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Arrangement of Acridine Orange in the Poly-*α*, L-glutamic Acid–Acridine Orange Complex

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The temperature dependencies of the absorption and circular dichroism spectra of the poly-α,L-glutamic acid-acridine orange complex were observed at the temperatures in the range of 23—95 °C. The circular dichroism spectra were resolved into several Gaussian components by means of a computer online system. Thus, the circular dichroism spectrum can be resolved to five components. The shorter-wavelength bands, consisting of one positive and one negative element, were assigned to the transitions polarized parallel to and perpendicular to the axis of the α-helix of polypeptide respectively. On the other hand, the longer-wavelength bands, having equal magnitudes but signs opposite to each other, were assigned to the transition of the acridine orange dimer bound to polymer. The fifth band, at the shortest wavelength was uncertain. On the basis of these results, the arrangement of acridine orange molecules bound to poly-α,L-glutamic acid was discussed. Thus, two kinds of arrangements of acridine orange molecules were postulated. One is a left-handed super-helix around the right-handed α-helix of the polypeptide in the acidic pH region, and the other is a right-handed super-helix around the core of the α-helix in the alkaline pH region when the molar ratio of glutamyl residues to the dye is kept around unity. In these two complexes, there are dimeric acridine orange molecules bound to the polymer; in the latter complex, the super-helix consists of dimeric acridine orange molecules. Two types of arrangements of acridine orange molecules around the polypeptide were offered for the elucidation of the circular dichroism spectra of the complex of acridine orange with poly-α,L-glutamic acid in an aqueous solution.

Stryer and Blout studied the optical rotatory properties of dyes bound to polypeptides.[1] They made the interesting observation that an extrinsic Cotton effect in the wavelength region corresponding to the absorption bands of bound dye was obtained only when the dye combined with the helical form of poly-α,L-glutamic acid (PLGA). They also explained their results in a manner consistent with the dye-aggregation concept. Thus, they postulated three types of models for the elucidation of the Cotton effects observed in the complex of acridine orange (AO) with PLGA. According to their concept, the acquired optical rotatory power of the bound dye molecule can be only one of two types, configurational or conformational. A configurationally-induced Cotton effect could arise from the interaction of the symmetric dye chromophore with the local asymmetric environment of the α-carbon atom of the polypeptide. On the other hand, a conformationally-induced Cotton effect might result from the interaction among several symmetric dye chromophores which have been oriented to one another in the axial chirality sense by binding to the asymmetric polypeptides.

A conformationally-induced Cotton effect could arise from the aggregates of the dye molecules, which superimpose on the α-helix of polypeptide to form a superhelix in a single-screw sense or which form a tangential helix to the α-helix of polypeptide. Their data favor the conformational type of induced Cotton effect.

On the other hand, Yamaoka and Resnik[2] assumed that the induced Cotton effect in the PLGA–AO complex was caused by a vicinal dissymmetry around the asymmetric carbons in PLGA, since the induced Cotton effect was observed even at very high values of the ratio of the glutamyl residue to the dye (R/D). This assumption of Yamaoka's agrees with the configurationally-induced Cotton effect proposed by Stryer and his collaborator.[1]

In a previous paper,[3] we reported that the induced Cotton effect in the PLGA–AO complex arose from an interaction between the dimerically-bound AO molecules and that the induced Cotton effect arose from a helical arrangement of the dye along the main chain of the polypeptide.

The structure of this complex has not yet been established, although the structure was discussed in another report.[4] In this paper, we wish to report on our study of the arrangement of AO molecules on PLGA by means of some analyses of the effect of the temperature on their circular dichroism and on the absorption spectra. We will consider the origin of the induced Cotton effects on the basis of curve resolution for the observed circular dichroism bands. Furthermore, we will show the possible models of the PLGA–AO complex both in the case of pH=4.5 at small R/D (the ratio of the glutamyl residues to the dye molecules) values and in the case of the alkaline pH region at a R/D value of around unity. The induced circular dichroism bands can be explained by these models.

Experimental

The materials and the procedure are the same as those described previously.[5] The temperature in the optical cell was measured by means of a Takara Thermistor, Type SPD-1D, and allowed to equilibrate for 5 min before each

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[1] L. Stryer and E. R. Blout, *J. Amer. Chem. Soc.*, **83**, 1411 (1961).

[2] K. Yamaoka and R. A. Resnik, *J. Phys. Chem.*, **70**, 4051 (1966).

[3] Y. Sato, M. Hatano, and M. Yoneyama, This Bulletin, **46**, 1980 (1973).

[4] R. E. Ballard, A. J. McCaffery, and S. F. Mason, *Biopolymers*, **4**, 97 (1966).

[5] Y. Sato, M. Yoneyama, and M. Hatano, This Bulletin, **45**, 1941 (1972); M. Hatano, M. Yoneyama, and Y. Sato, *Biopolymers*. **12**, 895 (1973).

reading. The computer used for the curve fitting was a JEC-6 Spectrum Computer of the Japan Electron Optics Lab. Co., Ltd.

All the data presented below are reduced to the molar basis of the total AO concentration and are expressed by the molar extinction coefficient, ε, and the molar ellipticity, $[\theta]$. The molar ellipticity of the band at 222 nm is obtained on the basis of the molar quantity of the glutamyl residue.

Results

Effect of Temperature on the PLGA–AO Complex. Although Eyring and his collaborators[6] observed the effect of the temperature on the optical rotation of the PLGA–AO complex, they did not analyze this phenomenon. Therefore, we analyzed the temperature effect on the absorption and circular dichroism spectra of the PLGA–AO system with the variation in the value of the R/D ratio and in that of the pH of the system.

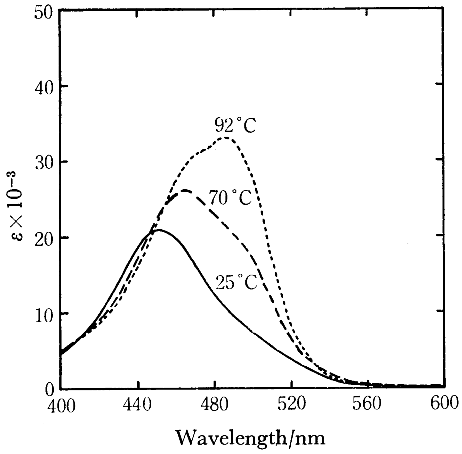

Fig. 1. Temperature dependence of absorption spectrum of PLGA–AO system. R/D=10, pH=4.5, $[\mathrm{AO}]=2\times10^{-5}$M.

When the PLGA–AO solution was gradually heated, the absorption spectrum changed, as is shown in Fig. 1. This variation may be due to a dissociation of AO molecules from the PLGA–AO complex or to a deaggregation of the AO molecules in the complex. This phenomenon was quite the reverse of the that anticipated. The course of the variation in the absorption spectrum was followed by measurements of the changes in the absorbance of the bands at 450 and 492 nm. The former band can be assigned to a band due to the aggregated AO, and the latter band, to the monomeric AO.[7] The temperature-dependent variations of the absorption bands at 450 and 492 nm in the R/D ratios of 10 and 1 are shown in Figs. 2 and 3 respectively. From these figures it can be seen that there are two types of shapes in these curves; this may be due to a difference between the arrangements of AO molecules bound to PLGA in the R/D ratios of 10 and 1. In order to discriminate the types of the arrangements of AO, the course of the decrease in the molar ellipticities at the wavelengths of 435, 465, and 520 nm were observed; the variations in the ellipticities are summarised in Fig. 4. The circular dichroism (CD) bands at 435 and 465 nm may be ascribed to the aggregated AO in a helical fashion, and that at 520 nm, to the dimerically-bound AO.[3,4] The shapes of the temperature-dependent variation curves at 435 and 465 nm are similar to each other, but the shape at 520 nm is different. This indicates that the dimeric AO molecules are not in a helical arrangement. Furthermore, the variations in the residue molar ellipticities of PLGA samples with various degrees of polymerization were measured in the presence or in the absence of AO molecules; the results are shown in Figs. 5 and 6 respectively. From Fig. 5, it may safely be said that a complete helix-coil transition of PLGA does not occur in this temperature range. This may be because the glutamyl residues acquire a somewhat negative heat of ionization with

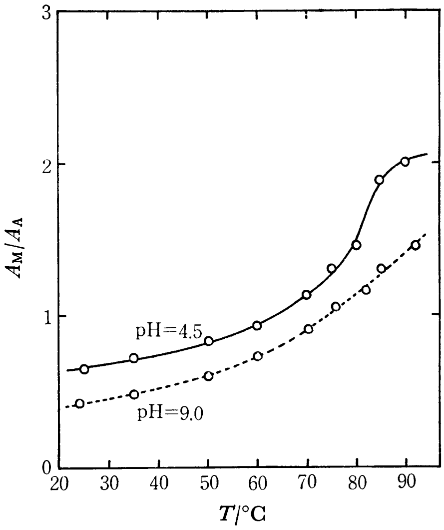

Fig. 2. Variation of ratio of absorbance at 492 nm (A_M) to absorbance at 450 nm (A_A) with changing temperature. R/D=10, $[\mathrm{AO}]=2\times10^{-5}$ M.

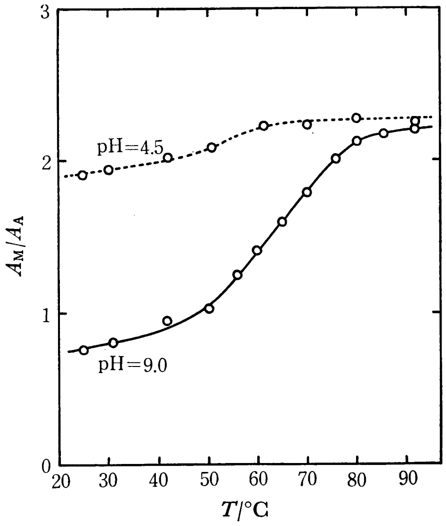

Fig. 3. Variation of ratio of absorbance at 492 nm (A_M) to absorbance at 450 nm (A_A) with changing temperature. R/D=1, $[\mathrm{AO}]=2\times10^{-5}$ M.

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[6] E. J. Eyring, M. Kraus, and J. T. Yang, *Biopolymers*, **6**, 703 (1968).

[7] V. Zanker, *Z. Phys. Chem.*, **199**, 225 (1952).

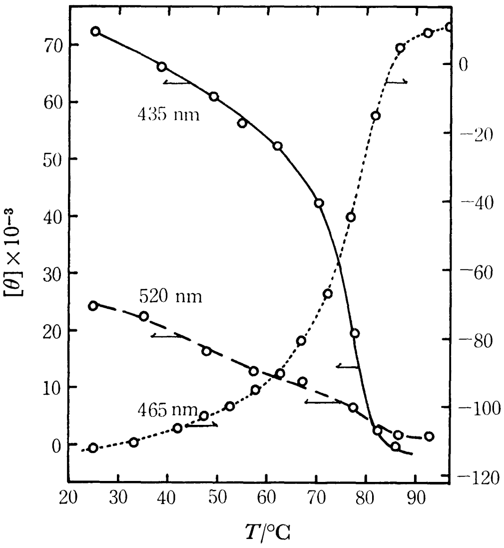

Fig. 4. Temperature dependence of the magnitudes of molar ellipticities of PLGA–AO system at each wavelength.
R/D=10, pH=4.5, [AO]=2×10^{-5} M.

an increase in the temperature.[8] On the other hand, Fig. 6 shows the occurrence of the helix-coil transition for the PLGA–AO system in the observed temperature range. This may reflect the dissociation of AO molecules from the complex, or it may suggest that a raising of the temperature disturbs the formation of an array of AO molecules on PLGA anions. In other words, it is suggested that the aggregation of AO molecules on PLGA favors keeping the α-helix structure of PLGA itself.

Effect of the Degree of Polymerization of PLGA on the Dye Aggregation in the PLGA–AO Complex. As has previously been reported,[3,5] the pH, the R/D ratio, and a neutral salt addition can all affect the extent of the dye aggregation on PLGA. Figure 7 shows the relation between f_H (helix fraction of PLGA in the PLGA–AO system) and the molar ellipticities of CD bands at 435, 465, and 520 nm. The values of f_H can be estimated from the residue molar ellipticities at 222 nm. The molar ellipticities at 435 and 465 nm increase with an increase in the f_H value, whereas that at 520 nm increases gradually with an increase in the f_H value up to 0.5 and then is almost constant in the case f_H values higher than 0.5. Figure 7 indicates that a higher f_H value than 0.2 is necessary to induce a Cotton effect in the visible region. The magnitudes of the molar ellipticities at 435 and 520 nm are dependent mainly on the helical aggregated and dimeric AO fractions respectively.[3,4] Figure 8 gives the relation between the degree of the polymerization of PLGA and the ratio of the molar ellipticity of the CD band at 520 nm to that at 435 nm. This indicates that none of all the AO molecules bound to PLGA

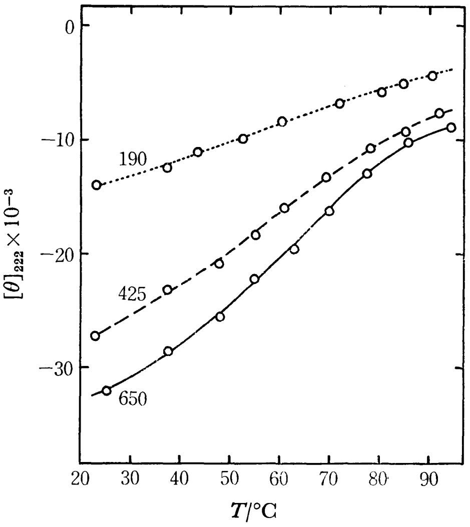

Fig. 5. Temperature dependence of the magnitudes of molar ellipticities of PLGA at 222 nm.
Numerical values in the figure shows the degree of polymerization of PLGA; pH=4.5.

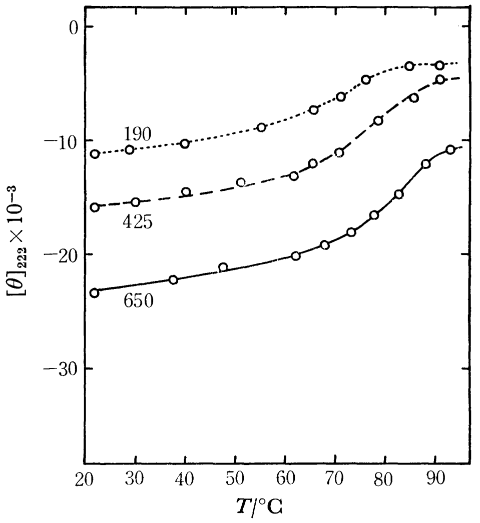

Fig. 6. Temperature dependence of magnitudes of molar ellipticities of PLGA–AO system at 222 nm. Numerical values in the figure show the degree of polymerization of PLGA; R/D=10, pH=4.5, [AO]=2×10^{-5} M.

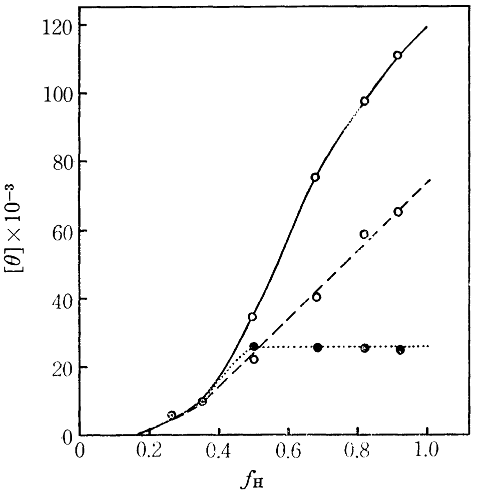

Fig 7. Plots of [θ] *vs.* helix fraction of PLGA (f_H) in the PLGA–AO systems having various R/D values.
pH=4.5. ---○---: at 435 nm, —○—: at 465 nm, (−[θ]); ···●···: at 520 nm.

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[8] R. Doty, A. Wada, J. T. Yang, and E. R. Blout, *J. Polymer. Sci.*, **23**, 851 (1957).

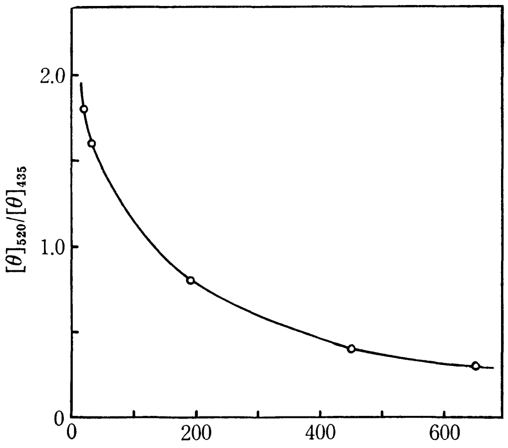

Fig. 8. Plots of ratio of magnitude of molar ellipticities at 520 nm to magnitude at 435 nm *vs.* degree of polymerization of PLGA (D.P.) in PLGA–AO system. R/D=10, pH=4.5, [AO]=2×10^{-5} M.

are dimeric, since the relation shown in Fig. 8 is not linear. This consideration is supported by the findings on neutral salt effects on the PLGA–AO system.[3]

Curve Fitting. In order to assign the CD bands observed in the PLGA–AO system, we resolved the CD bands by a conventional Gaussian fitting method. Figure 9 shows the most favorable curve fitting for the case of R/D=10 and pH=4.5, and Fig. 10, for the case of R/D=1 and pH=9.1. In both figures, the solid lines indicate the observed curves, whereas the dotted lines indicate Gaussian curves obtained by the use of a computer.

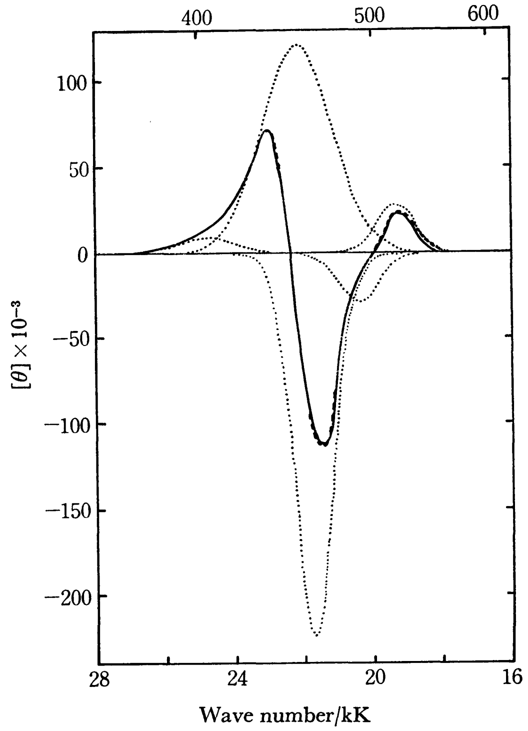

Fig. 9. CD spectrum of PLGA–AO system and its resolution into Gaussian bands; R/D=10, pH=4.5, [AO]=2×10^{-5}M. ······: Gaussian bands, ——: Observed spectrum.

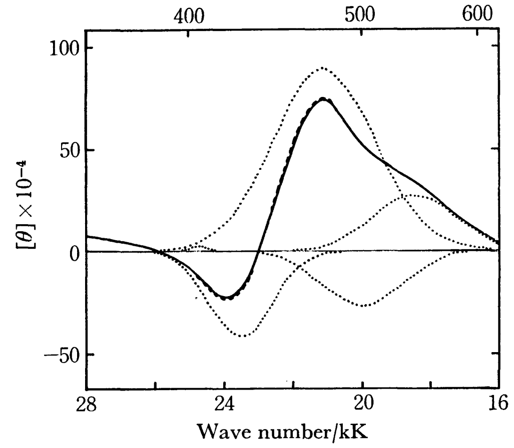

Fig. 10. CD spectrum of PLGA–AO system and its resolution into Gaussian bands; R/D=1, pH=9.1, [AO]=2×10^{-5} M. ······: Gaussian bands, ——: Observed spectrum.

Discussion

Aggregation of AO Molecules on PLGA. Ballard *et al.*[4] reported that the aggregation of AO molecules in the PLGA–AO complex has the form of a left-handed super-helix around the core of the right-handed α-helix of PLGA in a acidic pH region. On the other hand, we found that AO molecules are arranged to form a right-handed super-helix in the alkaline pH region at a R/D of around unity.[5] In a previous paper,[3] the CD band at 520 nm was assigned to the band due to the AO dimer bound to the helical PLGA in an acidic aqueous solution. Furthermore, the magnitude of the CD band at 520 nm is nearly constant at various R/D values, from 4 to 500, (as is shown in Table 1); therefore, it may safely be said that the PLGA–AO complex consists of both dimeric AO and helical AO molecules. The dimeric AO molecules contribute almost nothing to the rotational strength at 435 and 465 nm, since the increase in dimeric AO molecules is not accompanied by an increase in the magnitude at 435 and 465 nm.[3]

TABLE 1. SUMMARY OF RESULTANT CD FOR PLGA–AO SYSTEM

	Present work			a		b
R/D	4	10	20	100	400	500
λ(nm)	520	520	520	525	522	522
$[\theta]\times10^{-3}$	26	25	23.1	26.4	23.76	29.7

a) Ballard *et al.*, Ref. 4.
b) Yamaoka and Resnik, Ref. 2.

Arrangement of AO Molecules Bound to PLGA in the Acidic pH *Region.* In the acidic pH region, it was found, by using a variation method, that one AO molecule interacts with three glutamyl residues in PLGA.[9] Since AO molecules have a strong stacking tendency, the molecules are not randomly distributed among the available sites on a helical PLGA, but occupy sites adjacent to one another on the PLGA

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[9] B. C. Myhr and J. G. Foss, *Biopolymers*, **10**, 425 (1971).

and stack with each other.[10] The angle, v, between the direction of the long-axis electronic-transition moment of the AO molecule and the helix axis of PLGA may have values in the range from 5° to 40°.[4] If the thickness of the AO molecule is assumed to be 3.2 Å, and if the distance between the 3-dimethyl amino group and the 6-dimethyl amino group along the long axis of the AO molecule itself is 9.6 Å, the schematic model of the PLGA–AO complex shown in Fig. 11 can be used. In this case, the geometry of the binding sites of PLGA is that of α-helix, *i.e.*, 3.6 residues/turn, a vertical distance of 1.5 Å from each other, and a radius 5.4 Å.[11]

On the basis of the geometry of AO molecules in the PLGA–AO complex, the v angle can be determined to be near 15°; this seems plausible in view of Ballard's results[4] and the geometrical positions of the binding sites on PLGA. The short axis of the AO plane may be slightly tangentially-oriented with respect to the axis of the α-helix of PLGA, since this complex gives a positive CD band near 295 nm, where a transition polarized along the short axis of AO appears.[5]

A frequency interval, $\Delta\nu$, between the two transition energies which interact each other is given by:[12]

$$\Delta\nu = 2\mu^2 G/hcd^3 \qquad (1)$$

where G is the geometric factor for the dipole-dipole interaction with the limitting values of $+2$ and -1, h is Planck's constant, c is the velocity of light, and d is the distance between the two transition moments. The dipole strength, μ^2, of the visible absorption band of monomeric AO is 47×10^{-36} c.g.s.[12]

A Gaussian analysis, as is shown in Fig. 9, for the CD curve of the PLGA–AO complex in the acidic pH region suggests the presence of one positive CD band at 520 nm and one negative CD band at 490 nm. The magnitudes of the two bands at 520 and 490 nm are nearly equal to each other, but the signs are opposite. In the pair of bands, the frequency interval is 1100 cm^{-1}. The geometric factor, G, is assumed to be $+1$ because of the antiparallel sandwich type of AO dimer, and the distance from the center of one AO molecule to that of the nearest neighbor AO molecule in the PLGA–AO complex can be estimated to be 7.6 Å, which is a reasonable value.

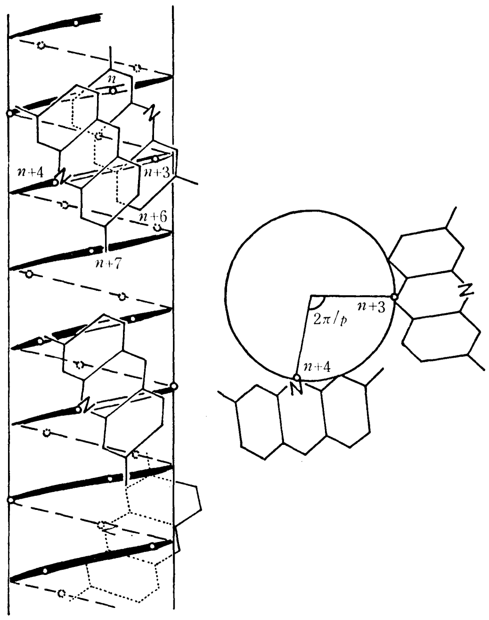

Fig. 11. Schematic model of PLGA–AO complex and the correlation between the positions of dimeric AO molecules in acidic pH.

In the PLGA–AO complex, two types of interaction between the transition dipoles of the AO molecules in the helical array bound to the helical PLGA can be expected. One is dependent on the radius of the helix, whie the other is dependent on the pitch. The radius-dependent rotational strength, R, is given by the following equation:[12]

$$R_{//} = -R_{\perp} = 2\pi a\mu_v\mu_t \sin^2(\pi/P) \qquad (2)$$

where a is the radial distance of each transition dipole from the helical axis and where $(2\pi/P)$ is the angle in radian about the axis between the two adjacent dipoles, which refer to Fig. 11. The rotational strengths, $R_{//}$ and $R_{\perp}$, arise from the resultant electronic transitions, which are polarized parallel and perpendicular to the helix axis respectively. The moments, μ_v, μ_t, and μ_r are, respectively, the vertical, tangential, and radial components of the dipole vector, $\boldsymbol{\mu}$, in the cylindrical coordinate frame of the helix. If the AO molecular planes lie at an angle of 15° to the axis of the helix, the pitch-dependent rotational strength can be neglected. Hence, the radial components of the interactions between the two excitation dipoles could be considered; the rotational strength was estimated to be 25×10^{-40} c.g.s., assuming the angle to be 15°. This value was obtained on the approximation that the dimeric AO molecules were attached to the glutamyl residues numbered (n, $n+3$, $n+6$) and ($n+1$, $n+4$, $n+7$) in the sequence along the polypeptide backbone of the α-helix of PLGA. This approximation was based on the finding that the distance between the dimeric AO molecules was nearly equal to that between the binding residues numbered $n+3$ and $n+4$. On the other hand, the experimental value of the rotational strength obtained from the CD magnitude of the bands at 520 and 490 nm was 23×10^{-40} c.g.s.; it was obtained using the following equation:

$$R = 0.696\times10^{-42}\sqrt{\pi}\,[\theta_k]\frac{\Delta_k}{\lambda_k} \qquad (3)$$

where $[\theta_k]$, Δ_k, and λ_k are the molar ellipticity, the half-band width, and the wavelength of the k-th CD band respectively. This experimental value agrees with the calculated one. Accordingly, the postulated scheme for the arrangement of AO molecules bound to PLGA may be plausible. As is shown in Fig. 11, two AO molecules attached to the glutamyl residues numbered (n, $n+3$, $n+6$) and ($n+1$, $n+4$, $n+7$) form the dimeric AO. Besides, the aggregated AO molecules have the form of a left-handed super-helix around the core of the α-helix of PLGA. These aggregated AO mole-

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[10] D. F. Bradley and M. K. Wolf, *Proc. Natl. Acad. Sci., U.S.*, **45**, 944 (1959).

[11] I. Tinoco, Jr., R. W. Woody, and D. F. Bradley, *J. Chem. Phys.*, **38**, 1317 (1963).

[12] B. J. Gardner and S. F. Mason, *Biopolymers*, **5**, 79 (1967).

cules have two CD bands, a negative CD band at 465 nm and a positive CD band at 435 nm, arising, respectively, from the transitions polarised parallel to and perpendicular to the axis of the α-helix of PLGA.4) As is shown in Fig. 9, there are a negative component at about 460 nm and a positive component at about 450 nm in the CD curve of the PLGA–AO complex. These components can be assigned to the transitions polarized parallel and perpendicular to the axis of the α-helix of PLGA.

Arrangement of AO Molecules Bound to PLGA in the Neutral and Alkaline pH *Regions.* In the neutral and alkaline pH regions, PLGA is substantially ionized and randomly coiled in an aqueous solution. However, we have found that the PLGA–AO complex has a helical structure even in the neutral and alkaline pH regions at a R/D of unity.5) In this case, the ring nitrogen cations of AO molecules bind to the carboxylate anions of PLGA side chains; these AO cations may be neutralized enough by the ion coupling to stack together. From the sign of the CD band near 450 nm (Fig. 10), it was considered that the arrangement of AO molecules bound to PLGA at R/D$=$1 and pH$=$9 may be such that their long axes form a right-handed super-helix around the PLGA chain. Thus, the formation of stacked dimeric AO molecules on PLGA is most plausible.

Over the same geometry of the α-helix of PLGA with that in an acidic aqueous medium, an array of aggregated AO, at R/D$=$1 and pH$=$9, may be arranged in such way as is shown in Fig. 12. In this case, one positive CD band and one negative CD band can be observed at 540 nm and at 500 nm respectively. In view of the steric hindrance between the dimethylamino groups of the AO molecules, the transition dipoles of the AO molecules may lie at an angle of about 51° to the axis of α-helix of PLGA. In this complex, AO molecules attached to the glutamyl residues numbered n, $n+3$, and $n+6$ may form two pairs of dimers. On the basis of these presumptions, the rotational strength arising from the interaction between dimeric AO molecules can be estimated to be 101×10^{-40} c.g.s. Therefore, the total rotational strength originating from dimeric AO molecules is 202×10^{-40} c.g.s. This value is very small compared with the experimental value. The rotational strength obtained from resolved CD curves using Eq. (3) amounts to 480×10^{-40} c.g.s. This extraordinarily large rotational strength may arise from an interaction between the neighboring AO molecules on PLGA numbered $n-1$, $n+1$, $n+2$, *etc.* In the case of an R/D of unity in the alkaline pH region, the dimeric AO molecules should form an aggregate more tightly and should interact with each other. Therefore, the interaction between the dimeric AO units must be considered to be stronger in this case than in the case of a large value of R/D in the acidic pH region.

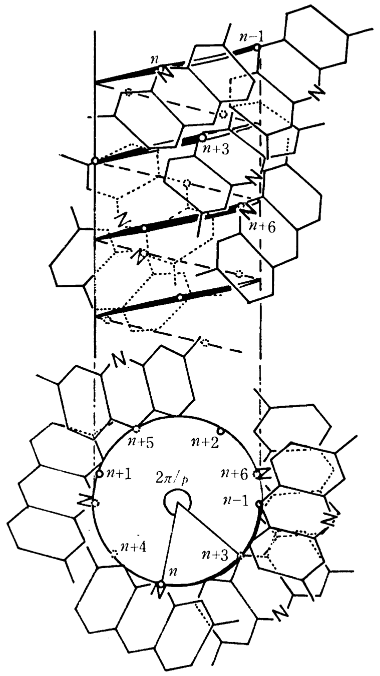

Fig. 12. Schematic model of PLGA–AO complex and the correlation between the positions of dimeric AO molecules at R/D$=$1, pH$=$9.

These closely-spaced AO molecules around the core of the α-helix of PLGA may also give an extraordinarily large rotational strength at 222 nm,5) one which results from the coupling of the spin-forbidden n-π* transition of polypeptide with some other allowed π-π* transition of the AO molecule.

The polarization directions of the AO molecules and dimeric AO could be assigned by means of a polarization technique using a liquid crystal, one which is oriented by the application of an electric field. This experimental technique and our results will be described elsewhere in the near future.