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The effect of membranes on the *in vitro* fibrillation of an amyloidogenic light chain variable domain SMA

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Summary

Light chain (or AL) amyloidosis is the most common form of systemic amyloidosis, characterized by the pathological deposition of insoluble fibrils of immunoglobulin light chain fragments in various organs and tissues, especially in the kidney and heart. Both the triggering factors and the mechanisms involved in the abnormal formation of the insoluble fibrillar aggregates from the soluble proteins are poorly understood. For example, although the fibrillar deposits are typically found associated with the extracellular matrix and basement membranes, it is not clear whether fibrils are initially formed intra- or extra-cellularly, nor it is understood what determines where the deposits will occur; i.e., site tropism. In the present investigation, we studied the interaction of a recombinant amyloidogenic light chain variable domain, SMA, with lipid vesicles. The nature of the interaction was dependent on the lipid composition and the SMA to lipid ratio. The most pronounced effect was found from vesicles composed of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine/1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate (DPPC/POPA), which dramatically accelerated fibril growth. Interestingly, spectral probes, such as intrinsic fluorescence and far-UV CD spectroscopy did not show significant conformational changes in the presence of the vesicles. The presence of cholesterol or divalent cations, such as Ca²⁺ and Mg²⁺, lead to decreased membrane-induced SMA fibrillation. Thus, membranes may have significant effects on light chain fibrillation and may contribute to the site selectivity observed in AL amyloidosis.

Keywords

lipid vesicles; amyloid; AL amyloidosis; light chain; fibril; membrane; aggregation; misfolding; cation; cholesterol

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Prof. Anthony L. Fink has passed away on March 2, 2008

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INTRODUCTION

Light chain amyloidosis (also known as AL or primary amyloidosis) is a monoclonal plasma cell dyscrasia associated with the pathological accumulation and deposition of monoclonal light chains in the form of insoluble fibrils. Chemical analysis of amyloid fibrils isolated from patient postmortem tissues revealed that the major constituent is the light chain variable domain (V_L), sometimes coupled with a short fragment of the constant domain. The fibrillar deposits are localized extracellularly in almost every organ or tissue within the body, but the predominant sites include the kidneys, heart, lungs, spleen and liver^{1;2}. Light-chain deposition disease (LCDD) is an analogous protein deposition disorder except that the deposits are amorphous rather than fibrillar^{3;4}. Occasionally, patients may have both AL amyloidosis and LCDD, with both types of specific deposits being comprised of same V_L , and sometimes in the same tissue⁵. In both disorders, the deposits are most commonly found associated with the basement membranes of the extracellular matrix (ECM)^{3;6;7}.

Although the role of amyloid plaques in the etiology of light chain amyloidosis remains controversial, the physiological damage is thought to originate from the bulk accumulation of fibrils that displace normal tissue, and eventually lead to the cell death. Currently, no effective means of reversing the disease is known, and death usually ensues in about one year.

SMA belongs to the kappa IV family of IgG light chains, and was originally extracted from lymph node-derived amyloid fibrils of an AL amyloidosis patient. SMA corresponds to the variable domain of the kappa IV family of IgG light chains. *In vitro* experiments with recombinant SMA (114 residues, 12.7 kDa) have demonstrated that the protein aggregated via partially folded intermediates to form ordered aggregates such as protofibrils and fibrils as well as disordered amorphous aggregates^{8;9}. Earlier studies suggested that insoluble fibrils and amorphous protein deposits play a role in the molecular pathogenesis of amyloid disease^{10;11}. However, it has become increasingly evident that certain nonfibrillar forms, such as soluble or insoluble oligomers, possess toxic properties^{12–15}.

An ongoing controversy with respect to the light chain amyloidosis has been whether fibrillation occurs only extracellularly, or it can be initiated intracellularly, e.g. in lysosomes. Internalization of amyloidogenic light chains has been reported in primary cardiac fibroblasts¹⁶, and mesangial cells¹⁷. The *in vivo* pathological deposition of insoluble light chain fibrils is associated with various tissues, walls of blood vessels, and basement membranes¹⁸. Therefore, it is reasonable to expect that the interaction between amyloidogenic light chains, such as SMA, and membranes may be involved in the regulation of the aggregation process. There is already evidence that surfaces and membranes may be crucial for fibril formation of the A β peptide^{19;20} and α -synuclein^{12;21}.

There has been only one reported investigation of the interactions of V_L with surfaces²². These preliminary investigations suggested that the outcome of the interactions of SMA with surfaces was very dependent on the nature of the surface. For example, the *in vitro* assembly of SMA on fresh mica has been investigated by using AFM²². Compared to the solution conditions, where amorphous aggregates formed predominantly at pH 5.0, fibrils grew on mica surfaces with much lower concentrations of the protein and at faster rates at the same pH. The surface-catalyzed fibrillation might be explained on the basis that fibrillation is nucleated on surfaces, and that a key aspect is the initial absorption of the protein to the surface.

In this report we investigate the hypothesis that membrane surfaces play an important role in controlling the fibrillation of amyloidogenic light chains. In support of this hypothesis we show that vesicles with certain types of lipid composition can significantly accelerate the SMA fibrillation.

RESULTS

Biological membranes are crucial cellular components with multiple roles, including maintenance of the electrochemical gradients and control the diffusion of ions and biomolecules. They also act as a supporting matrix for embedded enzymes and receptors. Biological membranes are a complex and heterogeneous assembly of nonpolar and amphiphilic molecules. Although phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the two major phospholipid components of the mammalian cellular membranes²³, their composition varies depending not only on the organism or the tissue of origin, but also on the cellular localization of a given membrane. For example, mammalian red cell membrane consists of lecithin (PC, 29.3%), sphingomyelin (SM, 25.5%), lysolecithin (LPC, 1.0%), phosphatidylethanolamine (PE, 27.6%), phosphatidylserine (PS, 14.9%), phosphatidylinositol (PI, 0.6%), and phosphatidic acid (PA, 1.1%)²⁴; rat liver plasma membrane is composed of PC (39.9%), SM (18.9%), LPC (5.9%), PE (17.8%), PS (3.5%), PI (7.5%), PA (<1.0%), and lysophosphatidylethanolamine (LPE, 5.7%)²⁵, whereas rat liver nuclear membrane has a totally different composition, with 60.0% of PC, 3.2% of SM, 1.5% of LPC, 22.7% of PE, 3.6% of PS, 8.6% of PI, <1.0% of PA and with no detected LPE²⁵. Furthermore, various quantities of cholesterol (10–20%) can be found in the mammalian cellular membranes.

To determine whether the phospholipid charge influences the binding of SMA to membranes, we examined a series of vesicles composed of DPPC, DPPA, DPPS, POPA, POPE, POPC, POPG, POPS, and binary mixtures of these lipids (with the mass ratio of any two components 1:1). Therefore, all the lipids used in this study (except to phosphatidylglycerol) were the major components of mammalian cellular membranes. PG, being a common component of the bacterial and plant membranes²⁶, is less common in animal membranes but is present in mitochondria and some tissues²⁷. With respect to head group charge, only PC and PE were neutral, all others were negatively charged. The fatty acid chains were also chosen to be representative of saturated (DP) or unsaturated (PO) fatty acids.

Amyloidogenic LCs have been shown to preferentially interact with caveolae in the membranes of mesangial cells¹⁷. Caveolae are specialized membrane domains enriched in specific lipids and characteristic proteins. They have similar lipid composition to lipid rafts, including high concentrations of cholesterol^{28–30}. Thus, in order to mimic disease-critical cell membranes more accurately, vesicles containing various amounts of cholesterol were also prepared.

Due to the fact that some combinations of liposomes were not stable, difficult to make homogeneous or unstable over long periods of time, we mostly used binary mixtures containing DPPC since they enhance the homogeneous stability of the vesicles. Liposomes containing cholesterol were prepared as ternary mixtures that also contained DPPC. Other parameters examined included the mass ratio of protein to lipid (5:1, 1:1, 1:5) and the size of the vesicles.

Fibrillation of SMA Modulated by Phospholipid Vesicles

Amyloid fibrils are known to bind the fluorescent dye thioflavin T (ThT) relatively specifically. This interaction leads to a large increase in the fluorescence intensity around 482 nm^{31;32}, which provides a convenient method to monitor the kinetics of fibrillation. It has been recognized that mildly destabilizing conditions are often necessary for natively folded proteins to form fibrils *in vitro* on a reasonable time scale³³, and low concentrations of denaturants or low pH have been the most frequently used conditions to induce fibril formation in various proteins. However, to mimic physiological conditions as close as possible, and out of concern for liposome stability, the incubation solutions for SMA fibrillation in the presence of vesicles were chosen to be neutral (pH 7.4) or weakly acidic (pH 5.6), as in the kidney, the most frequent site of the light chain deposition, and in the lysosomes, a possible site of the intracellular fibril formation. The spectra of ThT bound to fibrils at both pH values were almost identical. Under

native-like conditions (pH 7.4 PBS, 37 °C) or at slightly acidic conditions (pH 5.6, 37°C) with gentle stirring, SMA did not form fibrils within 250 hours in the *in vitro* fibril assay. The absence of fibrils in these experiments was also confirmed by EM. These findings did not agree with earlier published results³⁴ likely due to the more gentle stirring conditions used in the present study and due to the difference in the incubation vessel material. Agitation is well known to increase the rate of protein aggregation, and is attributed to interfacial phenomenon increasing the population of partially folded intermediates, leading in turn to self-association and aggregation.

Most of the vesicles investigated, including various binary mixtures of DPPS, POPE, POPC, POPG, POPS, DPPC did not exhibit significant effects on the kinetics of SMA fibrillation within a 200-hour period, as no significant increase in ThT signal was observed. EM images confirmed the lack of fibrils in all these cases. However, vesicles of certain lipid composition greatly accelerated fibril formation. These liposomes contained either POPA or DPPA; i.e. phospholipids with the smallest head group, phosphatidic acid. Typical results with DPPC/POPA liposomes are described below.

As shown in Figure 1A, the observed ThT curves for SMA under native-like conditions (pH 7.4 PBS, 37 °C) in the presence of a 1:1 mixture of DPPC/POPA show typical fibrillation kinetics. Initial lag phase is attributed to the nucleation preceding a relatively fast elongation/growth phase^{35;36}. Not unexpectedly, the rates of fibrillation increased with increasing relative concentration of lipid. Comparing the three mass ratios of protein to phospholipid, the least amount of lipid (5:1 protein/lipid) had the weakest acceleration effect as shown by longest lag time and lowest intensity for fibrillation (see Table 1). As the relative amount of lipid was increased, with the protein/lipid ratio of 1:5, the ThT fluorescence traces indicated a slightly shorter lag time, and higher fluorescence intensity. Higher concentrations of lipid were also tested. Unfortunately, the fluorescence was affected by the pronounced light scattering under these conditions. These technical difficulties precluded accurate determination of the lipid effects on the fibrillation kinetics. A protein/lipid ratio of 1:5 turned out to be optimal and was chosen for most of the subsequent ThT measurements. Despite the limited concentration range that was used, it is still clear that the rate and extend of the SMA fibrillation was substantially influenced by the vesicles in a concentration-dependent fashion. This conclusion was further confirmed by the analysis of SMA fibrillation at slightly acidic pH (Figure 1B, Table 2). Comparison of data presented in Figure 1 and in Table 1 and Table 2 revealed that the accelerating effect of phospholipids was rather similar at both pH values. More specifically, the rate of fibrillation at the lower pH was a bit slower, being characterized by smaller k_{app} values and longer lag times (cf. Table 1 and Table 2).

The size of liposomes (LUVs of 100–200 nm and SUVs of 20 nm) possessed minimal effect on SMA fibrillation. However, EM images showed that the smaller vesicles tended to coalesce after long times of incubation or storage (data not shown), while the size of larger vesicles did not change with time for up to 10 days of incubation (Figure 2). Therefore, for ThT fluorescence measurements where long incubation times were needed we used LUVs.

Effects of Cholesterol

To investigate the potential influence of cholesterol-containing vesicles on SMA fibrillation, DPPC/POPA vesicles were prepared containing various amount of cholesterol, namely at mass ratios of 10%, 20%, and 30%. The kinetics of SMA fibrillation monitored by ThT fluorescence in the presence of DPPC/POPA vesicles containing 10% cholesterol were very similar to those in the vesicles without cholesterol (Figure 3). However, as the relative amount of cholesterol increased to 20%, the rate of fibril formation decreased, as shown by the longer lag time and lower fluorescence intensity. When the cholesterol concentration was increased to 30% (~ 2

mM), no increase in ThT signal was observed, suggesting that fibril formation was inhibited in the presence of vesicles with high cholesterol content.

In order to investigate whether cholesterol might decrease the ThT fluorescence signal from SMA fibrillation by simply blocking the binding of ThT to SMA fibrils, two further experiments were performed. First, SMA fibrils were grown under mildly destabilizing conditions, (4 M urea or 2 M GdnHCl) at pH 7.4. These fibrils were similar to those grown under native conditions, based on EM images⁸. Thioflavin T fluorescence was measured manually by adding a 10 μ l aliquot of fibrils to 990 μ L of 20 μ M TFT solution in a 1 mL fluorescence cuvette. Then, the fibril solution in the cuvette was titrated with 40 μ g/ μ l (i.e. ~ 100 mM) cholesterol in chloroform, and ThT fluorescence was measured after the addition of each cholesterol aliquot.

The titration curve (Figure 4A) shows that ThT fluorescence decreases with increasing amount of cholesterol, and was almost completely absent when the amount of cholesterol was 8 mM. Thus, the presence of cholesterol is sufficient to decrease the ThT signal from SMA fibrils. However, this concentration is around 4 times higher than the cholesterol concentration that completely quenched the ThT signal from the fibrils, suggesting that cholesterol may indeed be inhibiting fibrillation (note, however, that the ThT concentration was 5 times higher in the latter experiment).

The effect of cholesterol on ThT fluorescence most likely arises from cholesterol competing with ThT for the same binding sites on the fibrils, although it could also stem from fluorescence quenching by the cholesterol.

To test whether the cholesterol-induced decrease in the ThT fluorescence signal on SMA incubation was in fact due to inhibition of fibril formation, the relative amount of SMA fibrils grown in the presence of DPPC/POPA vesicles containing cholesterol at 10%, 20%, 30% for about 200 hour incubation was measured as follows. First, the three samples were spun down by ultracentrifugation at 150,000 g for 10 minutes. For each supernatant, the protein concentration was measured with the Lowry Assay (Figure 4B), allowing estimation of the amount of protein in the pellet; hence, fibril formation could be quantitatively measured. For the sample containing 10% cholesterol, the protein concentration in the supernatant was close to zero, indicating that essentially all the protein was in the pellet and hence fibrillar. Increasing the cholesterol concentration in the incubation solution led to an increase in the protein concentration in the supernatant. For the sample containing 30% cholesterol, most of the protein was in the supernatant, suggesting that little fibrillation occurred under these conditions. Thus, cholesterol decreases the ThT fluorescence signal as a consequence of both blocking the binding of ThT to SMA fibrils and by inhibiting the fibril formation.

These results suggest that when membranes contain cholesterol at the high range of physiological levels, such as 30%, SMA fibrillation would be inhibited, although membranes of the appropriate lipid composition with little or no cholesterol could accelerate fibrillation.

Effects of Calcium

Calcium ion is an important factor in many protein-membrane interactions. There have been no studies to determine how Ca^{2+} might affect the interaction of light chains with membranes. Thus the effect of Ca^{2+} at physiologically relevant concentrations from 1 μ M to 1 mM was determined on SMA fibrillation in the presence of vesicles. The kinetics of SMA fibrillation in the presence of DPPC/POPA/cholesterol vesicles was measured using ThT fluorescence, with a lipid composition ratio of 9:9:2 and a 1:5 protein/lipid mass ratio (Figure 5A). The lag time of SMA fibrillation increased as a function of increasing Ca^{2+} concentrations (Figure 5B).

With concentrations above 0.5 mM, SMA fibrillation was completely inhibited, suggesting that calcium levels could be important in AL amyloidosis.

To investigate the specificity of the Ca^{2+} effect on SMA fibrillation in the presence of vesicles, other divalent cations, such as, Mg^{2+} and Zn^{2+} were also tested. Similar effects were found with them, indicating that the response to Ca^{2+} might be due to a general divalent ion effect, rather than a specific effect of Ca^{2+} . An ionic strength effect is unlikely, since the buffer contained >100 mM NaCl.

Binding of DPPC/POPA /Cholesterol Vesicles Induced Slight Changes in the Structure of SMA

In order to investigate if interaction with the liposomes affected the conformation of SMA, the far-UV CD spectra of SMA in the presence of various concentrations of DPPC/POPA/cholesterol vesicles with a lipid composition ratio of 9:9:2 were measured after a short time of incubation (Figure 6). Freshly prepared solutions of SMA show a far-UV CD spectrum characterized by a minimum in ellipticity at 217 nm, corresponding to β sheet structure, as expected for a β -sandwich fold. The circular dichroism spectra indicated no pronounced changes in the secondary structure of the protein with various ratios of DPPC/POPA/cholesterol vesicles, and that the protein remained in its predominantly β -sheet configuration, as shown by similar ellipticity at 230 nm, but slightly changed ellipticity around 195 and 217 nm. However, there was a weak correlation between increased lipid and decreased β -sheet.

Intrinsic Trp fluorescence was analyzed under conditions described above (Figure 7). The intrinsic fluorescence of SMA is determined by two Trp residues, Trp35 and Trp50. In the native state, the fluorescence of Trp35 is completely quenched by the spatial proximity of the disulfide bridge and the intrinsic fluorescence results only from the solvent-exposed Trp50 with a λ_{max} around 350 nm. If major changes in SMA conformation occur the intensity or λ_{max} of the intrinsic fluorescence would change due to the increasing contribution of Trp35, but not necessarily would be sensitive for any subtle conformation changes. However, neither λ_{max} nor emission intensity changed much, indicating that the addition of DPPC/POPA/cholesterol vesicles did not induce significant conformational changes that increase the exposure of Trp35 in SMA (Figure 7).

Thus intrinsic fluorescence and far-UV CD spectroscopy did not show significant changes in SMA conformation after a short time of incubation in the presence of DPPC/POPA/cholesterol vesicles.

Confirmation of Fibrillation by FTIR

The kinetics of SMA fibrillation in the presence of various liposomes was also followed as a function of longer incubation times using FTIR. The amide I region ($1600\text{--}1700\text{ cm}^{-1}$), emanating from the carbonyl stretch vibration, has been used to estimate protein secondary structure content^{37–39}. The ATR-FTIR spectra of hydrated thin films of freshly prepared SMA at neutral pH revealed a strong absorbance band at 1640 cm^{-1} , no significant changes occurred during 10 days of incubation at $37\text{ }^{\circ}\text{C}$ with gentle shaking (Figure 8A). In contrast, the spectra of SMA in the presence of DPPC/POPA/cholesterol LUVs with a 1:5 protein/lipid mass ratio and a lipid composition ratio of 9:9:2 incubated under similar conditions, revealed the transformation from its native state to amyloid fibril structure as a function of increased incubation time (Figure 8B). Compared to the native state, the major changes of the amide I band were the gradual decrease in native β -structure around 1640 cm^{-1} and the gradual increase in the intensity of a band at 1631 cm^{-1} , which is characteristic of fibrils. The FTIR data show that the liposomes induced SMA fibrillation during the 10 day incubation, Figure 8C,

confirming the accelerating effect of DPPC/POPA/cholesterol vesicles on SMA fibrillation as monitored by ThT, Figure 8D.

Electron Microscopy

Our previous studies indicated that the aggregation and fibrillation of the immunoglobulin light chain SMA involves several different aggregation intermediates with a variety of morphologies⁹. As shown in Figure 9A, SMA dissolved in PBS buffer, with a concentration of 0.5 mg/ml, assembles into filaments *in vitro*, as reported previously^{8;9;34;40}. The effect of phospholipid vesicles on the morphology of SMA aggregates was further examined by electron microscopy. The addition of the 10% cholesterol-containing DPPC/POPA LUVs with the same SMA concentration led to the formation of similar fibrils, with somewhat more parallel lateral aggregation (Figure 9B). Fibrils were observed more clearly with such vesicles with a 1:5 protein/lipid mass ratio.

DISCUSSION

Previous investigations suggested that the outcome of interactions of the amyloidogenic light chain SMA with surfaces is highly dependent on the charge properties of the surface²². For example, no fibrils were observed on hydrophobic or positively charged surfaces, whereas negatively charged surfaces induced effective SMA fibrillation. This might be explained by electrostatic effects, considering that SMA has an isoelectric point of 8.3, and thus is significantly positively charged at neutral or acidic pH. With respect to the lipid bilayer membranes, the most important factor influencing protein binding is expected to be the lipid composition, especially the chemical nature of phospholipid head groups. Although the lipid composition varies widely among different membranes, all cell membranes contain a substantial proportion of phospholipids, predominantly phosphoglycerides. In addition, cholesterol is an integral component of all eukaryotic cell membranes and is essential for normal cellular functions. Cholesterol is not distributed uniformly within cell membranes, and is present in much higher concentrations in rafts and caveolae. The average cholesterol/phospholipid ratio of plasma membranes is ~ 0.5–0.7 (mol/mol)^{41;42}.

The location of light chain amyloid deposits in AL patients, typically on basement membranes, suggests that membranes may be important in triggering deposition of these proteins. Our data suggest that vesicles with acidic phospholipids and limited cholesterol content bring about very large increases in the efficiency of SMA fibrillation, both at pH 7.4 and pH 5.6. The latter pH would be encountered in lysosomes, which have been suggested to be involved in light chain amyloidogenesis⁴³. Our data suggest that the accelerating effect is similar at both pH values, probably because there is no significant change in the protein charge at these conditions, and suggesting that electrostatic interactions are important for the protein-membrane interactions. Interestingly, neither the size of the vesicles, nor the protein/lipid ratio had much effect on the kinetics of fibrillation. However, the amount of fibrils formed, as measured by ThT fluorescence signal, was very dependent on the protein/lipid ratio. Since the physiological ratio is likely to be highly in favor of excess lipid, we anticipate that acidic membranes, both within cells, and extracellularly, would be expected to catalyze light chain fibrillation.

The cholesterol addition to the vesicles affected the efficiency of SMA fibrillation with cholesterol concentrations above 10% possessing an inhibitory effect. It is also interesting to note that the presence of cholesterol decreased the intensity of the ThT fluorescence signal from SMA fibrils; this is most likely due to competition between ThT and cholesterol molecules for the same binding sites on the fibrils.

The inherent fibril accelerating effects of acidic vesicles were also offset by the presence of Ca^{2+} and other divalent ions. The most plausible explanation is that the cations bind to the

negatively charged vesicles and minimize the electrostatic attraction for SMA. The lack of evidence for vesicle-induced structural changes in SMA indicates that the SMA-vesicle interactions did not induce significant conformational changes in the protein, consistent with a simple electrostatic surface interaction.

In summary, our results demonstrated that membranes and membrane surfaces might have substantial effects on the fibrillation of amyloidogenic light chains. Since our data clearly indicate that small differences in lipid composition and cation concentrations can have a large effect on the kinetics of fibrillation, it suggests that the localization of amyloid deposits in light chain deposition diseases will be influenced by the nature of the membrane surfaces the protein encounters, as well as the intrinsic stability of the light chain.

EXPERIMENTAL PROCEDURES

Materials

Thioflavin T (ThT) was obtained from Sigma. All lipids were purchased from Avanti Polar Lipids, Inc. ANS was from Kodak.

Expression and Purification of Recombinant V_L SMA

The recombinant V_L domain SMA was purified from JM83 *E. coli* cells transformed with the plasmid pkIVsma004, generously provided by Dr. Fred Stevens, Argonne National Lab. The overexpressed protein was purified using the procedure of Stevens et al.⁴⁴ with minor modifications. Briefly, the recombinant protein was extracted from the periplasm using osmotic shock via treatment with ice-cold TES followed by distilled water. The periplasmic extract was dialyzed against 4 changes of 20 volumes of 10 mM acetate buffer, pH 5.6, and loaded onto a fast-flow SP-Sephacrose column. The column was washed with 10 mM acetate buffer, pH 5.6, and the protein eluted using 10 mM phosphate buffer, pH 8.0. The fractions were assayed by SDS-PAGE, and fractions containing the recombinant protein were pooled, filtered through 0.22 μ m filters, and stored in glass vials. Typical yields were 7–8 mg of purified protein per liter of cells. Protein concentrations were measured via optical density at 280 nm using the extinction coefficient of E^{0.1%} = 1.8 calculated from the sequence. The purified protein was stored in 10 mM phosphate buffer (pH 8.0) at 4 °C and used within 2 weeks of the initial purification. The purity of the protein preparations was assayed by SDS-PAGE and by electrospray mass spectrometry and was > 95%.

Preparation of Phospholipid Vesicles

Chloroform solutions of the lipids were evaporated using nitrogen gas and frozen; the resulting thin lipid films were hydrated with aqueous medium, with the temperature of the hydrating medium above the gel-liquid crystal transition temperature of the lipid; the hydrated lipid sheets detach during agitation and self-close to form large, multilamellar vesicles (LMV). Once these particles were formed, vesicles were prepared with two different sizes, SUVs (around 20 nm) and LUVs (100–200 nm). Reducing the size of the particles was achieved with sonication or extrusion. Sonicated SUVs of DPPC, DPPA, DPPS, POPE, POPG, POPS, and binary mixtures of these (1:1 mass ratio of any two components), were prepared as described previously²². LUVs of all the lipids were prepared by 10 cycles of freeze-thaw and extruded through a polycarbonate membrane of desired pore size.

Intrinsic Fluorescence Measurements

Fluorescence measurements were carried out on a FluoroMax-3 fluorescence spectrometer (Jobin Yvon-Spex). Emission spectra between 300 and 440 nm were collected with the excitation at 295 nm. Spectra were collected at neutral pH using 0.05 mg/ml protein samples

in PBS after one hour of incubation at 37 °C. The conformation of SMA was monitored as a function of vesicle concentrations by recording changes in tryptophan fluorescence intensity upon excitation at 295 nm and emission at 350 nm.

Circular Dichroism Spectra

CD spectra were collected on an AVIV model 62DS spectrometer between 260 and 190 nm for the far-UV region, with a step size of 0.5 nm and an averaging time of 2 s and collecting 5 repeat scans. Cells of 0.01 cm path length were used for far-UV CD measurements with protein concentrations of 2 mg/mL.

FTIR spectra

Data were collected on a Thermo-Nicolet Nexus 670 FTIR spectrometer equipped with a MCT detector and an out-of-compartment germanium trapezoidal internal reflectance element (IRE). The hydrated thin films were prepared and analyzed as described previously³⁷. Typically, 256 interferograms were co-added at 1 cm⁻¹ resolution. ATR-FTIR spectra were collected followed by Fourier transformation of the sample spectra using a clean crystal spectrum as a background. The water vapor spectrum was collected by reducing the air purge and subtracted from the protein spectrum until the spectra were featureless in the region between 1700 and 1600 cm⁻¹. ATR-FTIR spectra for SMA were collected at pH 7.4 in PBS. Buffer spectra were subtracted from the sample spectra. Data analysis was performed with GRAMS32 (Galactic Industries).

In Vitro Fibril Formation Assays

Fibril formation was monitored using a fluorescence assay based on the enhanced fluorescence of the dye Thioflavin T (ThT) on binding to amyloid fibrils⁴⁵. The application of this method is based on the characteristic properties of the thiazole Thioflavin T. As a free dye, ThT has very little fluorescence, with an excitation and emission maxima at 342 nm and 430 nm, respectively. Upon binding to amyloid structures (including SMA fibrils), spectral properties of ThT undergo characteristic alterations to a new excitation maximum at 450 nm and a new emission maximum at 482 nm with a dramatic increase in fluorescence intensity.

The *in vitro* kinetics of SMA fibril formation show the classic sigmoidal behavior, usually attributed to a nucleation-dependent polymerization. For simplicity in comparing the fibrillation kinetics, the intensity of ThT fluorescence was plotted as a function of time and fitted by a sigmoidal curve described by the following equation using SigmaPlot⁴⁶:

$$Y = y_i + m_i x + \frac{y_f + m_f x}{1 + e^{-(x - x_0)/\tau}}$$

where Y is the fluorescence intensity, x is time, and x_0 is the time to 50% of maximal fluorescence. Thus, the apparent first-order rate constant, k_{app} , for the growth of fibrils is given by $1/\tau$, and the lag time is given by $x_0 - 2\tau$.

Amyloid fibrils were grown from purified protein (40 μM) in PBS. The kinetics of SMA fibrillation were measured by thioflavin T fluorescence, either by automatically monitoring with multi-well plate reader, or by manual measurement at a series of incubation time points. For the latter case, a filtered protein sample (using 0.22 μ syringe filters) was incubated under the desired conditions in a 1.8 mL flat-bottomed screw-capped glass vial with moderate stirring using a Teflon-coated microstir bar. Typical fibril growth experiments involved incubating the protein at 37 °C with constant stirring and removing aliquots (10 μL) over time for analysis by ThT binding and TEM (see below). Fluorescence spectra were collected using a SPEX/Jobin-Yvon Fluoromax-3 spectrofluorometer. At each time point, Thioflavin T binding assays were conducted by adding sample aliquots (10 μL) to 990 μL of 20 μM ThT in PBS in a 1 mL

fluorescence cuvette. Fluorescence emission was monitored with excitation at 450 nm using a 5 nm band-pass on both the excitation and emission monochromators. Fluorescence intensities were reported at 482 nm.

Transmission Electron Microscopy

Transmission electron micrographs were collected using a JEOL JEM-100B microscope operating with an accelerator voltage of 80 kV. Typical nominal magnifications ranged from 27,000x to 67,000x. Samples were deposited on Formvar-coated 300 mesh copper grids and negatively stained with freshly prepared 1% aqueous uranyl acetate.

Abbreviations

SUV, small unilamellar vesicle
 LUV, large unilamellar vesicle
 ATR FTIR, attenuated total reflectance Fourier-transform infrared spectroscopy
 ThT, thioflavin T
 EM, electron microscopy
 CD, circular dichroism
 LC, light chain
 SDS-PAGE, SDS-polyacrylamide gel electrophoresis
 AFM, atomic force microscopy
 ECM, extracellular matrix
 DPPA, 1,2-Dipalmitoyl-sn-glycero-3-phosphate
 DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine
 DPPS, 1,2-dipalmitoyl-sn-glycero-3-[phospho-L-serine]
 POPA, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate
 POPE, 1,2-Dipalmitoyl-sn-glycero-3-phosphoethanolamine
 POPS, 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-L-serine]
 POPG, 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-RAC-(1-glycerol)].

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Figure 1.

The effect of membranes on SMA fibrillation kinetics. SMA fibril formation was monitored at pH 7.4, 37°C with agitation, using Thioflavin T, with emission at 482 nm and excitation at 450 nm. The vesicles were a 1:1 molar ratio of DPPC/POPA. The ratio of protein/lipid was varied from 5:1 (mass ratio) (circles), 1:1 (triangles), to 1:5 (squares). The filled triangles represent the corresponding data for incubation of SMA in the absence of vesicles.

Figure 2. Stability of DPPC/POPA/cholesterol LUVs. Electron micrographs of negatively stained preparations of DPPC/POPA/cholesterol LUVs at time zero (**A**) and after incubation for 10 days at 37 °C with constant stirring (**B**). Comparison of plots A and B reveals that LUVs for 10 days at 37 °C with constant stirring.

Figure 3.

The effect of cholesterol on the vesicle-induced fibrillation of SMA. DPPC/POPA/cholesterol vesicles at a 1:5 protein/lipid mass ratio, containing 10% (circles), 20% (triangles), 30% (squares) cholesterol, respectively, were incubated at pH 7.4, 37°C with agitation. Data for the SMA fibrillation in the presence of DPPC/POPA only (i.e., at 0% cholesterol) is represented by black circles and dashed line for comparison. Fibrillation was monitored by ThT fluorescence.

Figure 4.

The effect of cholesterol on the ThT fluorescence of SMA fibrils. A: Thioflavin T fluorescence of SMA fibrils, titrated with 40 $\mu\text{g}/\mu\text{l}$ cholesterol. B: The Lowry Assay for the three samples after 200 h incubation in Figure 3A: the arrows at increasing absorbances represent the protein sample in the presence of DPPC/POPA vesicles containing 10%, 20%, 30% mass ratio of cholesterol, respectively.

Figure 5.

The effect of Ca^{2+} on SMA fibrillation in the presence of vesicles. a: Ca^{2+} concentration dependence of SMA fibrillation in the presence of DPPC/POPA/cholesterol vesicles at a component mass ratio of 9:9:2, pH 7.3, 37°C monitored by ThT fluorescence. The arrow shows the direction of increasing Ca^{2+} concentration. b: Lag times of SMA fibrillation in the presence of DPPC/POPA/cholesterol vesicles (10% cholesterol) as a function of Ca^{2+} concentration. The data show that increasing Ca^{2+} leads to slower fibrillation. By 0.5 mM no fibrillation occurred.

Figure 6.

The interaction of SMA with vesicles does not affect its secondary structure. The figure shows the effect of various ratios of protein to lipid (by mass) using DPPC/POPA/cholesterol (9:9:2) vesicles on the far-UV CD spectra of SMA in PBS. SMA alone, (solid line); 5:1 (protein/lipid mass ratio) dotted line; 2:1 short dashed line; 1:1 (dash dot dot line); 1:2 (long dashes); 1:5 (dash dot dash line).

Figure 7.

The interaction of SMA with vesicles does not affect its tertiary structure. SMA intrinsic tryptophan emission spectra were measured with excitation at 295 nm for 4 μ M SMA with various ratios of DPPC/POPA vesicles in PBS after 1 hour of incubation at 37 °C. The protein/lipid mass ratios cover the range 10:1 to 1:10. The solid line represents SMA in the absence of vesicles. Long, medium and short dash lines correspond to 10:1, 5:1 and 2:1 protein/lipid. Dotted, dash-dot and dash dot dot correspond to 1:1, 1:2 and 1:10 protein/lipid.

Figure 8.

Monitoring SMA fibrillation with ATR FTIR. A: Amide I region of the spectra of SMA as a function of incubation time (pH 7.4, 37°C). B: The FTIR spectra of SMA in the presence of DPPC/POPA/cholesterol LUVs (with a 1:5 protein/lipid mass ratio and a lipid composition ratio of 9:9:2, i.e. 10% cholesterol), as a function of incubation time (days). C: The spectra show the gradual transformation of SMA from its native state to a crossed β -pleated sheet conformation during 10 days of incubation at 37°C. D: The corresponding kinetics of SMA fibrillation in the absence and presence of DPPC/POPA/Cholesterol vesicles monitored by thioflavin T fluorescence.

Figure 9. Electron micrographs of negative-stained preparations of SMA in the absence (A) and presence (B) of DPPC/POPA/cholesterol vesicles (with a 1:5 protein/lipid mass ratio and a lipid composition ratio of 9:9:2, i.e. 10% cholesterol)

Table 1

The effect of various lipid contents on the kinetics of SMA fibrillation at pH 7.4

Mass ratio of SMA to lipid pH 7.4	k_{app} (1/h)	Lag time (h)
5:1	0.105	38.9
1:1	0.106	36.6
1:5	0.107	35.3

Table 2

The effect of various lipid contents on the kinetics of SMA fibrillation at pH 5.6

Mass ratio of SMA to lipid (pH 5.6)	k_{app} (1/h)	Lag time (h)
5:1	0.068	47.8
1:1	0.070	47.5
1:5	0.081	44.8