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Supramolecular assembly containing hydrophobic α-helical oligopeptide molecules

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Hydrophobic α-helical oligopeptides, Boc-(Ala-Aib)$_n$-OMe ($n = 4$ and 8) and Boc-(Ala-Aib)$_n$-Ala-Cr ($n = 4$ and 8), where Aib and Cr represent α-aminoisobutyric acid and a crown-ether unit respectively, were synthesized. These peptides formed a voltage-dependent ion channel by aggregation of the oligopeptides orthogonal to the bilayer lipid membrane. The electric current increased with increasing the transmembrane potential and the chain length (n) of the oligopeptides. The addition of K^+ or Cs^+ in the presence of Boc-(Ala-Aib)$_4$-Ala-Cr induced a stronger voltage-dependent electric current than in the presence of Boc-(Ala-Aib)$_4$-OMe. Monolayers of hydrophobic α-helical oligopeptides, Boc-(Lys(Z)-Aib)$_8$-OMe, Boc-(Ala-Aib)$_8$-OMe, and Boc-(Leu-Aib)$_8$-OBzl, were formed at an air/water interface. It was found that the oligopeptide molecules oriented the α-helical axis parallel to the interface. In particular, the monolayer of Boc-(Leu-Aib)$_8$-OBzl formed a condensed state, a two-dimensional (2-D) crystal, by compression of the monolayer, in which the helices are oriented and associate by interdigitation of side chains. Under a monolayer of biotinyl lipid molecules, a 2-D crystal of streptavidin (SAv) was formed, leaving two unoccupied coordination sites for binding biotin. A double-layer of 2-D crystal of SAv was synthesized by using as the linker molecule a suitably designed bisbiotinyl α-helical peptide, Bio-(Lys-Aib-Aib)$_6$-Bio, with Bio representing a biotinyl group. An amphiphilic α-helical oligopeptide, H-(Ala-Aib)$_8$-OBzl, was synthesized and dispersed in water by sonication. Circular dichroism spectroscopy, transmission-electron microscopic observation and dynamic light scattering measurement indicated that the α-helical oligopeptides associate together into a spherical form with an average diameter of 70 nm probably consisting of a bilayer structure. Copyright © 1996 Elsevier Science Ltd.

(Keywords: amphiphilic α-helix; hydrophobic α-helix; ion channel)

INTRODUCTION

It is well-known that proteins form supramolecular structures by self-assembly of oligopeptide fragments and take various conformations and structures, such as α-helix, 3_{10}-helix and β-sheet. Typical examples are the folded structure of ion-channel protein[1] and the four-helix bundle structure of cytochrome $b562$[2]. It is considered that the key factors determining the supramolecular structure are embedded in the amino acid sequence. The search for these key factors has been the focus of interest in protein chemistry[3]. However, it might be possible to construct protein-like higher-order structure of oligopeptide molecules exhibiting novel functionalities, provided that the molecular template to control the aggregation of peptide molecules under various conditions is established. In the present investigation, the construction of supramolecular assemblies under various conditions, e.g., an ion channel in bilayer lipid membrane (BLM), a two-dimensional (2-D) crystal at air/water interface, and a vesicular assembly in aqueous dispersion, of hydrophobic α-helical peptides were attempted.

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PREPARATION

Peptide synthesis

The molecular structures of synthetic oligopeptides are shown in *Figure 1*. Peptide synthesis was carried out by the conventional liquid-phase method as shown typically in *Figure 2*. Fragment condensation was repeated by using DCC and HOBt as coupling reagents ultimately to obtain Boc-(Ala-Aib)$_n$-OMe ($n = 4$ and 8) and Boc-(Ala-Aib)$_n$-Ala-Cr ($n = 4$ and 8). The synthesized peptides were purified by either recrystallization from a methanol/diethyl ether mixture or by chromatography through a Sephadex LH-20 column with methanol as eluant.

BLM experiments

A 0.25 mm thick Teflon film with an aperture of either 0.15 or 0.3 mm diameter was clamped between two halves of a Teflon trough. The aperture was precoated with hexadecane/hexane (6/4 v/v) mixture. Azolectin was purified by the method reported by Kagawa and Racker[4]. The azolectin membrane was formed by the method reported by Montal and Mueller[5]. The electrolyte solution was 1 M, unbuffered KCl, CsCl, or LiCl. Before measurements, an electric potential of AC 200 mV (peak-to-peak, 1 kHz) was applied for 30 min across the

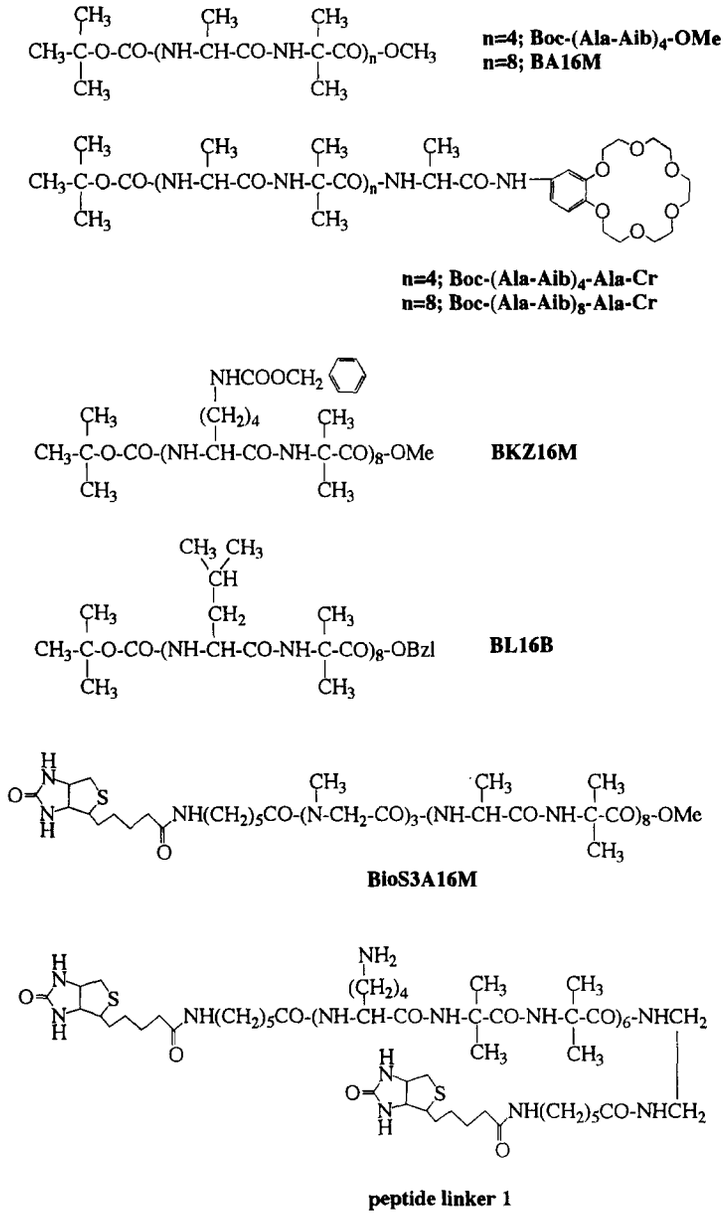

Figure 1 Molecular structure of synthetic α-helical oligopeptides

membrane, which made a virtually solvent-free planar bilayer membrane[6]. The oligopeptides were added to both aqueous phases.

π–A isotherm

BKZ16M and BA 16M were dissolved in chloroform/methanol (9/1 v/v) mixture and BA 16M was dissolved in chloroform at the concentrations of $(1.5–3) \times 10^{-4}$ M. The *π–A* isotherm was recorded at a constant rate of reducing area with a Langmuir trough having the surface area of 900 cm^2. The oligopeptide solution was spread on the aqueous phase by using a microsyringe, and equilibrated for 15 min before compression. Double-distilled water was used for the subphase.

2-D crystallization of SAv

After BioS3A 16M was spread on 1.5 M NaCl solution to a surface molecular area of 1800 $Å^2$ per molecule, an aqueous solution containing 1 mg of SAv or inactive SAv was injected into the subphase (500 ml) and the mixture was incubated for 1 h at 30°C, followed by cooling down to 20°C. A compression was begun at the rate of 0.17 $cm^2 s^{-1}$. The hysteresis experiments were carried out in the following order: (1) compression to 8 $mN m^{-1}$; (2) immediate relaxation of pressure to attain the largest surface area; (3) incubation for 20 min at 20°C; (4) compression at the rate of 0.17 $cm^2 s^{-1}$ to the smallest surface area.

Bilayer system of SAv

The protein bilayer was formed at the air/water interface in a small trough (10 cm^2) by the following procedure. (1) The biotinyl lipid was spread on the aqueous subphase (0.5 M NaCl) at a concentration of 500 $Å^2$ per molecule. (2) An SAv solution was injected into the subphase and temperature was maintained at 30°C for 30 min, followed by cooling to 20°C. The subphase was replaced with a new aqueous solution. With procedures (1) and (2), the first SAv layer was formed over the whole area of interface. (3) A 4.2×10^{-8} M aqueous solution of the peptide linker 1 was added to the subphase, and was incubated at 20°C for 120 min. The subphase was

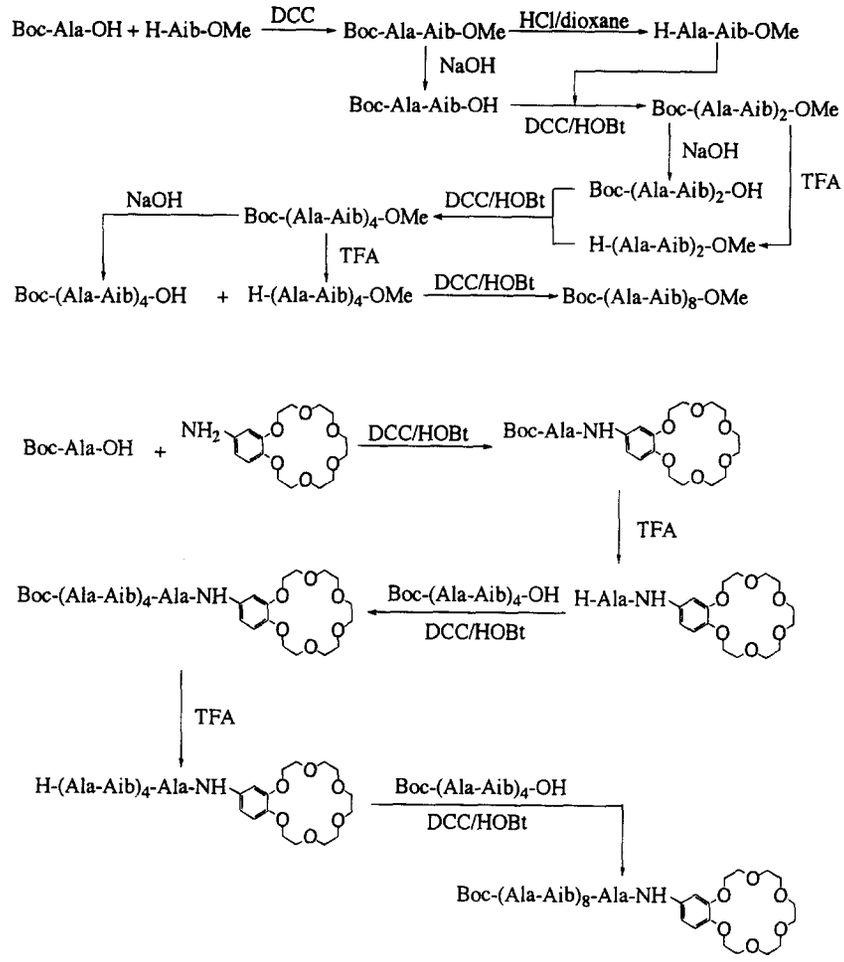

Figure 2 Synthetic scheme of Boc-(Ala-Aib)$_n$-OMe (n = 4 and 8) and Boc-(Ala-Aib)$_n$-Ala-Cr (n = 4 and 8)

replaced with brine. (4) The aqueous solution of FITC-SAv was injected into the subphase. The domain formation of the labelled SAv was observed using a fluorescence microscope.

Surface plasmon resonance (SPR)

The thickness of the adsorbed layer can be estimated by SPR[7]. The experimental procedure is as follows: (1) adsorption of biotin thiol and 11-mercaptoundecanol on a gold substrate evaporated onto a slide glass (incubation of the glass with 0.5 mM thiol mixture in 1:4 molar ratio for 16 h); (2) substitution of the aqueous phase with 1 mM of SAv solution and incubation for 30 min; (3) substitution with 9.1×10^{-5} M aqueous solution of the peptide linker 1 and incubation for 15 min; (4) substitution with 1 mM aqueous SAv solution and incubation for 14 h. Each operation was followed by careful rinsing with water.

Aqueous dispersion of the amphiphilic peptide

A chloroform/methanol (9/1 v/v) solution of H-(Ala-Aib)$_8$-OBzl was evaporated, and a thin peptide film was dispersed by sonication with a probe-type sonicator. The dispersion was eluted twice through a polycarbonate film with pores of 0.4-mm average diameter to obtain a transparent dispersion.

RESULTS AND DISCUSSION

Ion-channel formation of hydrophobic peptides

We investigated the mechanism of a voltage-dependent ion-channel formation in BLM, and reached a conclusion that hydrophobic α-helical oligopeptides are appropriate as channel-forming materials[8]. On the basis of this molecular design, Boc-(Ala-Aib)$_n$-OMe (n = 4 and 8) was synthesized in which Aib represents an α-aminoisobutyric acid residue. Also, for cation-selective ion-channel formation, an alanine 3-aminobenzo-18-crown-6 amide was connected to the C terminal of the above hydrophobic alternating copolypeptide to obtain Boc-(Ala-Aib)$_n$-Ala-Cr (n = 4 and 8), Cr representing a crown-ether unit.

Current/voltage (I/V) response across BLM containing Boc-(Ala-Aib)$_n$-OMe (n = 4 and 8) is shown in *Figure 3*. The I/V curve at higher voltages can be described by an exponential function of voltage, where current increases exponentially at higher voltages than the critical voltage, V_c, where the curve stands nearly vertical. V_c was about 150 mV for the octapeptide, but decreased to about 50 mV for the hexadecapeptide.

When the BLM was kept under slightly higher voltage than V_c in the presence of oligopeptides, a stepwise change of conductance was observed. The stepwise current diagram represents opening and closing of channel, and is characteristic of channel formation[6]. The channel conductance and opening frequency of the octapeptide was much lower than those of the hexadecapeptide. The higher ability of the hexadecapeptide in channel formation can be explained in terms of the tendency for aggregation in BLM with the helix axis orienting perpendicularly to the bilayer membrane.

Current/voltage response across BLM in the presence of Boc-(Ala-Aib)$_4$-Ala-Cr was investigated and compared with Boc-(Ala-Aib)$_4$-OMe in *Figure 4*. Boc-(Ala-Aib)$_4$-Ala-Cr formed an ion channel at lower applied voltage than Boc-(Ala-Aib)$_4$-OMe. When a voltage higher than V_c was applied to BLM containing Boc-(Ala-Aib)$_4$-Ala-Cr, stepwise conductance changes characteristic of a single-channel formation were observed. However, when the added salt was changed from KCl to LiCl, the single channel was not formed. These results support that the crown-ether group of Boc-(Ala-Aib)$_4$-Ala-Cr facilitates the formation of a voltage-dependent ion channel and acts as an ion-binding site of the ion channel. It is notable that current fluctuations due to the formation of a single ion-channel were observed in the presence of K^+ at an applied voltage as low as 20 mV in the case of BLM containing Boc-(Ala-Aib)$_8$-Ala-Cr.

BLM containing Boc-(Ala-Aib)$_4$-Ala-Cr showed current fluctuations owing to the formation of a single ion-channel in the presence of Cs^+ at 140 mV, but did not in the presence of K^+ under the same conditions. Because the oligopeptide tends to aggregate upon formation of sandwich-type complexes with Cs^+, a bundle structure of helices is formed in lipid membrane, acting as ion channel. Conversely, Boc-(Ala-Aib)$_8$-Ala-Cr showed a higher potential for ion-channel formation on complexation with K^+ than Cs^+. Since the aggregation of the hexadecapeptide is not influenced by the nature of cation, it is concluded that the higher the binding constant with cation ($K^+ > Cs^+$), the higher the channel conductance.

The channel-forming potency of hydrophobic helical oligopeptides was increased by connection of crown-ether unit to the C-terminal region of the oligopeptide. The crown-ether unit plays important roles in formation and activity of ion channel: (1) an easy access of cations to the channel by ion binding; (2) increasing primary amphiphilicity of the peptide by cation binding at the C-terminal region, resulting in transmembrane orientation of the oligopeptide; and (3) promotion of oligopeptide aggregation for ion channel by forming sandwich-type complex.

Monolayers of hydrophobic helical peptides

Hydrophobic helical oligopeptides associate together in the protein, forming a 'helix bundle', which is one of the major structural motifs of naturally occurring globular proteins. It is therefore important to study interactions between helices in relation to the formation of a structure or the stability of the protein[9,10]. The monolayer technique enables investigation of intermolecular interactions because it detects intermolecular forces acting in a two-dimensional array of molecules. When amphiphilic molecules are spread at the air/water interface, they show various states such as gas-analogous fluid-expanded, fluid-condensed, and solid-condensed phases, depending on the surface pressure[11]. These phases can be analysed on the basis of surface pressure–area (π–A) isotherm which reflects electrostatic interactions or molecular packing between amphiphilic molecules in each phase. In the present investigation, the monolayers of three kinds of hydrophobic helical oligopeptides, i.e., Boc-(Ala-Aib)$_8$-OMe, Boc-(Lys(Z)-Aib)$_8$-OMe and Boc-(Leu-Aib)$_8$-OBzl, were prepared, and the relationship between the structure of hydrophobic helical oligopeptides, especially the structure of side chains, and the surface properties of monolayers was investigated.

The π–A isotherms of the peptides spread at the air/water interface are shown in *Figure 5*. The π–A isotherm of Boc-(Ala-Aib)$_8$-OMe (BA 16M) showed an inflection and weak irregular bumping at a surface area of *ca* 240

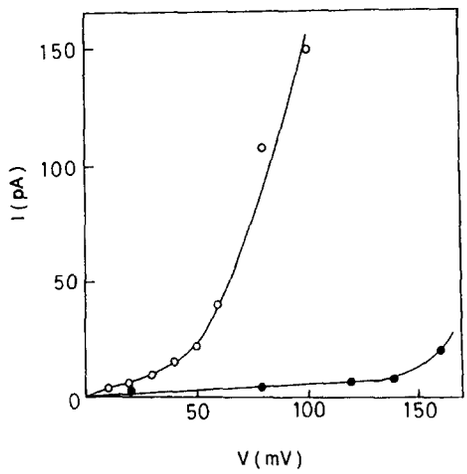

Figure 3 Current/voltage response of Boc-(Ala-Aib)$_n$-OMe. (●) $n = 4$ and (○) $n = 8$ in a planar lipid bilayer membrane. The peptide concentration is 3×10^{-6} g ml^{-1}, [KCl] = 1 M. The diameter of a hole in Teflon film is 0.3 mm

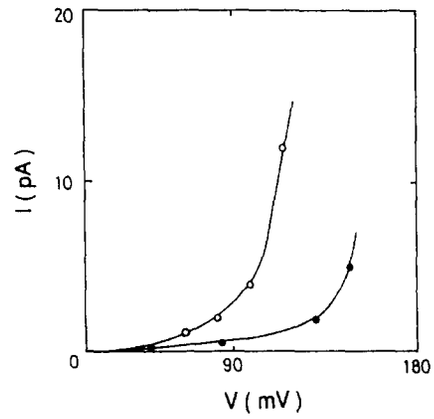

Figure 4 Current/voltage response of (●) Boc-(Ala-Aib)$_4$-OMe and (○) Boc-(Ala-Aib)$_4$-Ala-Cr in a planar lipid bilayer membrane. The peptide concentration is 3×10^{-6} g ml^{-1}, [KCl] = 1 M. The diameter of a hole in Teflon film is 0.15 mm

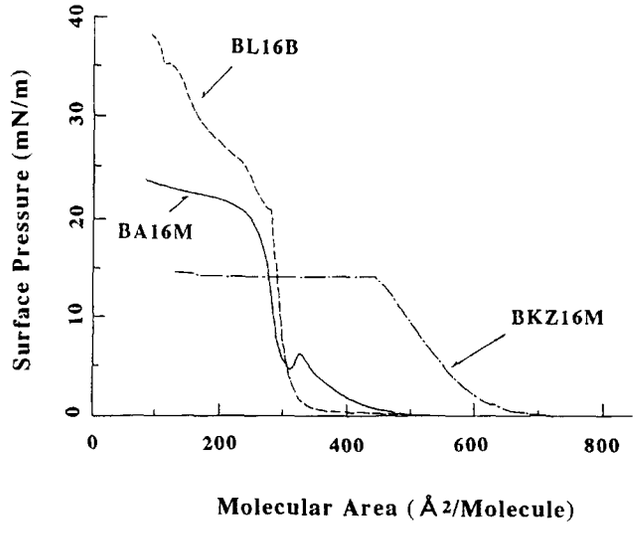

Figure 5 π–A isotherms of BKZ16M (–·–), BA16M (—) and BL16B (- - -) on the surface of double-distilled water at 20°C

and 230 Å^2 per molecule, respectively, indicating that the helix axis of the hexadecapeptide is oriented parallel to the interface. A small peak was observed at *ca* 300 Å^2 per molecule, which was ascribed to the phase transition from liquid to solid state. The monolayer of Boc-(Ala-Aib)$_8$-OMe, which carries short and wedge-like side chains, reached a solid state (2-D crystal) from a liquid state on compressing the oligopeptide monolayer.

The monolayer of Boc-(Lys(Z)-Aib)$_8$-OMe (BKZ16M), which carries long and flexible side-chains, reached a liquid state on compressing the oligopeptide monolayer.

The monolayer of Boc-(Leu-Aib)$_8$-OBzl (BL16B) easily reached a solid state (2-D crystal) in which the helices are firmly held together by interdigitation of side chains.

These observations are summarized in *Figure 6*.

Two-dimensional crystallization of proteins

The ordered structure of the monolayer should have a template effect on the second layer, inducing an ordered structure of the latter. For instance, it has been reported that the protein streptavidin (SAv) forms 2-D crystal underneath the monolayer of biotinyl lipids spread at an air/water interface[12]. The biotin and SAv system is a well-known biological receptor/ligand pair, with a strong, specific interaction[13]. In the present investigation, the monolayers of hydrophobic helical oligopeptides carrying a biotinyl group at the N terminal were prepared at the air/water interface. Using the monolayers as templates, the 2-D crystallization of SAv was attempted.

The π–A isotherms of BioS3A16M with active or inactive SAv were measured, and the results are shown in *Figure 7*. The addition of active SAv increased the molecular area more strikingly than the addition of inactive SAv, indicating the specific binding of SAv to BioS3A16M at the interface. No hysteresis was detected in the complex formation.

When a fluorescent protein, FITC-SAv, was added, domains of the FITC-SAv were formed which displayed distinct fluorescent anisotropy on irradiation of polarized excitation light. The anisotropy is caused by the microscopic orientation of the fluorescence probe attached to the SAv molecule and strongly suggests that the SAv molecules are highly ordered in the domain. Therefore, the domain should be constituted by the 2-D crystal of SAv. This situation is schematically shown in *Figure 8*.

Protein bilayer system

We focused our attention on the fact that in the 2-D crystal of SAv, two of the four binding sites for biotin in the SAv molecule are occupied by biotin groups of the lipids and the remaining two are free for further binding and exposed to the aqueous phase. In the present investigation, a bilayer system of SAv was constructed by using a linker molecule to connect the first and the second SAv layers, which possesses a suitable chain length with an appropriate rigidity to avoid coordination of two terminal biotinyl groups with neighbouring binding sites of SAv in the first template layer.

When the linker peptide Bio-(Lys-Aib-Aib)$_6$-NHCH$_2$CH$_2$-NH-Bio (peptide linker 1) was used in procedure (3) described in the Preparation section, bright domains were observed by fluorescence microscopy, after incubation of an SAv bilayer system at 20°C for 15 h. The bright domains were not observed in the control experiment which was carried out without procedure (3). This verifies that the bright domains are due to the second SAv layer formed underneath the first SAv layer with intervention of the peptide linker 1 (*Figure 9*). It is notable that the bright domains of the second SAv layer showed fluorescence anisotropy, indicating the formation of a 2-D crystal of SAv.

The protein bilayer was examined by SPR and the thickness of the adsorbed layer after each operation is summarized in *Table 1*. The SPR measurement revealed that the thickness of the first SAv layer was 34 Å. However, the average thickness of the second SAv layer was 10 Å. This low value indicates a partial adsorption of SAv on the first SAv layer. The partial adsorption can be explained by coordination of two terminal biotins of a linker peptide with binding sites of SAv.

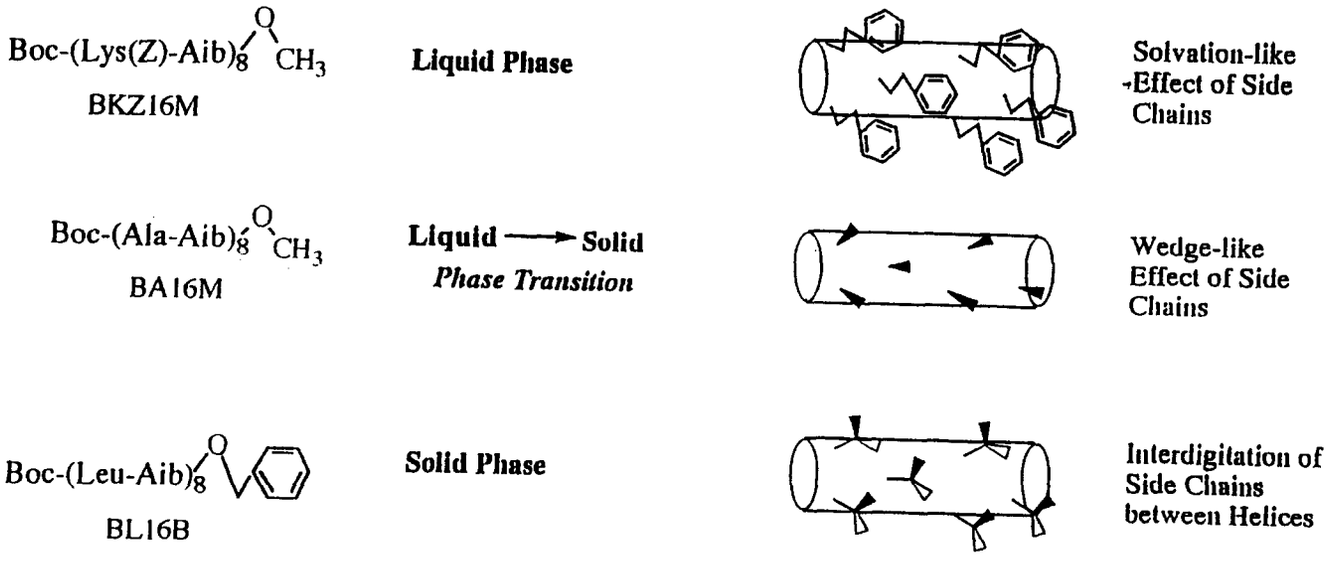

Figure 6 Schematic presentation of side-chains of oligopeptides influencing monolayer properties. Isobutyl groups of BL16B make the oligopeptide packing tightly by interdigitation. Methyl groups of BA 16M work wedge-like upon compression to induce the phase transition in the monolayer. The flexible and bulky side-chains of BKZ16M behave like solvent to keep the monolayer at a liquid state

The length of the helix part of peptide 1 is 27 Å and the radius of the helix rod should be larger than 5 Å, assuming an α-helical structure[14]. The distance between the biotin-binding sites of the SAv has been reported to be 20 Å[15], and that of neighbouring SAv molecules in the 2-D crystal has been reported to be *ca* 45 Å from 2-D projection map of the crystal[16]. Therefore, both biotin moieties of the same peptide linker will not occupy simultaneously the two binding sites of the same SAv molecules or of the neighbouring SAv molecules in so far as the perfect helical conformation of the linker peptide is maintained upon binding to SAv. Simultaneous occupation of neighbouring binding sites by the linker peptide is only possible if the helical structure is disturbed.

Vesicular assembly of helical oligopeptide

In phospholipid bilayer membranes, biologically active oligopeptides with molecular weights of a few thousands have an α-helical conformation and associate to form an ion channel, through which ions are translocated across the membrane. It is thus considered that helical oligopeptides may form regular structures, such as vesicles, tubules and bilayers, by self-assembly.

Towards the goal of constructing such a regular structure, the oligopeptide should take a stable conformation. It was found in the earlier part of this investigation that Boc-(Ala-Aib)$_8$-OMe formed a solid monolayer when deposited on an air/water interface. It is therefore considered that Boc-(Ala-Aib)$_8$-OMe has a very stable structure and can be packed in a regular array.

Amphiphilicity is another requirement for oligopeptides to form self-assembly in aqueous medium. It has been pointed out that the balance between hydrophilic groups and hydrophobic groups in lipid-like molecules is the key factor for determining the shape of the self-

Table 1 The thickness (Å) of the SAv bilayer determined by SPR (error of each value is ±1 Å)

+SAv	+Peptide linker 1	+SAv
34	36	46

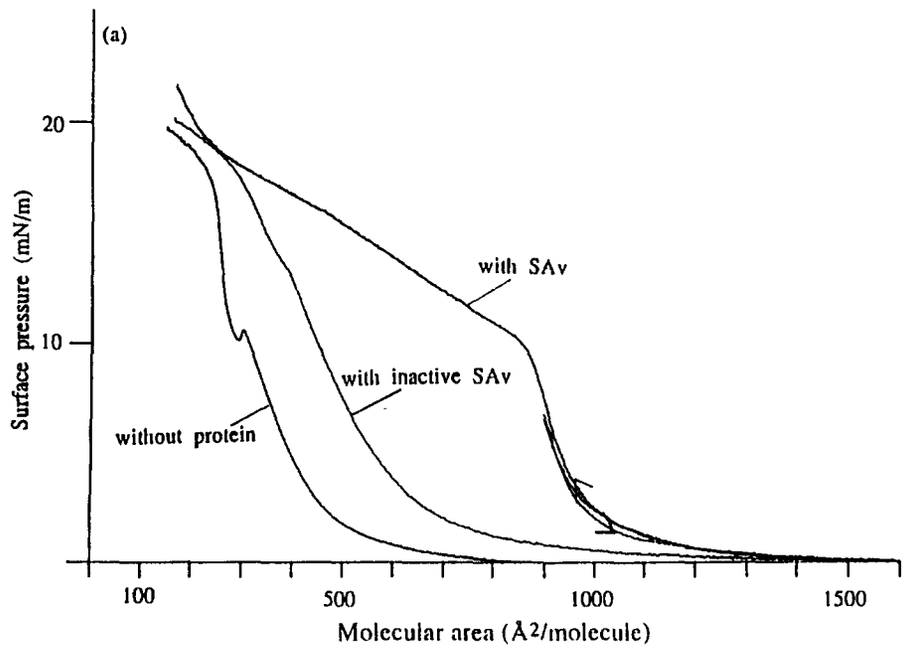

Figure 7 π–A isotherms of BioS3A16M interacting with SAv or inactive SAv or in the absence of SAv. The curves of compression, expansion and recompression in this order are shown together with arrows in the presence of active SAv

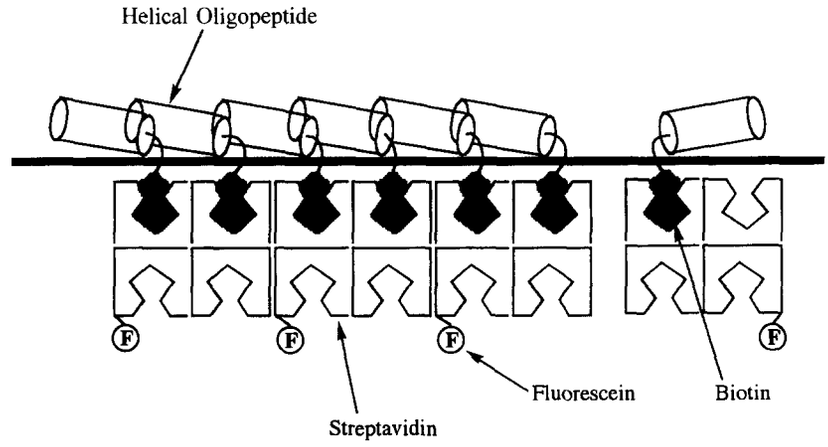

Figure 8 Schematic representation of the complex of BioS3A16M and FITC-SAv at the air/water interface

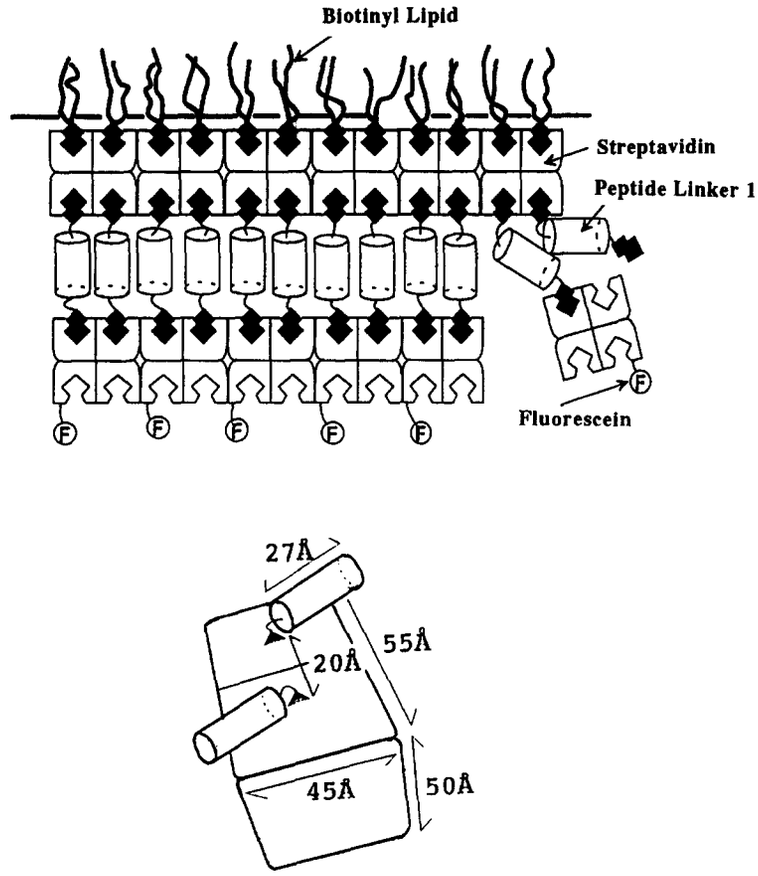

Figure 9 Schematic representation of the SAv bilayer system

assembly system[17]. Therefore, the Boc group of Boc-(Ala-Aib)$_8$-OMe was removed and the C-terminal group was changed from methyl ester to benzyl ester to strengthen the primary amphiphilicity of the oligopeptide; we then explored the possible formation of vesicular aggregate by self-assembly of the amphiphilic α-helical oligopeptide molecules.

The hexadecapeptide in ethanol shows a double-minimum pattern of circular dichroism (c.d.) spectrum which is typical of the α-helix conformation. Conversely, in aqueous dispersion, the negative Cotton effect at longer wavelength shifted to 225 nm and became stronger than that at shorter wavelength. This change is characteristic of association by hydrophobic α-helices[18]. It is therefore concluded that the oligopeptide takes an α-helix conformation and that the helices associate tightly in aqueous dispersions.

Transmission electron microscope observation of the aqueous dispersion of the oligopeptide revealed that particles with diameters of *ca* 50 nm were formed.

The size distribution of the particles was determined by dynamic light scattering method. The distribution is very narrow and the most frequent value of diameter is 75.7 nm.

Taking into account all experimental results available to date, it is considered that H-(Ala-Aib)$_8$-OBzl forms a vesicular assembly in aqueous dispersion. We propose the name peptosome (after liposome).

REFERENCES

1 Guy, H. R. and Conti, F. *Trends Neurosci.* 1990, **13,** 201
2 Weber, P. C. and Salemme, F. R. *Nature* 1980, **287,** 82
3 Shively, J. E., Paxton, R. J. and Lee, T. D. *Trends Biochem. Sci.* 1989, **14,** 246
4 Kagawa, Y. and Racker, E. *J. Biol. Chem.* 1971, **246,** 5477
5 Montal, M. and Muller, P. *Proc. Natl Acad. Sci. USA* 1972, **69,** 3561
6 Menestrina, G., Voges, K.-P., Jung, G. and Boheim, G. *J. Membr. Biol.* 1986, **93,** 111
7 Torrettaz, S., Stora, T., Duschl, C. and Vogel, H. *Langmuir* 1993, **9,** 1361
8 Otoda, K., Kimura, S. and Imanishi, Y. *Biochim. Biophys. Acta* 1992, **1112,** 1
9 Wada, A. *Adv. Biophys.* 1976, **9,** 1
10 Hol, W. G. J. *Prog. Biophys. Molec. Biol.* 1985, **45,** 149
11 Birdi, K. S. 'Lipid and Biopolymer Monolayers at Liquid Interfaces', Plenum, New York, 1989, Ch. 3
12 Blankenburg, R., Meller, P., Ringsdorf, H. and Salesse, C. *Biochemistry* 1989, **28,** 8214
13 Buackland, R. M. *Nature* 1986, **320,** 557
14 Lavigne, P., Tancrede, P., Lamarche, F. and Max, J.-J. *Langmuir* 1992, **8,** 1988
15 Hendrickson, W. A., Pahler, A., Smith, J. L., Satow, Y., Merritt, E. A. and Phizackerley, R. P. *Proc. Natl Acad. Sci. USA* 1989, **86,** 2190
16 Darst, S. A., Ahlers, M., Meller, P. H., Kubalek, E. W., Blankenburg, R., Ribi, H. O., Ringsdorf, H. and Kornberg, R. D. *Biophys. J.* 1991, **59,** 387
17 Cullis, P. R. and De Kruijff, *Biochim. Biophys. Acta* 1979, **559,** 399
18 Lau, S. Y. M., Tanega, A. K. and Hodges R. S. *J. Biol. Chem.* 1984, **259,** 13253