, as in the case with diluted non-ionic aggregate systems, $S'(Q)$ is reduced to unity. Therefore, Eq. (5) is expressed by the following form.

$$I(Q) = I_0 P(Q) \tag{6}$$

When the cross-section of a rod-like aggregate is circular, $P_i(Q)$ of an aggregate with aggregation number i can be calculated by the following equation,

$$P_i(Q) = \int_0^{\pi/2} \frac{\sin^2(Qleft(Hi/2)\cos\beta)}{Q^2(Hi/2)^2\cos^2\beta} \frac{4J_1^2(Qr\sin\beta)}{Q^2r^2\sin^2\beta}\sin\beta \mathrm{d}\beta \tag{7}$$

where i is the number of stacked circular disks (that is, the aggregation number), H is the thickness (Å) of the circular disk, r is its radius and J_1 is the first-order Bessel function.

When we assume that the aggregates are in a polydispersed state, $P(Q)$ can be expressed as:

$$P(Q) = \frac{\sum_{i=1}^{\infty} C_i i P_i(Q)}{\sum_{i=1}^{\infty} C_i i} \tag{8}$$

where C_i ($= CD_i$) is the concentration (mol/l) of an aggregate with aggregation number i.

Thus, the scattering intensity $I(Q)$ is expressed as:

$$I(Q) = AP(Q) \tag{9}$$

where $A \equiv$ scaling factor (averaged values of [observed scattering intensity]/$P(Q)$).

In the present analysis, we have assumed that the thickness, H, of a circular disk corresponds to the distance between the β-sheet-type peptide chains [1]. Molecular parameters used for calculations of the intensity profiles are listed in Table 1.

The present analysis has been carried out by use of Eq. (9) as follows. In the calculation of $P(Q)$, the values of H and r, listed in Table 1, were fixed and the number-average aggregation number (N_n) was varied as a fitting parameter. The scaling factor A, which can be calculated from the observed scattering data at all Q, was not used as an adjustable parameter in fitting the scattering intensity data. Thus, the $I(Q)$ value has been determined from the A value and the $P(Q)$ as a function of N_n, implying that $I(Q)$ can be calculated as a function of the aggregation number N_n. Finally, the value of N_n, which minimizes the error calculated from $I(Q)$ and the observed scattering intensity, was obtained.

Results and discussion

The X-ray powder diffraction patterns for the series of BOCN_pZ ($N_p = 4$, 6 and 8) in the solid state have been examined. It has been confirmed that lattice spacings are obtained in common at 4.68–4.69 and 14.92–15.07 Å for BOC6Z and BOC8Z and that these can be assigned to the distance between β-sheet type peptide chains and to the spacing between the side chains, respectively [5]. These observations reveal that the oligopeptides in the solid state take up a β-sheet structure. However, for BOC4Z, we found that the lattice spacings characteristic of a β-sheet disappear, indicating that the critical residue number required to take up a β-sheet form in the solid state is five or six.

In order to examine the type (antiparallel or parallel) of β-sheet structure, the IR spectra of these oligomers were measured in the solid state. The IR bands at 1690–1692 cm^{-1} (weak) and 1626–1628 cm^{-1} (very strong), which were observed in common for BOC6Z and BOC8Z, are assigned to the $\nu_{||}(0,\pi)$ and $\nu_{\perp}(\pi,0)$ amide I modes, respectively, that are characteristic of an antiparallel β-sheet [6]. In the IR spectrum of solid BOC4Z, bands for the amide I mode, which are characteristic of an antiparallel structure, were not observed, supporting the result obtained from the X-ray powder diffraction patterns.

However, the concentration dependence of the amide I mode region for the BOCN_pZ (4, 6 and 8)–benzene systems has also been examined. The results showed that these oligomers (even BOC4Z) do take up an antiparallel β-sheet form upon aggregation.

Table 1 Molecular parameters for BOCN_pZ used in SAXS analysis

Material	BOC6Z	BOC8Z
Residue number	6	8
H (Å)[a]	4.69	4.68
r (Å)[b]	11.5	13.0

[a] H: Height of a disk (from X-ray powder diffraction pattern of the solid [1])

[b] r: Radius of a disk [1]

SAXS spectra of the BOCN_pZ–benzene systems

In this present study, analysis of SAXS intensity spectra has been made for benzene solutions of BOCN_pZ ($N_p = 4$, 6 and 8) oligomers, in order to examine the effect of the bulky BOC group on the aggregational behavior, and results have been compared with those for N-acetyl-L-glutamic acid oligomeric benzyl esters (AN_pZ, $N_p = 4$, 6 and 8) [1].

The following assumptions [2] have been made for analysis of the SAXS data.

1. Based on the IR spectra of the BOCN_pZ ($N_p = 4$, 6 and 8)–benzene systems [2], the oligomeric molecules which form the aggregates take up an antiparallel β-sheet structure, which can be regarded as a flat disk.
2. In an aggregate, intermolecular hydrogen-bonded networks are formed between different disk-like molecules, leading to a one-dimensional linear model of the rod-like aggregate, in which the disk-like β-sheet molecules are stacked antiparallel to each other (Fig. 1).
3. The aggregates are in a poly-dispersed state.

For the BOC4Z–benzene solutions (0.008–0.016 mol/l), the scattering intensities were not clearly identified. However, the concentration (C) regions of the BOC6Z and BOC 8Z–benzene solutions, which provided scattering intensities, are $C > 0.012$ mol/l and $C > 0.004$ mol/l, respectively.

The SAXS spectra of the BOCN_pZ ($N_p = 6$ and 8)–benzene solutions were analyzed with the aggregation number (N_n) as a fitting parameter, thus assuming polydispersity and constancy for the remaining molecular parameters. The observed and best-fit scattering intensity profiles for the BOCN_pZ system are shown in Fig. 2A and B. The closeness of fit between the observed and calculated results is excellent, confirming that the observed SAXS spectra can be explained by a one-dimensional linear model. The fitting parameters [number-average aggregation number (N_n)], extracted number-average molecular weight (M_n) and monomer–monomer bond energy (αkT) are listed in Table 2.

From the SAXS spectral results, we conclude that the BOCN_pZ ($N_p = 6$ and 8) oligomers in benzene also form rod-like aggregates in the concentration regions

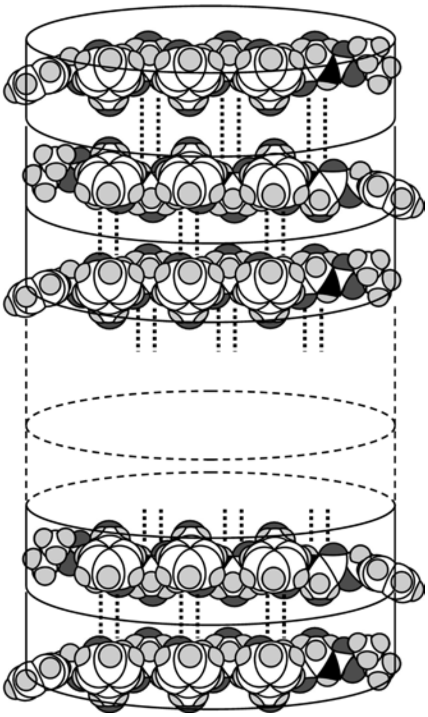


Fig. 1 Schematic model of the BOC6Z aggregate. Dotted lines between the β-sheet monomers imply intermolecular hydrogen bonding

$C > 0.012$ mol/l (BOC6Z) and $C > 0.004$ mol/l (BOC8Z), in which the β-sheet oligomers are one-dimensionally stacked with respect to each other in an antiparallel manner through intermolecular hydrogen bonding.

The aggregation number (N_n) and the monomer–monomer bond energy (hydrogen bond energy) (αkT) of the aggregates in the BOCN_pZ–benzene systems, obtained from the present SAXS analyses, should be compared with those in the AN_pZ ($N_p = 4$, 6 and 8)–benzene system (Table 2) [1].

In this present study, for the BOC4Z–benzene solutions, the fact that the scattering intensity spectra were not able to be detected indicates that aggregates of BOC4Z, which would have been detected by the SAXS instruments used in this study, if they had been present, are not formed in this concentration range. However, our previous SAXS results [1], for the A4Z–benzene solutions (0.009–0.017 mol/l), revealed that formation of A4Z–aggregates does occur and values of N_n and αkT were calculated as equal to 17 and 30–32 kJ/mol, respectively.

In this present study, SAXS measurements have also confirmed that aggregation does not occur in a benzene solution of BOC6Z (0.006 mol/l). However, the A6Z–benzene solution (0.006 mol/l), which was used in our previous SAXS analysis, furnished values of $N_n = 28$ and $\alpha kT = 36$ kJ/mol. Comparison of the N_n and αkT values of BOC8Z with those of A8Z (Table 2) shows the former values to be smaller.

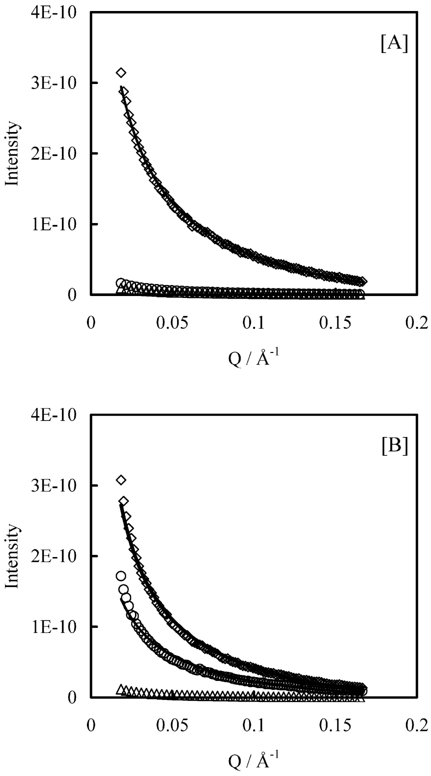


Fig. 2 SAXS profiles of the BOCN_pZ–benzene solutions. **A** BOC6Z, △ 0.006 mol/ l, ○ 0.012 mol/l, ◇ 0.023 mol/l. **B** BOC8Z, △ 0.005 mol/l,○ 0.007 mol/l, ◇ 0.018 mol/l

Table 2 Aggregation number (N_n), monomer–monomer bond energy (αkT) of the aggregates in the BOCN_pZ ($N_p = 4$, 6 and 8) and AN_pZ ($N_p = 4$, 6 and 8)–benzene systems

	C (mol/l)	N_n	αkT (kJ/mol)	$\alpha kT/N_p$
BOC4Z	0.008–0.050	–[a]	–[a]	–[a]
A4Z[b]	0.009–0.017	17	30–32	7.5–8
BOC6Z	0.006	–[a]	–[a]	–[a]
A6Z[b]	0.006	28	36	6
BOC8Z	0.007	24	34	4.3
A8Z[b]	0.007	50	38	4.8

[a] No scattering intensity (no aggregates)
[b] Cited from our previous paper [1]

Thus, the SAXS results indicate that it is more difficult to form aggregates in the BOCN_pZ systems than in the AN_pZ systems. This difference must be due to the bulky BOC group, which hinders the formation of aggregates.

Conclusion

Aggregates containing the bulky BOC group have a smaller aggregational number and intermolecular hydrogen bonding energy than those containing the *N*-acetyl group.

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