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Chemistry

Spontaneous assembly of a self-complementary oligopeptide to form a stable macroscopic membrane

(β-sheet/insoluble filaments/ionic bonds/origin of life/zuotin)

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ABSTRACT **A 16-residue peptide [(Ala-Glu-Ala-Glu-Ala-Lys-Ala-Lys)$_2$] has a characteristic β-sheet circular dichroism spectrum in water. Upon the addition of salt, the peptide spontaneously assembles to form a macroscopic membrane. The membrane does not dissolve in heat or in acidic or alkaline solutions, nor does it dissolve upon addition of guanidine hydrochloride, SDS/urea, or a variety of proteolytic enzymes. Scanning EM reveals a network of interwoven filaments ≈10–20 nm in diameter. An important component of the stability is probably due to formation of complementary ionic bonds between glutamic and lysine side chains. This phenomenon may be a model for studying the insoluble peptides found in certain neurological disorders. It may also have implications for biomaterials and origin-of-life research.**

Peptides of alternating hydrophilic and hydrophobic amino acid residues have a tendency to adopt a β-sheet structure. The complete sequence of (Ala-Glu-Ala-Glu-Ala-Lys-Ala-Lys)$_2$ (EAK16) was originally found in a region of alternating hydrophobic and hydrophilic residues in zuotin, a yeast protein that was initially identified for its ability to bind preferentially to left-handed Z-DNA (1). Previous studies with alternating amphiphilic-peptide polymers—e.g., poly(Val-Lys), poly(Glu-Ala), poly(Tyr-Glu), poly(Lys-Phe), poly(Lys-Leu)—and oligopeptides [(Val-Glu-Val-Orn)$_{1-3}$]-Val (2–7) have shown that these polymers can adopt β-sheet structures and can aggregate, depending upon pH, salt, and time. However, self-complementary EAK16 is distinctive in that it forms an insoluble macroscopic membrane.

MATERIALS AND METHODS

Peptides. The Glu-Ala-Lys peptides were synthesized by a peptide synthesizer (Applied Biosystems), purified by reverse-phase HPLC, and eluted by a linear gradient of 5–80% acetonitrile/0.1% trifluoacetic acid. The peptide stock solutions were dissolved in water (1–5 mg/ml) or in 23% acetonitrile (10 mg/ml). The concentrations of the peptides were determined by dissolving dried peptide in water (wt/vol) and centrifuging the solution. A portion of the solution was then analyzed by hydrolysis with internal controls. The sequence of the peptides was confirmed by microsequencing. The composition of the peptides was confirmed by hydrolytic analysis. Ala-Glu-Ala-Lys-Ala-Glu-Ala-Glu-Ala-Lys-Ala-Lys (EAK12) and EAK16 are acetylated and aminated at the N- and C-terminal ends, respectively. Blocking of both N and C termini of EAK16 appears nonessential for membrane formation.

CD Measurement. CD spectra were gathered on an Aviv model 60DS spectropolarimeter with 60HDS software for data processing. Because EAK16 contains both positively and negatively charged residues, the peptide itself can serve as a buffer. CD samples were prepared by diluting stock peptide solution (1–5 mg/ml) in water.

Membrane Preparations. The membranes were prepared as follows: 5–10 μl of the stock solution of EAK16 peptide (1–5 mg/ml) was added to 0.5–1.0 ml of phosphate-buffered saline (150 mM NaCl/10 mM sodium phosphate, pH 7.4) with 0.00001% Congo red in a 24-well-microtiter plate. The membrane was photographed under an inverted optical microscope with a rule underneath it as a size reference. The samples for scanning EM were prepared by first incubating the membranes in 5% glutaraldehyde at 4°C for 30 min and then dehydrating them sequentially with 20, 50, 70, 90, and 100% ethanol and liquid CO_2. The specimen was examined by using scanning EM at ×400–×20,000 magnification.

RESULTS AND DISCUSSION

Properties of EAK16. CD studies of EAK16 indicate a typical spectrum of β-sheet formation with a minimum ellipticity at 218 nm and a maximum ellipticity at 195 nm (Fig. 1). Because of this form, the molecule has hydrophobic alanine side chains on one side and self-complementary pairs of positively charged lysine- and negatively charged glutamic acid-side chains on the other surface. EAK16 spontaneously associates to form a macroscopic membrane, whereas the 12-amino acid peptide Ala-Glu-Ala-Lys-Ala-Glu-Ala-Glu-Ala-Lys-Ala-Lys (EAK12) of similar composition can associate to a much smaller extent. This result suggests that alternating pairs of complementary ionic bonds may be important or that the structure has parallel β-sheets. Five other peptides with various compositions and lengths, including a single unit of the repeat Ala-Glu-Ala-Glu-Ala-Lys-Ala-Lys (EAK8), did not form a membrane under the same conditions (Table 1).

Macroscopic Membrane Formation. The spontaneous assembly of EAK16 was first observed serendipitously in Dulbecco modified Eagle's medium/calf serum when it was being tested for toxicity. EAK16 did not affect the growth rate of nerve growth factor-differentiated rat PC-12 cells and was apparently nontoxic (data not shown). However, a transparent membrane was seen when viewed under ×100 magnification phase-contrast microscopy; the membrane was also visible in phosphate-buffered saline (Fig. 2*A*). The membrane can be stained by Congo red (Fig. 2 *B* and *C*), a dye that preferentially stains β-pleated sheet structures and is commonly used to visualize abnormal protein deposition in tissues (10). However, other peptides listed in Table 1 did not form visible macroscopic membranes when tested.

Stability of the Membrane. Once the membrane is formed, it is stable and resistant to digestion with several proteases—including trypsin, α-chymotrypsin, papain, protease K, and pronase—at a concentration of 100 μg/ml, even though EAK16 contains potential protease-cleavage sites (Table 1).

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Abbreviation: EAK16, (Ala-Glu-Ala-Glu-Ala-Lys-Ala-Lys)$_2$.
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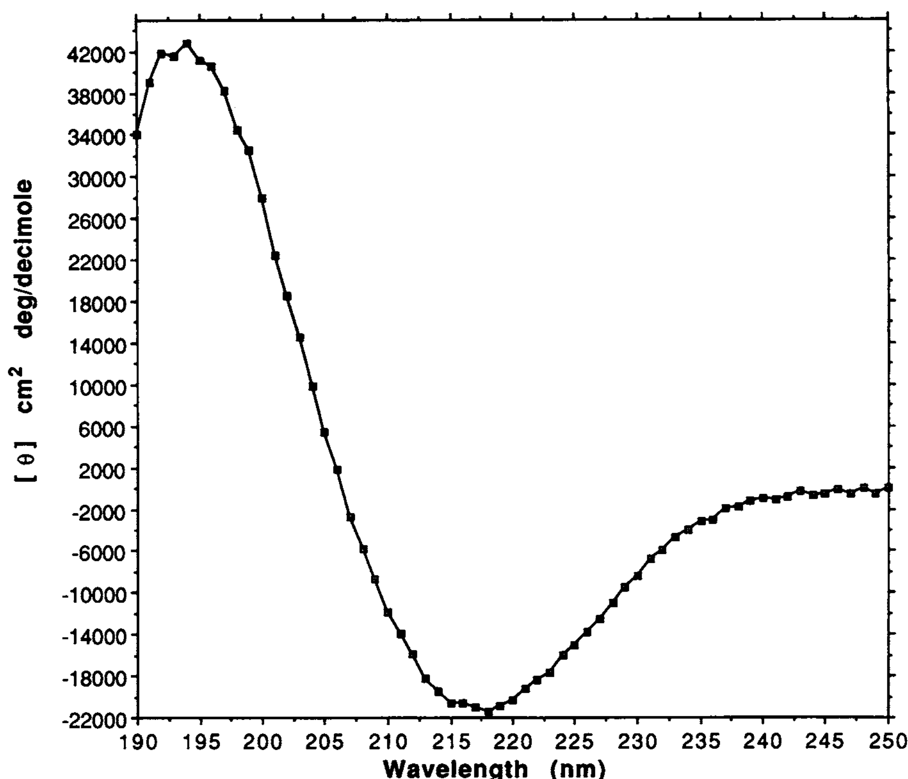

FIG. 1. CD spectrum of the EAK16 peptide. The EAK16 peptide was dissolved in water (10 μM) before taking the CD spectrum. A typical β-sheet CD spectrum with a 218-nm minimum and a 195-nm maximum is detected.

The membrane is stable in 1% SDS at 90°C for >4 hr. These observations are consistent with other studies that showed that the β-sheet CD spectrum was not significantly changed by heating the EAK16 solution to 90°C, by various pH (1.5, 3.0, 7.0, and 11), or by 0.1% SDS, 7 M guanidine hydrochloride, or 8 M urea (S.Z. and C.L., unpublished work). The membrane is mechanically stable and can be transferred from one solution to another by using a solid support but can be broken by cutting, tearing, or shearing.

Effect of Salts. Salt appears to play an important role in this spontaneous-assembly process. A variety of cations were tested. The order of effectiveness in inducing membrane formation appears to be $Li^+ > Na^+ > K^+ > Cs^+$. Cs^+ largely produces precipitates rather than a structural membrane. In aqueous solution, Li^+ has the largest hydrated radius (3.4 Å), whereas Na^+ (2.76 Å), K^+ (2.32 Å), and Cs^+ (2.28 Å) have smaller hydrated radii (11). The formation of the membrane seems to correlate with the order of the enthalpies of the monovalent metal ions (11). On the other hand, NH_4^+ and Tris·HCl seem not to induce EAK16 to form a membrane. Under our conditions, divalent metal ions primarily induced aggregates rather than membrane formation. At present, it is not known if the metal ions act as the catalyst or are themselves incorporated into the membrane, although the latter seems more likely.

There are numerous examples of monovalent metal ions that promote and stabilize other structures. One of these is the association of four guanosine nucleotides in nucleic acids called a G-quartet (12, 13). In this case, the order of the effectiveness is $K^+ > Na^+ > Cs^+ >>> Li^+$ for G-quartet formation (12). Brack and Orgel (2) reported that poly(Val-Lys) in water at pH 2.3 could be changed from a random coil to a stable β-sheet in high-molecular-weight aggregates in the presence of 100 mM NaCl. Furthermore, poly(Phe-Lys) and poly(Tyr-Lys), but not nonalternating peptides of similar compositions, can associate to form high-molecular-weight complexes in the presence of salt (4, 6). An additional example of salt-induced peptide aggregation is the β-amyloid

Table 1. Peptides used in this study

Peptide	Sequence*	DMEM†	PBS†	Water†	Structure‡	Ref.
EAK16	Ac-HN-AEAEAKAKAEAEAKAK-CONH$_2$	++++	++++	–	β	This study
EAK12	Ac-HN-AEAKAEAEAKAK-CONH$_2$	++	+	–	α, β	This study
EAK8	H$_2$N-AEAEAKAK-COOH	–	–	–	RC	This study
β-Amyloid-(1–28)	H$_2$N-DAEFRHDSGYEVHHQKLVFFAEDVGSNK-COOH	–	–	–	RC, α, β	8
β-Amyloid-(25–35)	H$_2$N-GSNKGAIIGLM-CONH$_2$	–	–	–	RC	This study
Substance P	H$_2$N-RPKPQQFGLM-CONH$_2$	–	–	–	ND	This study
Spantide	H$_2$N-(D)RPKPQQ(D)WL(D)L-CONH$_2$	–	–	–	ND	This study

EAK12 and EAK16 are acetylated and aminated at N- and C-terminal ends, respectively. Blocking of both N and C termini of EAK16 appears nonessential for membrane formation. A volume of 5–10 μl of dissolved peptide was applied to the Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), or water. Formation of the membrane-like structure was first observed under a phase-contrast microscope and then by naked eye. Sustance P, Spantide, and β-amyloid-(1–28) are commercially available from Bachem. The (D) in Spantide indicates D-amino acids incorporated into the peptide. Substance P, Spantide, and β-amyloid-(25–35) are aminated on the C-terminal ends.

*One-letter amino acid code is used.

†The + and – denote the presence or absence of the membranous structure, respectively.

‡α, α-Helix; β, β-sheet; RC, random coil; ND, structures not determined.

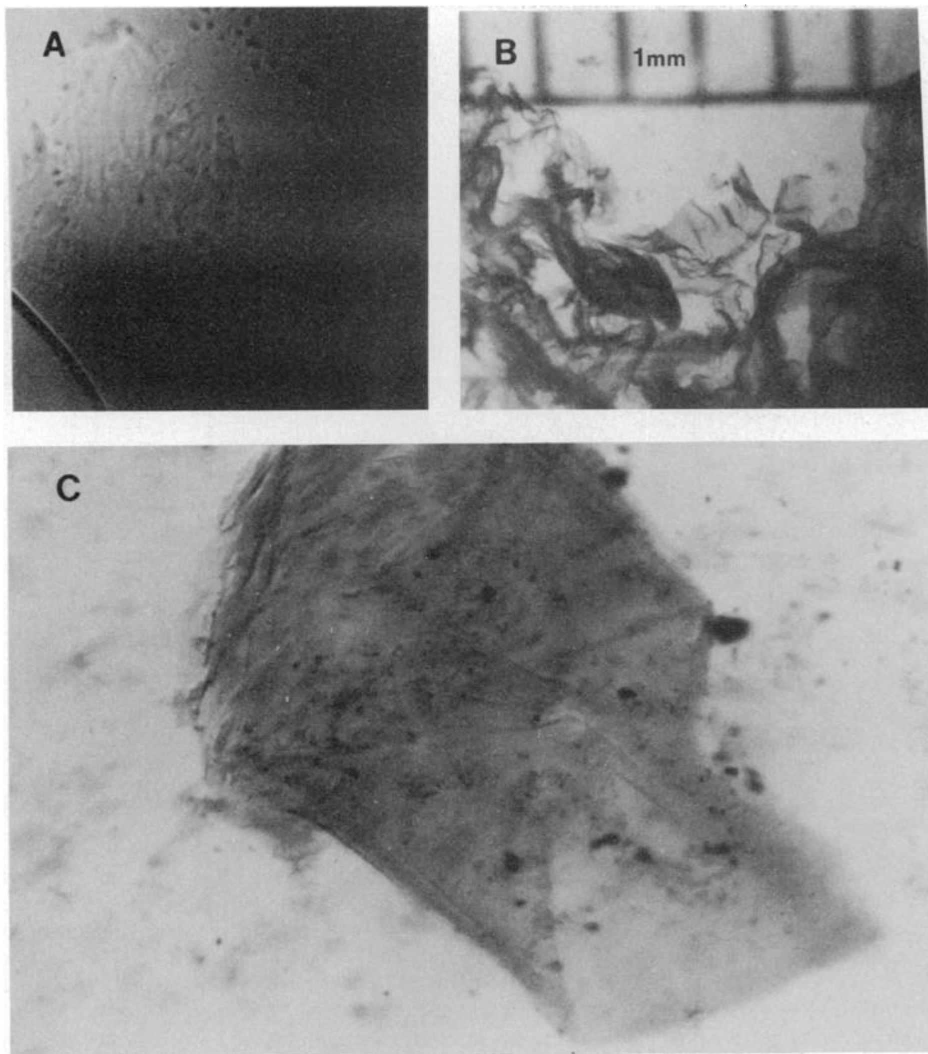

FIG. 2. Photographs of the membrane. (*A*) The structure was formed in phosphate-buffered saline and transferred to a glass slide. The colorless membranous structures are isobuoyant; therefore, the image is not completely in focus. (×75; Nomarski microscope.) (*B*) The structure stained bright red with Congo red (9) and can then be seen by the naked eye. (×15; each scale unit = 1 mm). (*C*) A portion of a well-defined membranous structure with layers is clearly visible; the dimensions of this particular membrane are ≈2 × 3 mm. (×20.)

protein found in the plaques of Alzheimer disease. The β-amyloid protein has 43-amino acid residues and is highly soluble in water (up to 30 mg/ml), but it is poorly soluble (0.5 mg/ml) in phosphate-buffered saline (14).

Scanning EM. The architecture of the membrane appears to resemble high-density felt. At low magnification (×20–×100), the structure looks like a flat membrane. However, structural details are revealed by scanning EM at high

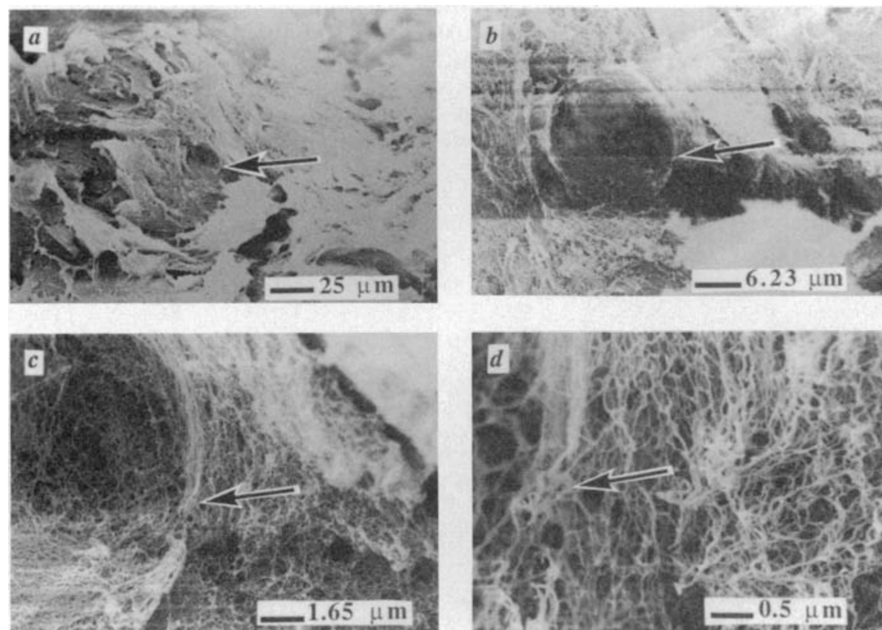

FIG. 3. Serial photography of scanning EM. The diameter of the filaments are ≈10–20 nm, and the distance between fibers are ≈50–80 nm. Arrows mark the same location. (*a*, ×300; *b*, ×1200; *c*, ×4500; *d*, ×15,000.)

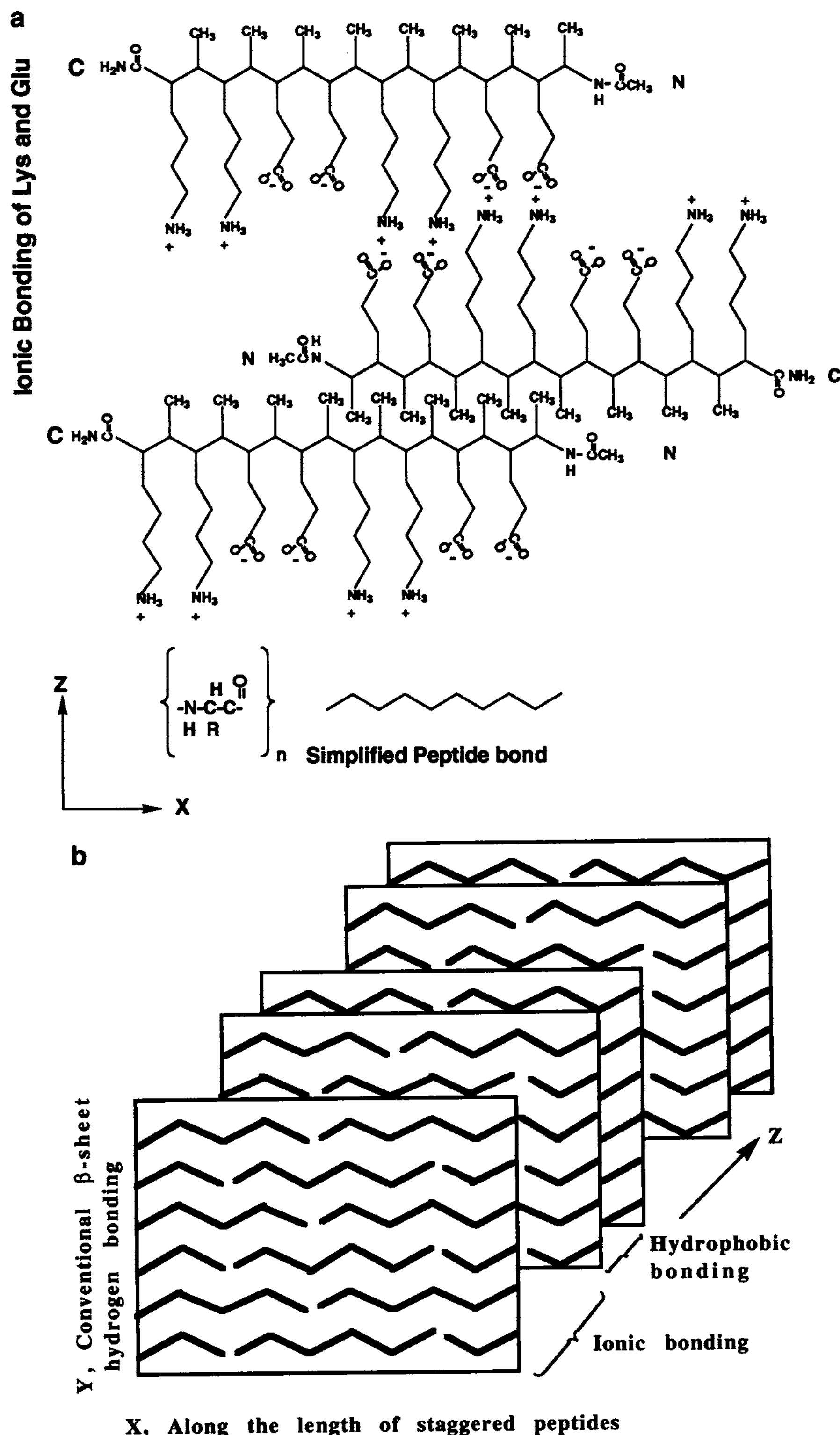

FIG. 4. Proposed model of the membrane. (*a*) View perpendicular to the β-sheet, which is the *y* axis. Three molecules of EAK16 peptide form three layers of an antiparallel β-sheet, held together on one side by hydrophobic bonding between alanine side chains facing each other and the charged lysines and glutamic acid side chains facing each other to form ionic bonds. The structure can also be drawn as a parallel β-sheet. In either case, the peptide would be staggered along *x*, as shown in the diagram. (*b*) Stacking of β-sheets. The staggered peptides are oriented along the *x* axis. The *z* axis has the complementary ionic bonds between lysine and glutamic acid, as well as the hydrophobic bonds between alanines referred to in *a*. The *y* axis contains the conventional β-sheet hydrogen bonds. Similar interactions are found in sawfly silk fibroin containing 70–80% alanine and glutamine but without the ionic pairing (21).

magnification (Fig. 3). The membrane appears formed from interwoven individual filaments. The self-complementary individual EAK16 oligopeptides probably interact strongly to form a stable structure promoted by the hydrated salt ions. Other examples of oligopeptides forming insoluble filaments are found in several pathological diseases—e.g., the neurofibrillary tangles found in Alzheimer disease plaques form salt-dependent aggregates from β-amyloid protein with an

extremely stable β-sheet structure that stains with Congo red (15). At high magnification, the aggregated Alzheimer filaments have a diameter of ≈10–15 nm (14–17), similar to the EAK16 filaments. The scrapie prion protein likewise stains with Congo red and forms aggregated filaments that are extremely stable and resistant to proteases (18, 19). Peptides that spontaneously assemble to form an insoluble filament have also been reported in liver cirrhosis, where intracellular inclusions are found due to a mutation that occurs at the A β-sheet region of the Z α_1-antitrypsin (20).

Proposed Structure of the Membrane. EAK16 contains two-unit repeats of alternating hydrophilic and hydrophobic residues, where every other residue in the peptide is alanine. At neutral pH, the four glutamic acids and four lysines are negatively and positively charged, respectively. Because of the β-sheet structure, all of the charged amino acids lie on the same side and, thus, have complementary patterns with pairs of positively and negatively charged ionic groups. The peptides may be staggered, which would contribute to stability in the direction along the molecule (Fig. 4 *a* and *b*, *x* axis). The alanines on one side of the β-sheet form hydrophobic bonds, as in silk fibroin (21), and glutamic acids and lysines on the other side form complementary ionic bonds (Fig. 4 *a* and *b*, *z* axis). However, the peptides could also be organized in register, without staggering. The results of x-ray diffraction studies will be published elsewhere.

Implications of the Self-Complementary Peptide Structure. Spontaneous formation of such a macroscopic membrane has some implications for biology and for biomaterial research. Because of the extreme stability of the EAK16 membrane in serum, where it was originally discovered, its high resistance to proteolytic digestion, simple composition, apparent lack of cytotoxicity, and easy synthesis in large quantities, such materials might be useful for biomaterial applications. These could include slow-diffusion drug-delivery systems, artificial skin, and separation matrices.

The similarity of the EAK16 filaments to the insoluble proteins found in various pathological diseases (14–20) suggests that it might be a useful model system for exploring those aspects of structures and sequences that produce such unusual properties as extreme insolubility, resistance to proteolytic digestion, and spontaneous assembly. Drugs that inhibit self-assembly of the peptides may be useful for the treatment of these diseases.

It is of great interest that oligopeptides containing simple self-complementary repeats can spontaneously assemble to form relatively ordered macroscopic structures, independent of an external assembly mechanism or instruction code. Brack and Orgel (2) suggested that alternating peptides with a tendency to form β-sheets may be able to form "membrane-like aggregates." Our observation of a macroscopic membrane spontaneously assembled from EAK16 is consistent with the hypothesis that such simple molecules may form larger and more complex structures, which may have been important in the origin of life. Miller and Urey (22) have shown that amino acids are readily synthesized from CH_4, NH_3, H_2, and H_2O molecules and that amino acids can be condensed to form oligopeptides in prebiotic conditions (for review, see ref. 23). Further, Yanagawa *et al.* (9) reported that a 12-residue peptide of glycines can also form 30- to 50-μm microscopic aggregates of different shapes and textures, dependent on salt and other conditions and similar to our observations. We speculate that oligopeptides with self-complementary sequences, as in the example of EAK16, might serve as templates to condense tetra- or octapeptides of similar sequence to form longer oligopeptides. These oligopeptides could then spontaneously assemble to form membranes, yielding compartmentalization and eventually establish an enclosed environment for a primitive metabolism (24).

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