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Spectroscopic Study on Direction and Inclination of Helical Peptides in Monolayers Formed at the Air/Water Interface and on a Gold Substrate

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Monolayers at the air/water interface and self-assembled monolayers (SAMs) on a gold substrate were prepared from helical peptide derivatives carrying a 9-ethyl-3-carbazolyl group and a disulfide group. π–A Isotherms and circular dichroism spectra of Langmuir–Blodgett monolayers on a quartz substrate showed that the helical peptides were lying at the air/water interface. The molecular arrangement in the monolayer was analyzed by fluorescence quenching using in situ fluorescence spectroscopy. Computer simulations for the quenching experiment under the assumption of the mixed arrangement of parallel and antiparallel orientations explain the experimental data. On the other hand, the helical peptides in the SAMs were found to take standing orientation and a parallel arrangement by Fourier transform infrared reflection–absorption spectroscopy and fluorescence spectroscopy.

Peptide molecules can be designed and synthesized to adopt specific secondary structures.[1—4] It is interesting to assemble the peptide molecules therefore to construct novel molecular systems which function on the basis of their highly ordered structure.[5] For example, Ghadiri et al. have reported peptide nanotubes as artificial ion channels where cyclic β^3-peptides form a tubular structure by intermolecular hydrogen bondings.[6] Fujita et al. have prepared peptide monolayers at the air/water interface.[7] Worley et al. have synthesized poly-(γ-L-benzyl glutamate) with lipoic acid at the N-terminal, and formed the peptide self-assembled monolayer (SAM) on a gold substrate under an electrostatic field.[8] Fujita et al.[9] and Miura et al.[10] have prepared the oriented α-helical peptide SAMs vertically to gold substrate by utilizing the self-assembling property of the helical peptides. These peptide monolayers have been investigated on the conformation and inclination (molecular orientation with respect to the surface) at the air/water interface or on a metal substrate. However, the adequate information about the direction of the helical peptides (parallel or antiparallel arrangement) in the monolayers is not obtained yet.

In the present work, the direction and inclination of peptide molecules in monolayers at the air/water interface and in SAMs on a gold substrate were investigated by various spectroscopic methods. All the peptides have a sequence of alternating L-alanine (Ala) and α-aminoisobutyric acid (Aib) because of their high preference for adopting an α-helical structure.[11] A 9-ethyl-3-carbazolyl (ECz) group was covalently linked at the peptide terminal as a fluorescence probe.[12—14] The structures of the peptide derivatives and the dialkyl control compound carrying an ECz group are shown in Fig. 1 together with their abbreviations.

Thiol and disulfide compounds are frequently used for covalent connections to a gold substrate via an S–Au linkage, forming highly ordered SAMs.[15] In order to obtain the peptide SAMs, a disulfide group was covalently linked to the peptide terminal. The disulfide group was also used as a fluorescence quencher. ECz-A12-SS and SS-A12-ECz are tridecapeptides carrying an ECz group and a disulfide group at the terminals, however, in a reverse way about the N- or C-terminal. ECz-A20-SS is a henicosapeptide analogous to ECz-A12-SS with respect to the positions of the functional groups. ECz-A12 is a tridecapeptide which carries an ECz group at the N-terminal but lacks a disulfide group at the C-terminal. ECz-C11-SS is a disulfide compound composed of a dialkyl chain for the peptide chain.

Monolayers at the air/water interface were prepared from these compounds and the molecular orientation was investigated by π–A isotherms and circular dichroism (CD) measurements of Langmuir–Blodgett (LB) monolayers transferred on a quartz substrate. The molecular arrangement was determined by in situ fluorescence spectroscopy and computer simulations. The peptide SAMs on the gold substrate were investigated on the orientation and arrangement of peptide molecules by Fourier transform infrared reflection-absorption spectroscopy (FTIR-RAS) and fluorescence spectroscopy.

Experimental

Chemicals. ECz-A12-SS, SS-A12-ECz, ECz-A20-SS, and ECz-C11-SS were synthesized by the method reported previously.[16] ECz-A12 was synthesized similarly and identified by ^{1}H NMR

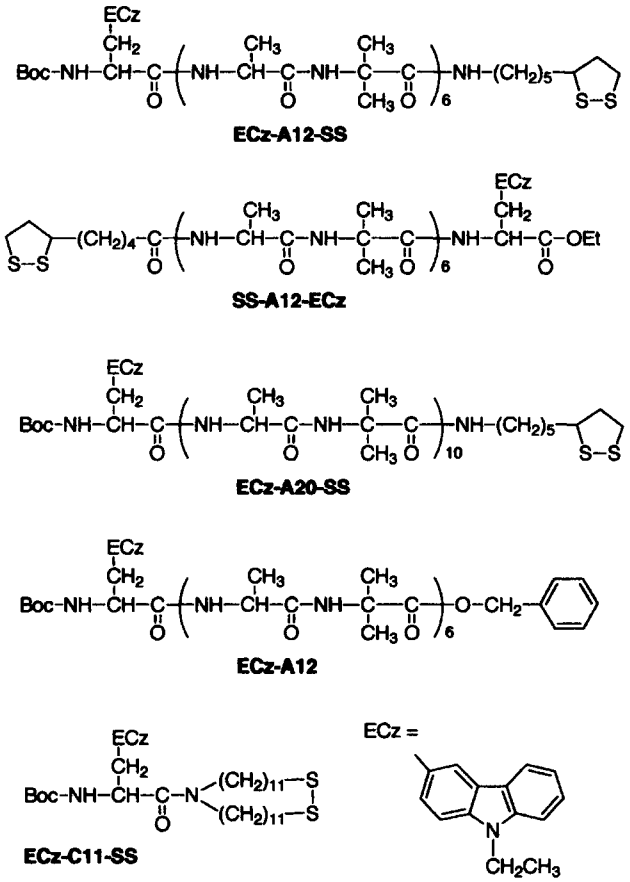

Fig. 1. Molecular structures of peptide derivatives and a dialkyl compound carrying an ECz group.

and mass spectroscopy together with analytical thin-layer chromatography (TLC). The solvent systems for TLC were (A) chloroform/methanol/acetic acid (90/10/3 v/v/v), (B) chloroform/methanol/ammonia water (13/5/1 v/v/v).

^{1}H NMR (270 MHz, $CDCl_3$) δ = 1.39—1.65 (66H, brm, NHCH-(CH_3)CONHC(CH_3)$_2$, (CH_3)$_3$C, NCH$_2$CH_3), 3.13—3.42 (2H, d, NHCHCH_2), 3.96, 4.25 (6H, m, NHCH(CH_3)CONHC(CH_3)$_2$), 4.37 (3H, brm, NCH_2CH$_3$, NHCHCH$_2$), 5.12 (2H, m, OCH_2), 5.53 (1H, s, NHCHCH$_2$), 7.22—8.08 (24H, brm, NHCH(CH_3)CONHC-(CH_3)$_2$, phenyl-H, carbazolyl-H). MS (FAB, matrix; *m*-nitrobezyl alcohol) *m/z* 1432 (Calcd for $C_{71}H_{104}N_{14}NaO_{16}$: [(M+Na)$^+$] *m/z* 1431.77). TLC: R_f(A) = 0.65. R_f(C) = 0.88.

Preparation of Monolayers at the Air/Water Interface. Peptide derivatives and the reference compound were dissolved in chloroform at a concentration of 5.0×10^{-4} M (1 M = 1 mol dm^{-3}). The solution was spread on an aqueous phase by using a microsyringe, and equilibrated for 10 min before compression. π–A Isotherm was recorded at a constant rate of compression of 10 cm^2 min^{-1} with a Langmuir trough (FSD110, USI Co., Ltd., Japan). The bicomponent monolayers of ECz-A12 and ECz-A12-SS or SS-A12-ECz were prepared similarly but from a chloroform solution containing both compounds. LB monolayers were prepared by transferring monolayer on the water subphase to a quartz substrate by the horizontal lifting method.

Preparation of SAMs. A slide glass was washed thoroughly with methanol and distilled water followed by H_2SO_4 (97%) : H_2O_2 (30%) = 7 : 3 (a piranha solution) for 3 h. A substrate coated with gold was prepared by vapor deposition of chromium and then gold (99.99%) onto the slide glass. The thickness of the chrominium and gold layers, monitored by a quartz oscillator, were approximately 100 and 1000 Å respectively. The gold substrate was used for a self-assembling experiment within a few days after preparation. The gold substrate was incubated in an ethanol solution of a disulfide compound (0.1 mM) for 24 h. After the incubation, the substrate was rinsed rigorously with ethanol and dried in a stream of dry nitrogen gas.

Fluorescence Spectroscopy of Peptide Monolayers. In situ fluorescence spectroscopy for the monolayers at the air/water interface was performed by a home-made apparatus reported previously.[17] The light came from a Xe lamp (500 W, JASCO Co., Ltd., Japan), and a monochromatic light (half width of 10 nm) was obtained by a home-made spectrometer. The incident light was focused on an area of 0.2 cm^2. Fluorescence from the surface was guided by a fiber optics to a spectrometer (Unisoku Co., Ltd., Japan) with a multichannel photodiode array (C3966 PC-IMD, Hamamatsu Photonics Co., Ltd., Japan). Fluorescence spectra of the SAMs on the gold substrate were recorded on a fluorescence spectrometer (F4010, Hitachi Co., Ltd., Japan) in a front-face mode with a 15° angle between a gold substrate and detector.[18] All measurements were performed at room temperature.

CD Measurement. A CD spectrum of the peptide LB monolayer was measured at room temperature on a CD spectrometer (J-600, JASCO Co., Ltd., Japan) by lining up eight quartz plates on which the peptide monolayer was deposited.

FTIR-RAS. FTIR spectrum was recorded on a Fourier transform infrared spectrometer (Magna 850, Nicolet Japan Co., Ltd., Japan) at room temperature. For RAS measurements, a reflection attachment (model RMA-1DG/VRA, Harrick Co., Ltd., NY) was used, and a *p*-polarized beam was obtained through an Au/AuBr wire-grid polarizer (Hitachi Co., Ltd., Japan). The incident angle was set at 85° from the surface normal. The number of interferogram accumulations was 500. Molecular orientation of the peptide monolayer on the gold substrate was determined on the basis of the amide I/amide II absorbance ratio in the FTIR-RAS spectrum under the assumption of a uniform peptide-thin layer.[19—23]

Results and Discussion

Orientation of Helical Peptides in Monolayers at the Air/Water Interface. The π–A isotherms of the ECz-A12-SS, SS-A12-ECz, ECz-A20-SS, ECz-A12, and ECz-C11-SS monolayers are shown in Fig. 2. The isotherms of the ECz-A12-SS and SS-A12-ECz monolayers seem to be nearly the same, which present a plateau in the region of molecular area 250—120 Å^2/molecule. A molecular area of a peptide lying parallel to the water surface was calculated to be about 270 Å^2/molecule by using the length of the tridecapeptide (28 Å) for a fully helical conformation and the diameter of the helix (9.6 Å).[10] This area corresponds to the starting point of the plateau region. It is thus considered that the peptides lying flat on the water subphase change the physical state upon further compression in the plateau region. The isotherm of the ECz-A12 monolayer also has analogous profile but its plateau region shifts to a little larger molecular area, indicating a larger molecular size on the water subphase. The isotherm of the ECz-A20-SS molecule shifts to a larger molecular area because of the larger cross-section area of the henicosapeptide. However, the isotherm did not show a distinct plateau region, suggesting that the

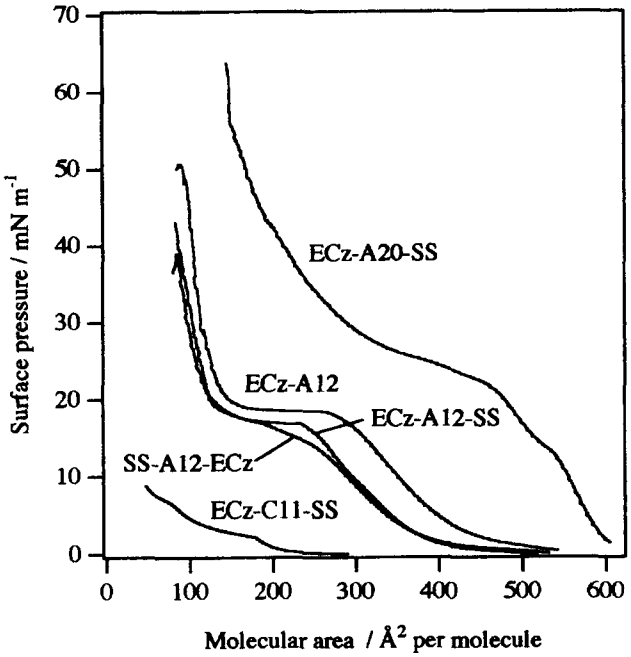

Fig. 2. π–A Isotherms of ECz-A12-SS, SS-A12-ECz, ECz-A20-SS, ECz-A12, and ECz-C11-SS on the water subphase.

peptide layer does not form a regular structure under high surface pressures. The ECz-C11-SS monolayer shows low surface pressure even in a compressed phase, indicating that the monolayer is not stable.

The changes of the physical state of the tridecapeptide monolayers upon compression were investigated by CD measurements of the LB monolayers of ECz-A12 (Fig. 3). The monolayer on the water subphase was transferred at two different molecular areas, 268 and 124 Å^2/molecule, which correspond respectively to the first and last molecular area in the plateau region upon compression. It is obvious that the transfer of the monolayer was unsuccessful at 268 Å^2/molecule. On the other hand, the CD spectrum of the monolayer transferred at 124 Å^2/molecule showed a double-minimum pattern, which is characteristic of an α-helical structure.[24,25] The magnitude at 208 nm is stronger than that at 222 nm, suggesting that the helices are lying parallel to the quartz substrate and probably at the air/water interface.[26] It is thus considered that the helical peptide should mount over the packed monolayer while retaining the lying orientation upon compression.[27]

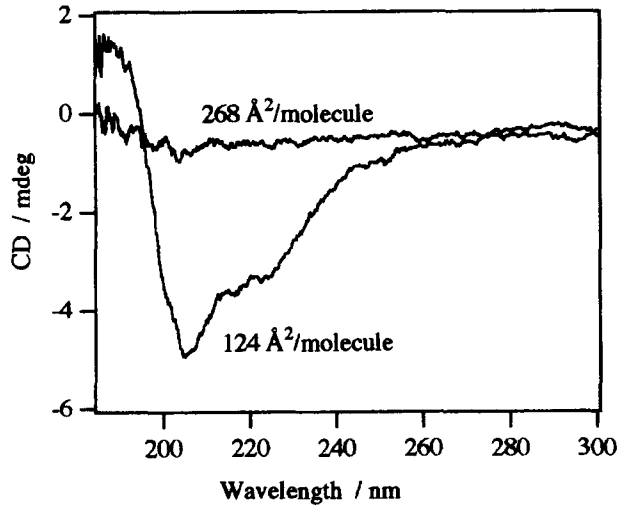

Fig. 3. CD spectra of Langmuir–Brodgett monolayers of ECz-A12 which were transferred at the molecular areas of 268 Å^2/molecule and 124 Å^2/molecule on a quartz substrate.

In Situ Fluorescence Spectroscopy of Monolayers at the Air/Water Interface. In situ fluorescence spectra of the ECz-A12 monolayer at various molecular areas are shown in Fig. 4. Monomer emission of ECz group (357 nm, 373 nm) was observed even at a compressed phase, indicating no excimer formation. Similar results were also obtained in the cases of the ECz-A12-SS, SS-A12-ECz, and ECz-A20-SS monolayers. The dependence of fluorescence intensity on the molecular area is shown in Fig. 5. The fluorescence intensity of the ECz-A12 monolayer increases remarkably from molecular areas less than 270 Å^2/molecule. This increase can not be explained by increase of the molecular number per unit area upon compression, but is explainable by the formation of bilayers at molecular areas less than 270 Å^2/molecule. The fluorescence quantum yield of ECz groups at the first layer on the water subphase is considered to be low because of an aqueous environment. However, the ECz groups at the second layer on the water subphase should fluoresce intensively in a nonaqueous environment. In the other monolayers, the emission was much weaker than that in the ECz-A12 monolayer, indicating that the quenching of ECz group by the disulfide group occurs in both monolayer and bilayer. The radius of the active sphere for the quenching of ECz group by the disulfide group was calculated to be about 10 Å from the Stern–Volmer plot for the quenching of 9-ethylcarbazole by DL-α-lipoic acid in toluene.[28] A disulfide group is thus considered to effectively quench the neighboring ECz groups. The fluorescence intensities of the ECz-

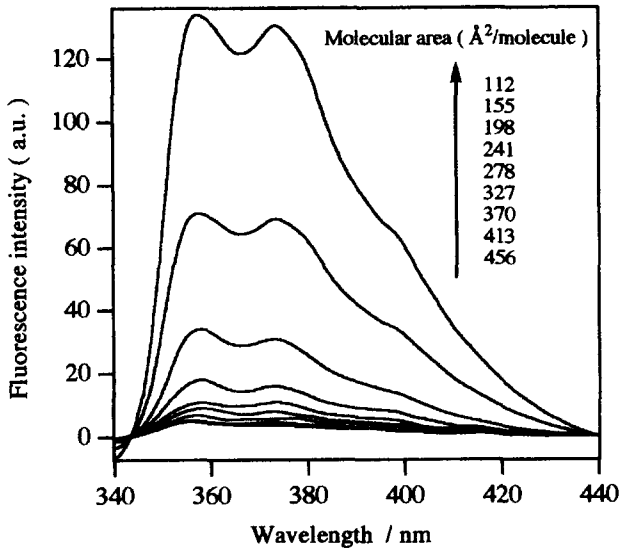

Fig. 4. In situ fluorescence spectra of the ECz-A12 monolayer on the water subphase at various molecular areas.

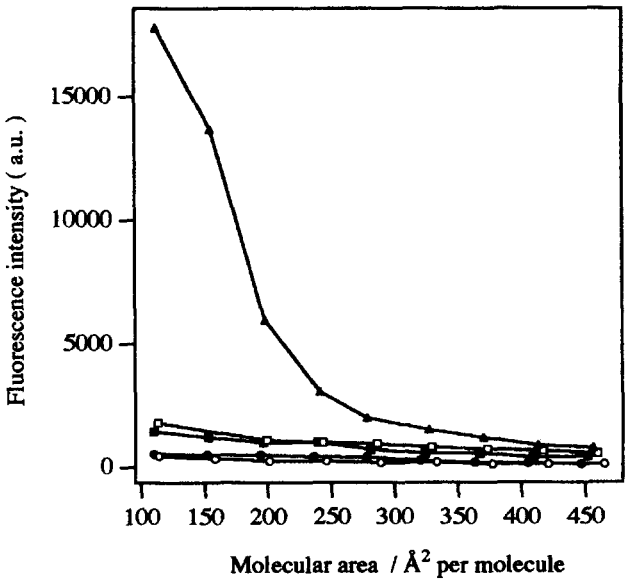

Fig. 5. The relation of fluorescence intensity (integration value from 350 to 400 nm) of the helical peptide monolayers with molecular surface areas: ECz-A12-SS (●), SS-A12-ECz (○), ECz-A12 monolayer (▲), ECz-A12/ECz-A12-SS (■), ECz-A12/SS-A12-ECz bicomponent monolayer (1/1 mol/mol, □), respectively.

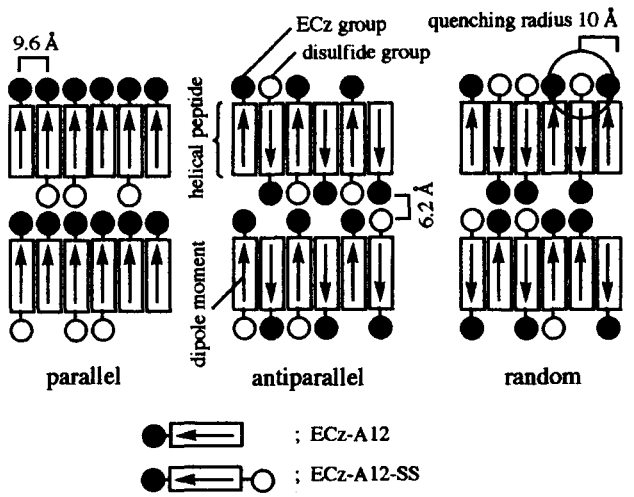

Fig. 6. Schematic illustration of computer simulation for the fluorescence quenching of ECz group by the disulfide group in the bicomponent monolayers (ECz-A12 and ECz-A12-SS) on the water subphase. The number of radiative ECz groups were calculated at three different arrangements.

A12-SS and SS-A12-ECz monolayers are substantially the same, suggesting that the arrangement of the helical peptides is not affected by the dipole direction.

In order to determine the arrangement of the peptide molecules at the air/water interface, the fluorescence quenching of ECz group by the disulfide group was simulated by calculation. A schematic illustration of the computer simulation is presented in Fig. 6. Three distinct arrangements of peptide molecules, that is, parallel, antiparallel, and random arrangements were assumed. The helical peptides (10500 molecules) were located on a two-dimensional lattice (250×42) according to the respective arrangement, and the number of radiative ECz groups was counted under an assumption that ECz groups within the quenching sphere of the disulfide group (radius 10 Å) are nonradiative. The lateral distance between functional groups (ECz-ECz or ECz-disulfide) was assumed to be 9.6 Å and the distance between functional groups of neighboring layers was assumed to be 6.2 Å where the two ECz groups are in the closest contact. The results of the computer simulations at 270 Å^2/molecule are summarized in Table 1. The simulated values for the random arrangement agree well with the experimental values. It is therefore considered that dipole–dipole interaction is not prevailing in the helical peptide monolayer probably due to hydration of helical peptides, resulting in a random arrangement.

At smaller molecular areas, the fluorescence from the disulfide containing peptide monolayers was quenched significantly in comparison with that of the ECz-A12 monolayer (Fig. 5). This could be explained by energy migration among ECz groups which occurs efficiently because the fluorescence quantum yield of ECz group in the second layer on the water subphase is high. The energy migration among ECz groups increases the encounter chance with the quencher in the peptide bilayer. We assume that the ECz group locating at the diagonal position of the neighboring layer is quenched by the disulfide group due to the effect of energy migration, which is reflected in the computer simulation by taking the quenching radius of 12 Å. The computer simulation for the quenching by the disulfide group in the bilayer of the helical peptides in a random arrangement was carried out taking the quenching radius to be 10 or 12 Å, and the results are summarized in Table 2. The simulated values for 12 Å quenching radius

Table 1. Computer Simulation Results and Experimental Values for Fluorescence Quenching of ECz Group by the Disulfide Group in the Monolayers of Peptide Derivatives

The fluorescence intensities were corrected by assuming the intensity of the ECz-A12 monolayer to be 100.

	Experimental values	Simulated values		
	at 270 Å^2/molecule	Parallel	Antiparallel	Random
ECz-A12	100	100	100	100
ECz-A12-SS	17.1	0	0	12.2
SS-A12-ECz	8.3	0	0	12.1
ECz-A12+ECz-A12-SS	33.1	49.8	11.8	41.2
ECz-A12+SS-A12-ECz	41.1	37.9	55.9	41.6

Table 2. Computer Simulation Results for Fluorescence Quenching of ECz Group by the Disulfide Group in the Bilayers of Peptide Derivatives by Taking the Quenching Radius of the Disulfide Group to be 10 or 12 Å
The fluorescence intensities were corrected by assuming the intensity of the ECz-A12 monolayer to be 100.

	Experimental values	Simulated values	
	at 135 Å^2/molecule	Quenching radius 10 Å	Quenching radius 12 Å
ECz-A12	100	100	100
ECz-A12-SS	3.3	6.6	0.8
SS-A12-ECz	2.5	6.6	0.9
ECz-A12+ECz-A12-SS	8.5	31.7	14.5
ECz-A12+SS-A12-ECz	10.4	32.0	14.9

agree with the experimental values better than the values by assuming 10 Å quenching radius, indicating that energy migration among ECz groups promotes the quenching by the disulfide group. The difference between the simulated and experimental values is, however, relatively large. This may be attributed to the simplicity of the model where a homogeneous arrangement is assumed in the monolayers and the quenching radius is three-dimensionally isotropic.

Orientation and Arrangement of Helical Peptides in Self-Assembled Monolayers. The surface coverage of ECz-A12-SS, SS-A12-ECz, ECz-A20-SS, and ECz-C11-SS on the gold substrate were 146, 115, 94.4, and 48.1 Å^2/molecule, respectively.[16] The molecular area of ECz-C11-SS is much smaller than those of the helical peptides because of the smaller cross section of the dialkyl group, and is nearly twice the molecular area of a single-chain *n*-alkanethiol.[29] The amide I/amide II absorbance ratios in the FTIR-RAS spectra of the ECz-A12-SS, SS-A12-ECz, and ECz-A20-SS SAMs were 2.34, 2.71, and 3.34, respectively. The tilt angles of the helices from the surface normal were calculated to be 44°, 41°, and 36°, respectively. The molecular areas were calculated using these tilt angles to be 121, 114, and 105 Å^2/molecule for the ECz-A12-SS, SS-A12-ECz, and ECz-A20-SS, respectively, assuming hexagonal packing of the helices and uniform orientation in the SAMs. The molecular areas of SS-A12-ECz and ECz-A20-SS determined by the FTIR-RAS coincide well with those obtained by the QCM measurements, indicating the formation of well-packed monolayers. On the other hand, the molecular area of ECz-A12-SS from the FTIR-RAS was slightly smaller than that from the QCM measurement (146 Å^2/molecule). Therefore, the ECz-A12-SS SAM should contain some defects or incompletely-packed regions. The disorder of the ECz-A12-SS SAM is considered to be due to the connection of the C-terminal of the helical peptide to the gold substrate through an S–Au linkage. Since the S–Au linkage (S^-–Au^+) has an ionic property, the negative charge of S^- should cause unfavorable interaction with the negative dipole end at the C-terminal of the helix.[30,31]

The fluorescence spectra of the SAMs are shown in Fig. 7. Monomer emission was dominant in the ECz-A20-SS SAM. However, second excimer emission (375—380 nm) was also observed in the ECz-A12-SS, SS-A12-ECz, and ECz-C11-SS SAMs.[32,33] The excimer formation suggests a disorder structure in the SAMs. Because short peptides do not adopt fully helical conformation and the alkyl chains are flexible, these spacers may allow the excimer arrangement of ECz groups.

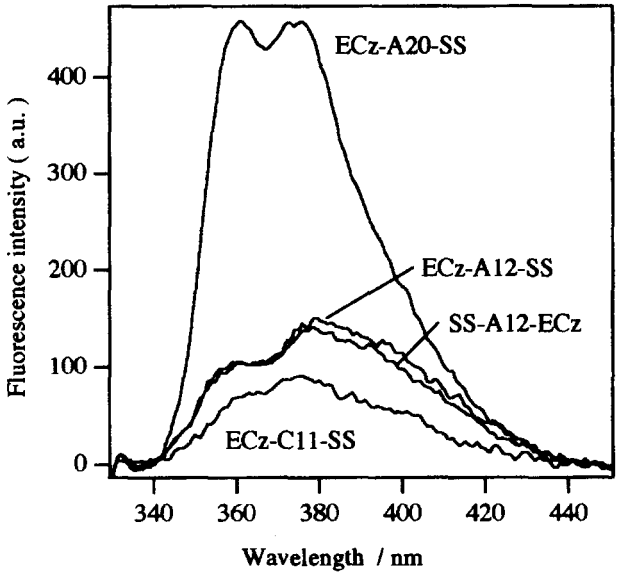

Fig. 7. Fluorescence spectra of the ECz-A12-SS, SS-A12-ECz, ECz-A20-SS, and ECz-C11-SS SAMs.

The fluorescence from the ECz-A12-SS, SS-A12-ECz, and ECz-C11-SS SAMs was quenched more than that from the ECz-A20-SS SAM. The distances of the ECz group from the gold substrate in the SAMs are calculated to be 21, 21, 33, and 17 Å for the ECz-A12-SS, SS-A12-ECz, ECz-A20-SS, and ECz-C11-SS SAMs, respectively. The degree of quenching is therefore correlated with the distance of the ECz group from the gold substrate. The quenching mechanism should be due to energy transfer from the photoexcited ECz group to gold considering the long distance between them. All these results indicate that the helical peptides take the upright orientation to the gold substrate in a parallel arrangement in the peptide SAMs.

Concluding Remarks

Monolayers at the air/water interface and SAMs on the gold substrate were prepared from helical peptide derivatives carrying an ECz group and a disulfide group. The helical peptides were spread on the water subphase taking parallel orientation to the surface. Upon compression, bi-

layer of helical peptides was formed. The interactions between helical peptides are not strong enough to regulate the parallel or antiparallel arrangement at the air/water interface. On the other hand, the parallel arrangement is possible for the peptide SAMs although dipole–dipole interaction should be strong in such a nonaqueous environment. It is therefore concluded that the parallel or antiparallel arrangement in the helical peptide layer can be regulated by using some suitable interaction and/or chemical method upon the molecular assembling.

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