Electrostatic lipid–protein interactions sequester the curli amyloid fold on the lipopolysaccharide membrane surface

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Curli is a functional amyloid protein in the extracellular matrix of enteric Gram-negative bacteria. Curli is assembled at the cell surface and consists of CsgA, the major subunit of curli, and a membrane-associated nucleator protein, CsgB. Oligomeric intermediates that accumulate during the lag phase of amyloidogenesis are generally toxic, but the underlying mechanism by which bacterial cells overcome this toxicity during curli assembly at the surface remains elusive. Here, we elucidated the mechanism of curli amyloidogenesis and provide molecular insights into the strategy by which bacteria can potentially bypass the detrimental consequences of toxic amyloid intermediates. Using a diverse range of biochemical and biophysical tools involving circular dichroism, fluorescence, Raman spectroscopy, and atomic force microscopy imaging, we characterized the molecular basis of the interaction of CsgB with a membrane-mimetic anionic surfactant as well as with lipopolysaccharide (LPS) constituting the outer leaflet of Gram-negative bacteria. Aggregation studies revealed that the electrostatic interaction of the positively charged C-terminal region of the protein with a negatively charged head group of surfactant/LPS promotes a protein–protein interaction that results in facile amyloid formation without a detectable lag phase. We also show that CsgB, in the presence of surfactant/LPS, accelerates the fibrillation rate of CsgA by circumventing the lag phase during nucleation. Our findings suggest that the electrostatic interactions between lipid and protein molecules play a pivotal role in efficiently sequestering the amyloid fold of curli on the membrane surface without significant accumulation of toxic oligomeric intermediates.

Amyloid fibrils are exquisite nanoscopic aggregates of misfolded proteins and are associated with a plethora of deadly neurodegenerative diseases such as Alzheimer’s, Parkinson’s, Huntington’s, and prion diseases (1–5). They are characterized by an ordered supramolecular architecture consisting of a common fold possessing a protease-resistant cross-β spine. An enlarging body of study has revealed that amyloid formation is an intrinsic property of a polypeptide chain regardless of initial structures (6, 7). Extensive studies have been carried out to understand the molecular mechanism of amyloid formation. However, the molecular basis for amyloid propagation and cytotoxicity remains elusive (8–10). A body of evidences has indicated that the oligomers are the key toxic species in the disease progression. Recent findings have revealed the existence of a variety of amyloid systems that play important functional roles in a wide range of organism from bacteria to humans (11, 12). These amyloids are termed as functional amyloids. Functional amyloids are known to be involved in biofilm formation in bacteria, non-Mendelian inheritance traits in yeast, melanin biosynthesis in mammals, and in long-term memory in Aplysia, Drosophila, and possibly in mammals (12). Understanding functional amyloidogenesis will provide insights into how organisms have evolved to control amyloid propagation and minimize the associated cytotoxicity.

Curli is a functional amyloid expressed in the extracellular matrix of enteric bacteria such as Escherichia coli and Salmonella spp. (13, 14). Curli biogenesis is involved in biofilm formation that mediates cell adhesion and interactions with host proteins that induce inflammatory responses (15–17). Unlike disease-associated amyloids, curli biogenesis has a tightly regulated pathway. The proteins encoded by csgBAC operon (curli-specific genes, csg) along with four accessory proteins encoded by the csgDEFG operon are essential for the curli assembly (18, 19). Curli is composed of two subunits, CsgA and CsgB. The solubility of these subunits inside the cell is maintained by CsgC and their secretion to the outer membrane is regulated by the accessory proteins encoded by csgDEFG operon (20–22). Both CsgA and CsgB are secreted as intrinsically disordered proteins (14, 23). On the cell surface, CsgA, the major subunit of curli is nucleated by membrane-anchored minor subunit CsgB (24). The first domain forms the secretary signal peptide that is responsible for targeting the proteins to the periplasm and is cleaved off in the periplasm (Fig. 1a) (25). Thus the mature CsgA and CsgB are devoid of secretary signal peptide. The subsequent 22 amino acids form the N22 domain, which helps CsgA to get transported onto the outer membrane; however, this domain is not essential for CsgB secretion (21, 26). The major amyloidogenic domain consists of imperfect

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This article contains supplemental Table S1 and Figs. S1–S6.

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2 The abbreviations used are: csg, curli-specific gene; AFM, atomic force microscopy; DCVJ, 9-(2,2-dicyanovinyl)julolidine; ThT, thioflavin-T; IAEDANS, 5-(((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid; GdnHCl, guanidine hydrochloride.
repeats (R1–R5) comprising of 19–24 amino acids that forms the amyloid core (26, 27). In vivo studies have shown CsgB is necessary for CsgA polymerization, whereas in vitro, CsgA aggregates even in the absence of CsgB (27, 28).

The mechanism by which the bacteria bypass the toxic events during curli amyloidogenesis is poorly understood. The mechanistic studies of a variety of disease-associated proteins have revealed that amyloid formation typically follows a nucleation-dependent polymerization with a characteristic lag, elongation, and saturation phases (29, 30). The transient oligomeric intermediates formed during lag phase are known to be highly toxic compared with the mature fibrils (31, 32). Additionally, amyloid formation on the lipid membrane can disrupt the membrane integrity and can cause cell death (32–35). Nevertheless, how enteric bacteria allow the curli assembly on its cell surface and bypass the toxic events remain poorly understood.

We hypothesize that the bacterial membrane surface might be involved in the recruitment of the amyloidogenic precursors and thereby resulting in the formation of amyloid fibrils without accumulation of toxic intermediates. Outer membrane surface of Gram-negative bacteria is composed of lipopolysaccharide (LPS) containing negatively charged glycolipid phospholipids (36). In this work, we aimed at dissecting the key molecular events of the intermolecular interactions between anionic lipids and curli subunits that allow the sequestration and subsequent polymerization into matured amyloid fibrils. Using a diverse range of tools, we first show that the electrostatic modulation between the protein and a model anionic detergent accelerates the aggregation, markedly leading to the disappearance of the typical lag phase during nucleation. Then we demonstrate that negatively charged LPS plays a pivotal role in promoting the heteronucleation during curli amyloid assembly.

**Results**

**A switch in the polymerization mechanism in the presence of an anionic detergent**

A line of evidences suggested that CsgB gets tethered onto the membrane, which nucleates the aggregation of CsgA (24, 26). Lipid A, a negatively charged glycolipid, forms the outer membrane of Gram-negative bacteria (36). To understand the molecular basis of CsgB membrane interactions, we began our studies using an anionic detergent namely SDS, which is a well-known anionic lipid mimic (37). Subsequently, we carried out our experiments in the presence of LPS.

The function of curli nucleator CsgB is directed by its C-terminal. Without R4 and R5 repeats, CsgB is secreted away from the bacterial cell surface. Hence, we speculated that both R4 and R5 might have membrane interaction. To verify whether R4 and R5 interact with membrane, we carried out experiments using CsgBΔR4 or CsgBΔR5 (19 amino acids are deleted from C-terminal of CsgB) and CsgB. In this study we refer to CsgBΔR5 as CsgBΔ, C-terminal His-tagged CsgBΔ, CsgB, and CsgA were expressed and purified (see “Experimental procedures”). The purity was checked by SDS-PAGE (supplemental Fig. S1a) and the proteins of interest were confirmed by probing against anti-His antibody (supplemental Fig. S1b). First we monitored the aggregation kinetics of curli subunits using thioflavin T (ThT). CsgA, CsgBΔ, and CsgB exhibited typical nucleation-dependent polymerization kinetics with lag times of ~120, ~100, and ~30 min, respectively (supplemental Fig. S1c). However, in the presence of preformed CsgB, seeds, a shortening of the lag phase in the CsgA aggregation was observed, which suggested that preformed CsgB oligomers can nucleate the CsgA polymerization (supplemental Fig. S1c). These results are in good agreement with previous reports (23, 38). Next, we investigated whether SDS at non-denaturing submicellar concentrations can modulate the course of CsgBΔ aggregation. A shortening in the lag time of CsgBΔ aggregation was observed with an increase in the SDS concentration ranging from 100 to 500 μM. Interestingly, in the presence of 500 μM SDS, CsgBΔ bypassed the nucleation phase as indicated by the absence of any apparent lag time (Fig. 1b). A complete elimination in the lag phase was also observed for CsgBΔ in the presence of 500 μM SDS (Fig. 1c). This observation suggests that the aggregation propensity of CsgBΔ/CsgB is accelerated in the presence of submicellar concentrations of SDS. This aggregation might follow a non-nucleation or an isodesmic polymerization mechanism as there is no strong concentration dependence on half-time (t1/2), when different protein concentrations were titrated with 500 μM SDS (supplemental Fig. S1, d and e).

We next monitored the secondary structural changes by using far-UV circular dichroism (CD) spectroscopy. CsgBΔ adopts a random-coil structure with minima at 200 nm. As a function of time, CD spectra showed an increase in the β-sheet-rich secondary structure (216 nm) at the expense of random coil (200 nm) and within 3 h there was a complete transition to the β-sheet (Fig. 1d). On the contrary, upon addition of SDS, CsgBΔ showed a rapid conformational conversion and the attained β-sheet structure within 30 min (Fig. 1e). The CD kinetics monitored at 200 nm validates that SDS induces spontaneous secondary structural changes during the fibrillation of CsgBΔ (Fig. 1f). We also monitored the backbone conformation of the amyloid state using vibrational Raman spectroscopy. The amide I Raman band at 1670 cm−1 exhibited by both (with and without SDS) fibrils indicated the formation of a cross-β structure, which is a hallmark of an amyloid (Fig. 1f) (39). Therefore, our ThT fluorescence assays together with the CD and Raman spectroscopic data show that the SDS–CsgBΔ interaction facilitates the conformational change and accelerates amyloid fibrillation by minimizing or eliminating the lag phase.

**SDS induces rapid oligomerization of CsgBΔ**

Next, we embarked upon studies aimed at elucidating the mechanism of CsgBΔ polymerization in the presence of SDS. To do this we have used 9-(2,2-dicyanovinyl)julolidine (DCVJ), a molecular rotor, which shows an enhanced fluorescence upon binding to the early oligomers of protein aggregates (40). DCVJ is weakly fluorescent in buffer, and as a function of CsgBΔ aggregation, a slow increase in DCVJ fluorescence was observed that was saturated by ~4 h (Fig. 2a and b). Upon addition of SDS to CsgBΔ, there was a rapid increase in DCVJ fluorescence and reached a plateau within ~30 min. The DCVJ fluorescence
results indicated that SDS facilitates rapid oligomerization of CsgBt. Next, we performed the glutaraldehyde cross-linking assay to monitor the formation of soluble oligomers. We observed that the SDS-treated sample has much a lower content of monomeric protein compared with the untreated sample (supplemental Fig. S2a). The depletion of the monomer/dimer population in the SDS-treated sample provided an additional evidence of early oligomerization (supplemental Fig. S2a). To gain insights into the nanoscale morphology of the transient intermediates, we next utilized atomic force microscopy (AFM). At early time points, CsgBt forms protofibrils of ~5 nm height (Fig. 2c). These protofibrils convert into matured fibrils upon prolonged incubation (Fig. 2d). Interestingly, the fibrils exhibited two distinct height distribution, 8–10 and 20–25 nm indicating that the fibrils are laterally associated to form bundles (supplemental Fig. S2b), as described previously for the CsgA fibrils (41). The bundle formation might be the possible reason for the high stability of curli and require formic acid to disintegrate these fibrils (14). In the presence of SDS, CsgBt formed spherical oligomers with an average height of 8–10 nm (Fig. 2e). The oligomers convert into amyloid fibrils upon overnight incubation (Fig. 2f). Therefore, both our AFM and cross-linking results corroborated our DCVJ data, which suggested that the SDS–CsgBt interaction leads to the formation of higher order species. The absence of the lag phase observed in the kinetics of ThT fluorescence, CD, and DCVJ fluorescence indicate that the SDS–CsgBt interaction leads to the rapid formation of β-rich obligatory oligomers and allows the protein to bypass the nucleation phase. We next aimed at delineating the physicochemical basis of the SDS-CsgBt interaction.

The C-terminal end of CsgB interacts electrostatically with the anionic head groups

It was shown previously that the C-terminal region of CsgB containing the R4 and R5 are essential for anchoring and proper localization of the protein onto the membrane and without these repeats CsgB is secreted away from the cell (26). To monitor whether the C-terminal segment is responsible for the initiation of aggregation, we incorporated a tryptophan (Trp) at the C-terminal end (Y129W). CsgBt is devoid of Trp and therefore we envisioned that the single Trp mutant at R4 will report the early events of the aggregation. This Y129W mutant of CsgBt displayed aggregation kinetics similar to that of CsgBt indicating that the mutation did not alter the aggregation propensity of the protein (supplemental Fig. S3a). We next recorded several fluorescence readouts to monitor the aggrega-
tion process. Trp is an environmentally-sensitive fluorescent probe (42). Before aggregation, Y129W showed an emission maxima at 345 nm, which suggested that the Trp is solvent exposed. However, during the course of aggregation, there was a progressive blue shift of 8 nm indicating that the C-terminal segment harboring Trp gets buried (supplemental Fig. S3b).

Interestingly, in the presence of SDS, Y129W exhibited a blue shift in the emission maxima (334 nm) that did not undergo any further shift upon aggregation (supplemental Fig. S3c). This result indicated that the structure formation at the C-terminal segment of CsgBt was very rapid in the presence of SDS during the oligomerization (Fig. 3a).

Next, we monitored the fluorescence anisotropy that provides the information about the rotational mobility of the fluorophore (42). Without SDS, Y129W exhibited a low fluorescence anisotropy of 0.06 (Fig. 3b). The low anisotropy value indicates that Trp resides in a highly flexible region of disordered CsgBt. During the aggregation, the anisotropy showed a typical nucleation-dependent kinetics and increased to 0.16. The increase in the fluorescence anisotropy is due to the disorder-to-order transition during aggregation that causes a restriction in the mobility of Trp. In the presence of SDS, the initial Trp fluorescence anisotropy is much higher (0.13) and reached a plateau (0.18) during the aggregation without a lag phase (Fig. 3b). The initial increase in the Trp anisotropy upon addition of SDS indicates the increase in the rotational hindrance arising due to the rapid oligomerization of CsgBt. Taken together, our Trp fluorescence results show that the C-terminal region of CsgBt harboring the R4 repeat interacts with anionic SDS. This region of CsgBt contains positively charged residues such as lysine (Fig. 1a). Therefore, we conjectured that the interaction between the C-terminal segment of CsgBt and SDS
is driven by the electrostatic interaction. To test this, we next carried out aggregation experiments in the presence of salt. The kinetics of aggregation in the presence of salt is much slower than in the absence of salt, indicating that higher ionic strength decelerates the aggregation of CsgBt even in the presence of SDS (Fig. 3c). Interestingly, with the same concentration of salt there is no change in the aggregation kinetics of CsgB, which indicates that the interaction of SDS with the R5 repeat is stronger than the R4 segment (supplemental Fig. S3d). As expected, CsgBt failed to aggregate in the presence of a positively charged detergent such as cetyltrimethylammonium bromide (supplemental Fig. S3e). This set of experiments revealed that the ionic interaction between CsgBt and SDS plays a key role in triggering the aggregation process.

C-terminal segment of CsgB is involved in the formation of critical oligomers

The ionic interaction of proteins with the membranes or detergents can neutralize the charge and facilitate the protein–protein interaction by increasing the local protein concentration on the surface. We next studied the formation of early oligomers that are formed by the interaction between the C-terminal segments of CsgBt. To monitor the protein–protein interaction we performed intermolecular fluorescence resonance energy transfer (FRET). For this set of experiments, we chose two C-terminal variants of CsgBt, Y129W (Trp as a donor) and Y129C was labeled with an acceptor dye, IAEDANS (5-(((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid). Trp and IAEDANS are known to be an efficient FRET pair (42). We used FRET efficiency as the readout of the intermolecular interaction between CsgBt molecules. In the absence of SDS, CsgBt exhibited a low FRET efficiency (~14%), whereas upon addition of SDS, the FRET efficiency increased sharply (~36%) indicating the formation of early oligomers that bring together the C-terminal segments of CsgBt within the range of Förster distance (Fig. 3d). To establish further that the C-terminal contacts are critical for the oligomerization, we carried out aggregation of CsgBt, Y129C in the presence and absence of a reducing agent, DTT (dithiothreitol). Upon addition of DTT, CsgBt aggregated similar to wild-type CsgBt, as expected (Fig. 3e). However, in the absence of DTT, the aggregation kinetics was much faster indicating that dimerization of the C-terminal segments through disulfide formation facilitates the oligomerization. In other words, the C-terminal dimer might be an on-pathway intermediate during the aggregation process. Taken together, our data suggested that the electrostatic interaction between SDS and the C-terminal segment of CsgBt results in the charge neutralization and leads to the formation of oligomeric precursors, which mature into amyloid fibrils. Next, we asked whether or not the SDS-
induced aggregates of CsgBt are capable of nucleating CsgA polymerization that is critical for curli biogenesis.

SDS-induced oligomers of CsgBt nucleates CsgA polymerization

CsgA aggregated with a lag time of ~2 h, however, in the presence of SDS, the lag time was shortened but the aggregation followed a typical nucleation mechanism (Fig. 4a). This result is in sharp contrast to SDS-induced aggregation of CsgBt, which exhibited a non-nucleation or an isodesmic polymerization mechanism. Even upon addition of a catalytic amount of CsgBt to CsgA (CsgA:CsgBt, 5:1) the aggregation kinetics was similar to that of CsgA (Fig. 4b). Interestingly, upon addition of SDS, CsgA:CsgBt aggregated rapidly without a lag phase and exhibited kinetics similar to SDS-induced aggregation of CsgBt (5:1) (Fig. 4b). This set of results indicated that at substoichiometric concentrations of CsgBt, SDS accelerates the polymerization rate of CsgA. Our next aim was to verify that even in the presence of catalytic amounts of CsgBt, SDS-induced CsgBt oligomers act as seeds for CsgA polymerization.

CsgB oligomerizes faster than CsgA

CsgA has a single Trp at the 109th position in the R3 region of the polypeptide chain. We monitored Trp fluorescence of CsgA in the presence of CsgBt. During the course of aggregation, there was a blue shift in the emission maxima from 345 to 337 nm indicating that the R3 region gets progressively buried (supplemental Fig. S5a). However, in the presence of SDS, the emission maxima underwent blue shift much faster and then remained constant during the late kinetics of the aggregation process (supplemental Fig. S5b). This result indicated that the solvent-exposed Trp in the disordered state gets buried at a much faster rate in the presence of SDS. Therefore, the time course of the spectral shift suggested that CsgA undergoes fibrillation relatively faster in the presence of SDS and CsgBt (supplemental Fig. S5b), but slower than SDS-induced aggregation of CsgBt (1.6 μM). The solid lines are the fits using the nucleation-dependent polymerization model.
On the contrary, upon addition of SDS, Y129W CsgBt showed a rapid change in the emission maxima and anisotropy of Trp at the onset of aggregation (Fig. 3, a and b). Therefore, our Trp fluorescence data validates that the rapid aggregation and the change in the secondary structural content of CsgA even in presence of the substoichiometric ratio of CsgBt is indeed due to the initial SDS-induced oligomers of CsgBt. These experiments suggested that SDS imparts an amyloid fold in the C-terminal end of CsgBt that leads to rapid oligomerization of the latter, which nucleates the polymerization of CsgA. Similarly, upon addition of substoichiometric amounts of CsgB and submicellar concentration of SDS to CsgA, the latter aggregated without exhibiting lag phase (Fig. 5a). The preformed SDS-induced oligomers of CsgB, even after diluting, are capable of nucleating aggregation of CsgA, suggesting that these obligatory oligomers are stable even upon dilution (supplemental Fig. S5d). Fig. 5, b–d, summarizes the kinetic parameter halftime ($t_{1/2}$) recovered from the aggregation of curli subunits in the absence and presence of SDS obtained from the time course of different probes. Overall, our studies indicated that the electrostatic interaction between detergent and protein molecules facilitates curli assembly.

Lipopolysaccharide modulates curli assembly

Next, we asked whether curli assembly proceeds through a similar mechanistic control in the presence of LPS, the outer leaflet of Gram-negative bacteria. LPS is composed of three domains: lipid A, core oligosaccharide, and O-antigen. Lipid A...
is a glycophaspholipid consisting of a β-1,6-linked disaccharide of glucosamine, which is phosphorylated and fatty acylated (36). The phosphate groups impose negative charge on lipid A. To monitor the CsgBt–LPS interaction, LPS vesicles of 100 nm (supplemental Fig. S6a) were used in concurrence with the other studies on LPS–protein interactions (43). First, we monitored ThT fluorescence to follow the aggregation of CsgBt in the presence of different concentrations of LPS vesicles. There is an increase in the rate of aggregation as a function of LPS concentrations, as expected from our SDS experiments. Upon addition of 50 μg (per 1.5 ml) of LPS, both CsgBt and CsgB aggregated without a lag phase (Fig. 6a and supplemental Fig. S6b), similar to what we observed with SDS. AFM imaging revealed that CsgBt undergoes spontaneous oligomerization in the presence of LPS (Fig. 6b) and eventually formed fibrils of different heights (Fig. 6c and supplemental Fig. S6c). The spherical oligomers formed in the presence of LPS is 8–10 nm, which is similar to the size of SDS-induced oligomers of CsgBt (Fig. 2e). Thus, the ThT assay and AFM results in the presence of LPS are in line with those obtained from SDS-induced aggregation. Next, we monitored aggregation of CsgA:CsgBt (5:1) in the presence of LPS that exhibited a much faster kinetics compared with that of without LPS (Fig. 6d). CsgA:CsgBt (5:1) aggregates were faster in the presence of LPS, suggesting that the interaction between the repeats and LPS promote the aggregation process without an apparent lag phase (Fig. 6e). Taken together, our results show that in the presence of LPS, the aggregation of curli-forming proteins is highly accelerated.
Discussion

Curli is a functional amyloid formed on the surface of Gram-negative bacteria. Within the bacteria, curli subunits exist as intrinsically disordered proteins and avoid aggregation by interacting with CsgC (20). In this work, we dissect the role of membrane in curli formation. Our aggregation studies on CsgBt, using an array of biophysical and biochemical tools indicated that SDS, a membrane mimetic, binds to the protein through electrostatic interaction and promotes amyloid formation without a lag phase. We also showed that LPS, a primary component in the outer membrane surface of Gram-negative bacteria, also modulates the aggregation kinetics in a similar way. Binding to SDS/LPS neutralizes the positive charge of the protein molecules that facilitates intermolecular association leading to amyloid formation. Proteins upon binding to a surfactant such as SDS, at submicellar concentration, can induce the formation of clusters that can further grow into micelles and drive protein association (44). This ionic interaction with a detergent or a lipid is known to increase the local protein concentration, which can also promote protein aggregation (45, 46). Our FRET measurements using the Y129C mutant of CsgBt, and cross-linking assay suggested that SDS promotes intermolecular interaction in the C-terminal segment of CsgBt and accelerates the aggregation of the protein, thereby minimizing the possibility of accumulation of toxic intermediates (supplemental Figs. S2a and Sb). In vivo study has previously demonstrated that the C-terminal of CsgB, especially R4 and R5 repeats are essential for proper localization on the membrane and curli assembly (26). R5 peptide fails to aggregate in vitro; thus it was speculated that the membrane-bound C-terminal end might be devoid of amyloid fold thus the membrane integrity is not compromised (26). The ionic interaction between the R4/R5 repeat of CsgB, and SDS/LPS induced the formation of structured oligomers, which allow efficient polymerization of CsgA (Figs. 5b and 6, d and e). The binding of the positively charged C-terminal segment comprising R4 and R5 to SDS/LPS neutralizes the polypeptide chains via electrostatic interactions and therefore promotes critical chain–chain association that was otherwise hindered because of charge repulsion. Our findings allowed us to depict a simplistic model that is shown in Fig. 7. The facile recruitment of curli subunits on the LPS surface and subsequent fibrillation can potentially prevent the prolonged incubation of intermediates on the membrane: a mechanism by which bacteria might overcome the cytotoxicity during the course of aggregation of curli subunits.

Functional amyloid proteins might have evolved to undergo facile aggregation without significant accumulation of toxic oligomeric intermediates. For instance, Pmel17, a human functional amyloid involved in melanin synthesis undergoes a rapid pH-induced amyloidogenesis that is proposed to preclude the formation of its toxic intermediates (47). On the contrary, α-Synuclein, Aβ (amyloid-β), and tau, which are implicated in neurodegenerative diseases, exhibit slow aggregation kinetics with a long lag phase (48). Mounting evidences suggest that the accumulation of amyloid precursors is the cause of cytotoxicity (32–35). Doughnut-shaped prefibrillar oligomers of many disease-related proteins are known to be membrane active species (32). The oligomeric intermediates formed by α-Synuclein, Aβ, islet amyloid polypeptide, polyglutamine, etc. are recognized by A11 antibody (49). CsgA oligomers are A11 active indicating that these oligomers possibly share structural similarity with the disease-associated amyloids (38). The minimum size of the transient intermediate of CsgA recognized by the A11 antibody is a monomer or dimer. CsgA dimers have been shown to be very stable and are observed even after treating the CsgA fibrils with formic acid (14). Our results indicate that in the presence of SDS the minimum oligomeric size of CsgB is a dimer that is consumed rapidly to form higher order fibrils. Taken together, our results show that the electrostatic interaction between CsgB and SDS/LPS-membrane promotes facile protein–protein interaction leading to the sequestration of amyloid fold that finally nucleates the polymerization of CsgA. This membrane-assisted process is likely to prevent the accumulation of toxic species during the course of aggregation. Plausibly, bacterial membranes provide a platform that accelerates the dimer formation of the nucleator protein and facilitates the aggregation of curli subunits by sequestering the toxic intermediates.

Experimental procedures

Materials

Potassium phosphate monobasic, potassium phosphate dibasic, SDS, DTT, DCVJ, and LPS from E. coli 0111:B4 were procured from Sigma. Guanidine hydrochloride (GdnHCl) was procured from Amresco. Isopropyl β-thiogalactopyranoside and antibiotics (ampicillin and chloramphenicol) were obtained from Gold Biocom (USA). IAEDANS was purchased from Invitrogen. All the products were used without any additional purification. Mouse His6 epitope tag primary antibody (MA1–21315), HRP-conjugated rabbit anti-mouse secondary antibody (PA1–28568), and metal-enhanced 3,3′-diaminobenzidine substrate kit were obtained from Thermo Fisher Scien-
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tific. NEB3016 strain was procured from New England Biolabs. Nickel-nitrilotriacetic acid resin was obtained from Qiagen and PD-10 procured from GE Healthcare Life Sciences.

Expression, purification, and labeling

His-tagged CsgA, CsgBt, and CsgB were overexpressed in NEB3016 strain. CsgA and CsgBt were purified as described previously with slight modifications (50). A cell pellet from 500 ml of culture was lysed with 50 ml of 8 M GdnHCl in 50 mM potassium phosphate buffer, pH 7.3, and kept under stirring for 1 day. The lysate was centrifuged and the supernatant was sonicated for 2 min (pulse on: 30 s, pulse off: 20 s) and incubated with 1.5 ml of nickel-nitrilotriacetic acid resin at room temperature for 1 h. After 1 h the lysate was loaded onto a polypropylene column and the beads were washed with 7 column volumes of 50 mM potassium phosphate buffer, pH 7.3 (buffer A). Followed by another wash with 7 column volumes of buffer A containing 12.5 mM imidazole. The protein was eluted with buffer A containing 125 mM imidazole and passed through 30-kDa (Millipore Amicon) filter. The filtrate was subjected to PD-10 desalting column to remove imidazole. For the Y129C mutant of CsgBt, 10 \( \mu M \) \( \beta \)-mercaptoethanol was added in all steps except during the PD-10 gel filtration. CsgB was purified as described previously (41) with slight modifications. The wash and elution buffers had 8 M urea and 150 mM NaCl in 50 mM potassium phosphate buffer, pH 7.3, and the eluent was directly subjected to PD-10 spin desalting column. The protein concentrations were estimated using \( \varepsilon_{280} \) of 10,810, 7,680, and 7,680 M\(^{-1}\) cm\(^{-1}\) for CsgA, CsgB, and CsgBt, respectively. Mutants of CsgBt were created by site-directed mutagenesis (primers sequences used are given in supplementary Table S1) and the concentration was determined using \( \varepsilon_{280} = 12,090 \text{ M}^{-1} \text{ cm}^{-1} \) for Y129W and \( \varepsilon_{280} = 6,400 \text{ M}^{-1} \text{ cm}^{-1} \) for Y129C. The molar extinction coefficients were obtained using Protein Calculator 3.4 (The Scripps Research Institute).

For labeling CsgBt Y129C with IAEDANS, the protein was purified using native buffer containing 10 \( \mu M \) \( \beta \)-mercaptoethanol. The PD column was done in 6 M GdnHCl, 30 \( \mu M \) DTT, buffer A, and the protein concentration was estimated and DTT was further added so that the final concentration for protein:DTT was 1:2. The resulting solution was stirred at room temperature for 30 min at 10 rpm. Then 10 eq of IAEDANS (according to protein concentration) were added from 200 mM stock prepared in DMSO and allowed to stir for 1 h at room temperature. Ten eq of IAEDANS were further added and incubated for 1 h. PD-10 column chromatography was done in buffer A. The protein concentration was estimated by measuring the absorbance at 280 and 340 nm. The concentration of labeled protein was calculated by using absorbance of IAEDANS at 340 nm (\( \varepsilon_{340} = 6100 \text{ M}^{-1} \text{ cm}^{-1} \)).

Steady-state fluorescence

All aggregation reactions were carried out at room temperature in 50 mM potassium phosphate buffer, pH 7.3, and the protein concentration used was 8 \( \mu M \) unless otherwise specified. All fluorescence experiments were performed on Fluoromax-4 spectrophotometer (Horiba Y). For the ThT fluorescence assay, the samples were mixed with 20 \( \mu M \) ThT and incubated at room temperature. The samples were excited at 450 nm and emission was collected at 485 nm after every 10 min. The aggregation kinetic traces were fitted using the nucleation-dependent polymerization model (52). The excitation wavelength used for Trp was 295 nm and emission spectra were collected in the range of 310–400 nm. The collected spectra were buffer corrected and normalized. The samples containing 5 \( \mu M \) DCVJ were excited at 450 nm and emission spectra were collected in the range of 470–550 nm. The steady-state fluorescence anisotropy of Trp was measured at 345 nm. The steady-state fluorescence anisotropy is given by the following equation,

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R_{ss} = (I_L - I_G)/(I_L + 2I_G)
\]

(Eq. 1)

where, \( I_L \) and \( I_G \) are the parallel and perpendicular fluorescence intensities, respectively, with respect to the excitation polarizer. The perpendicular components were always corrected using a G-factor. The reaction mixture was stirred only during the measurements. The protein concentration used for all experiments was 8 \( \mu M \) in 50 mM potassium phosphate, pH 7.3, except for CsgA:CsgBt (5:1) in which the concentration of CsgA was 8 \( \mu M \) and CsgBt used 1.6 \( \mu M \). The seeds of CsgBt were prepared from 8 \( \mu M \) CsgB fibrils (50 mM potassium phosphate, pH 7.3), which were prepared by incubating at 25 °C for 1 day under quiescent conditions. The fibrils were then subjected to bath sonication (37 kHz) for 1 min.

For FRET measurements, a mixture of 6 \( \mu M \) CsgBt, Y129W (donor) and 2 \( \mu M \) AEDANS-labeled protein (acceptor) was excited at 295 nm and the spectra were recorded in the range of 320–580 nm. The FRET spectra were corrected with respect to the direct excitation of AEDANS at 295 nm. The FRET efficiency was estimated using the following relationship (42),

\[
E = 1 - F_{DA}/F_D
\]

(Eq. 2)

where, \( F_{DA} \) and \( F_D \) are the donor (Trp) fluorescence intensities in the presence and absence of acceptor (AEDANS), respectively.

CD experiments

CD experiments were performed on an Applied Photophysics Chirascan CD spectrophotometer. The CD spectra were recorded every one-half hour and scanned from 190 to 250 nm using 1-mm path length cuvette. Three spectra were averaged, blank subtracted, and smoothed using Pro-Data viewer and then plotted using Origin software. Single point CD at 200 nm was collected at 10-min intervals to monitor the kinetics. The concentrations used were 8 \( \mu M \) CsgBt, CsgA:CsgBt (5:1) = 8 \( \mu M \), and 1.6 and 500 \( \mu M \) for SDS in 50 mM potassium phosphate buffer, pH 7.3. The samples were mixed properly before every measurement.

Cross-linking assay and Western blot analysis

To 8 \( \mu M \) CsgBt, in 50 mM potassium phosphate buffer, pH 7.3 (with and without 500 \( \mu M \) SDS), glutaraldehyde was added so that the final concentration of the latter was 0.01%. The reaction was incubated at room temperature for 10 min. SDS-PAGE loading dye with \( \beta \)-mercaptoethanol was added to the reaction mixture and heated for 5 min at 100 °C. The samples were run
on a 15% SDS-PAGE and transferred to a PVDF membrane (Amersham Biosciences) followed by blocking with 3% BSA in PBST (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, 0.05% Tween 20) for 1.5 h at room temperature. The samples were washed three times with PBST and then probed with 1:3000 diluted anti-His primary antibody for 1.5 h at room temperature. After washing, the blots were incubated with 1:10,000 HRP-conjugated rabbit anti-mouse secondary antibody for 1 h at room temperature. The blots were developed using metal enhanced 3,3′-diaminobenzidine substrate.

**AFM imaging**

Ten μl of sample was deposited on freshly cleaved and Milli-Q water-washed mica (muscovite grade V-4 mica from SPI, PA) and incubated for 5 min followed by washing with water. CsgB sample (0 h) was diluted 100 times with water and deposited on mica. Then mica was dried under a stream of nitrogen gas for 15 min. The images were acquired using Innova (Bruker) AFM in tapping mode. The collected images were processed and analyzed using WSxM 5.0 (51).

**Raman spectroscopy**

The Raman spectra were recorded on an inVia™ Raman microscope (Renishaw, UK). The aggregation reaction incubated overnight at 25 °C was centrifuged at 13,000 rpm for 15 min and the supernatant was discarded keeping ~100 μl of solution. The resultant solution was deposited on a glass slide and air dried. The sample was focused by 50× objective lens (Nikon, Japan) and was excited using 785 nm HPNIR laser. A 1200 lines/mm grating was used to disperse the scattered light. Data were acquired using Wire 3.1 software provided with the instrument. The spectra were baseline corrected using the cubic spline interpolation method for eliminating the tilt and anti-mouse secondary antibody for 1 h at room temperature. The spectra were baseline corrected using the cubic spline interpolation method for eliminating the tilt and anti-mouse secondary antibody for 1 h at room temperature. The collected images were processed and analyzed using WSxM 5.0 (51).

**Preparation of LPS vesicles**

One mg/ml of LPS was dissolved in 50 mM potassium phosphate buffer, pH 7.3. The mixture was sonicated in bath sonicator (37 kHz) for 15 min at room temperature. The sizes of the vesicles were determined using Malvern Zetasizer Nano-ZS90 instrument.

**Author contributions**—S. M. provided the direction of research. H. M. S. and S. M. designed the experiments. H. M. S. conducted the experiments and analyzed the results. H. M. S. and S. M. wrote the paper.

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