Cell adhesion on amyloid fibrils lacking integrin recognition motif

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Keywords: Amyloids, adhesion, protein self assembly, integrin, focal adhesions.

Abstract

Amyloids are highly ordered, cross-β-sheet rich protein/peptide aggregates associated with both human diseases and native functions. Given the well-established ability of amyloids in interacting with cell membranes, we hypothesize that amyloids can serve as universal cell adhesive substrates. Here, we show that, similar to the extracellular matrix protein collagen, amyloids of various proteins/peptides support attachment and spreading of cells via robust stimulation of integrin expression and formation of integrin-based focal adhesions. Additionally, amyloid fibrils are also capable of immobilizing non-adherent red blood cells through charge-based interactions. Together, our results indicate that both active and passive mechanisms contribute to adhesion on amyloid fibrils. The present data may delineate the functional aspect of cell adhesion on amyloids by various organisms and its involvement in human diseases. Our results also raise the exciting possibility that cell adhesivity might be a generic property of amyloids.

Introduction

The extracellular matrix (ECM) composed of proteins, minerals, and carbohydrates (1) provides physical support to individual cells and facilitates interactions between cells and links them into functional tissue (2). Depending on the source of the tissue, difference in the composition and organization of the ECM proteins dictate the physical properties of tissues. For example, bones consist of highly mineralized ECM that support and resist compression (3) while skin has dense interpenetrating matrix, which is elastic, strong, and regenerative (4). Cells within the tissue sense
the physicochemical properties of the ECM and bind to each other through a gamut of different cell adhesion molecules (CAMs), including integrins, cadherins and trans-membrane proteoglycans, and regulate their own functions (5). These interactions between cell and its surrounding ECM through integrins and other receptors directly control cell adhesion, proliferation and tissue organization (6).

Amyloids are unique protein folds responsible for both disease and function in host organisms (7). Even though the initial identification of amyloids emerged from their association with several human diseases including Alzheimer’s and Parkinson’s, many amyloids with native functions in host were also discovered from unicellular to multicellular organisms including mammals (7,8). For instance, in many organisms like E. coli, yeast and barnacle, amyloids help the host organism for their adhesion to surfaces (9-12). Recently in mammals, amyloids have been found to have functional role in melanin synthesis and hormone storage (13,14). Due to their self-replication capability (15,16), and their function as biological and chemical catalysts (17), amyloid folds are now considered as one of the primitive protein folds (18,19). Amyloid fibrils possess highly repetitive structure along with unique surface properties consisting of a combination of charged and hydrophobic surfaces depending on the sequence, making them sticky (20-22) and thereby enabling them to bind to both small molecules, as well as, large macromolecules/polymers (23-26). Various microorganisms, for their surface attachment and colonization, exploit this sticky property of amyloids. Amyloid fibrils have been reported to be a part of the biofilms in numerous microorganisms including harpins of X. campestris and P. syringae (27), pil from M. tuberculosis (28), chaplins from S. coelicolor (29) and hydrophobins from fungi (30). These amyloidogenic ECM components help these organisms to adhere on to surface and form colonies. The above studies suggest that amyloids possess many ECM-like features and may be capable of supporting cell adhesion. In this context, amyloid fibrils functionalized with cell adhesive RGD motifs were shown to support cell adhesion (31,32). Recent studies also suggest that amyloid fibrils alone (without any functionalization) are also capable of supporting cell adhesion owing to their unique nanotopographic features (33-37). However, it remains unclear if this cell adhesive property is dependent on the sequence composition, or is a consequence of the amyloid nature.

Here we demonstrate that irrespective of the sequence, amyloid fibrils are capable of supporting cell adhesion.

**Experimental procedures**

**Chemicals and reagents**- Unless specified, all chemicals and reagents were purchased from Sigma. Water was double-distilled and deionized using a Milli-Q system (Millipore Corp., Bedford, MA). All of the peptide hormones except human galanin and somatostatin were a kind gift from Prof. Roland Riek, ETH Zurich. Somatostatin was purchased from BACHEM and human galanin peptides was custom synthesized by USV Limited (Mumbai, India) with >95% of purity. Purity of all these peptides was further confirmed by MALDI-TOF mass spectrometry.

**Peptide/protein fibril formation**- To test the adhesion of cells on amyloid fibrils, the amyloid fibrils were prepared by dissolving the peptides of kassinin (2 mg/ml), GLP 1(0.25 mg/ml), rUCN (2 mg/ml), oCRF (2 mg/ml), glucagon (2 mg/ml), GIP (2 mg/ml), mUCN III (2 mg/ml) and Aβ(25-35) (1 mg/ml) in 5% D Mannitol with 0.01% sodium azide and incubated at 37°C with slight rotation. The peptides of somatostatin (2 mg/ml), hGRF (2 mg/ml), bombesin (2 mg/ml), VIP (2 mg/ml), helodermin (2 mg/ml), GRP (2 mg/ml), galanin (1 mg/ml) and Sub P (1 mg/ml) were also similarly dissolved in 5% D Mannitol with 0.01% sodium azide and incubated at 37°C with slight rotation. The peptides of somatostatin (2 mg/ml), hGRF (2 mg/ml), bombesin (2 mg/ml), VIP (2 mg/ml), helodermin (2 mg/ml), GRP (2 mg/ml), galanin (1 mg/ml) and Sub P (1 mg/ml) were also similarly dissolved in 5% D Mannitol with 0.01% sodium azide and incubated in presence of 400 μM LMW heparin at 37°C in 1.5 ml eppendorf tubes. α-synuclein (α-Syn) protein was expressed and purified according to the protocol described by Volles and Lansbury (38) in E. coli BL21 (DE3) strain. For α-Syn, 30 mg/ml of lyophilized protein was dissolved in 20 mM MES buffer, pH 6.0 and LMW α-Syn was prepared by passing the dissolved protein through 100 kDa cut off membrane as described before (39). The eppendorf tubes containing peptide/protein solutions were placed into an EchoTherm model
RT11 rotating mixture (Torrey Pines Scientific, USA) at 50 rpm inside a 37°C incubator. At suitable intervals, ThT, CD and EM were performed to analyze the aggregation.

**Circular dichroism spectroscopy (CD)** - CD is a commonly used technique to monitor the secondary structural transitions during protein/peptide aggregation studies (40). To study the conformational changes during the aggregation of protein/peptides, 15 μl of peptide solutions were diluted in 5% D-Mannitol to 200 μl such that the final peptide concentration was of 20 μM. For α-Syn, 10 μl of protein solution was diluted in 20 mM MES buffer, pH 6.0 to 200 μl such that the final concentration was of 15 μM. The protein/peptide solution was placed into a 0.1 cm path-length quartz cell (Hellma, Forest Hills, NY) and the spectra were acquired using a JASCO 810 instrument. All measurements were done at 25°C. Spectra were recorded over the wavelength range of 198-260 nm. Three independent experiments were performed with each sample. Raw data were processed by smoothing and subtraction of buffer spectra as per manufacturer’s instructions.

**Thioflavin T (ThT) binding** - ThT is an amyloid detection dye widely used to probe amyloid formation during protein aggregation (41). In order to track amyloid formation in the aggregating mixtures of protein/peptides, 10 μl aliquot of peptide/protein samples were diluted to 500 μl in 5% D-Mannitol containing 0.01% (w/v) sodium azide such that the final concentration of the peptide/protein was of 8 μM. For α-Syn, 10 μl of protein solution was diluted to 500 μl such that the final concentration was of 6 μM. These solutions were then mixed with 2 μl of 1 mM ThT prepared in 10 mM Tris HCl pH 8.0. Fluorescence was measured immediately after addition of ThT. The fluorescence experiment was carried out using Horiba-JY (Fluoromax 4), with excitation at 450 nm and emission from 460-500 nm. The intensity values at 480 nm were plotted. Three independent experiments were performed for each sample.

**Electron microscopy (EM)** - To examine the morphology of the protein/peptide amyloid fibrils under EM, aliquots of peptide/protein samples were diluted in distilled water to obtain a final concentration of ~50 μM. The diluted solutions were spotted on a glow-discharged, carbon-coated Formvar grid (Electron Microscopy Sciences, Fort Washington, PA) and incubated for 5 min. The grids were then washed with distilled water, and stained with 1% (w/v) aqueous uranyl formate solution. Uranyl formate solution was freshly prepared and filtered through 0.22 μm sterile syringe filters (Millipore). EM analysis was performed using a FEI Tecnai G2 12 electron microscope at 120 kV with nominal magnifications in the range of 26,000 to 60,000. Images were recorded digitally by using the SIS Megaview III imaging system. At least two independent experiments were carried out for each sample. The images obtained were analyzed in image J to calculate fibril diameter.

**Aggregation and amyloid formation by BSA** - It was previously suggested that BSA could form amyloid fibrils at low pH and in the presence of high salt condition (42,43). To further examine the conditions required for BSA amyloid formation, lyophilized BSA (Sigma, USA) was dissolved in PBS, pH 7.4 with a concentration of 21 mg/ml and the concentration was determined by UV absorption measurement at 280 nm, considering the molar absorptivity of BSA as 43824 M⁻¹cm⁻¹. The 300 μM stock solution of BSA was used for the further study. The 300 μM of BSA and 5M NaCl stock solutions were used to search the optimal conditions for BSA amyloid formation. The concentration of BSA in the reaction was varied from 5 to 100 μM using 20 mM Glycine-NaOH buffer, pH 3.0 and the NaCl concentration was varied from 0 to 300 μM. All solutions were incubated at 65°C and the aggregation and amyloid formation was monitored using ThT binding studies (Data not shown). The condition which showed highest ThT binding was considered as amyloid forming condition and was used for further studies. The secondary structural changes were determined by CD spectroscopy and morphological analysis was done using electron microscope. Further amyloid formation was confirmed using CR binding by UV spectral assay and birefringence study.

**Congo Red (CR) Binding Assay** - CR is a dye routinely used for detecting amyloid formation in
protein/peptide aggregation studies (44). In order to confirm the amyloid nature of BSA aggregates, CR binding studies were performed. For CR binding, a 10 µL aliquot of aggregated BSA (of concentration 100 µM) was mixed with 160 µL of PBS buffer containing 10% ethanol. Then 30 µL of 100 µM CR solution in PBS (containing 10% ethanol) was added. After 5 min incubation in dark at RT, CR absorbance was measured in the range of 300 to 700 nm using JASCO V-650 spectrophotometer. Similarly, the CR alone spectrum was also recorded as a control. Three independent experiments were performed for each sample.

CR-birefringence- To further confirm the binding of CR to amyloid fibrils, CR birefringence was performed. 50 µL of incubated solutions of protein/peptides aggregates was subjected to ultracentrifugation (Optima Max-XP, Beckman Coulter, US) at 90,000 rpm for 1 hr and was washed once by distilled water. The pellets obtained were then used for performing CR birefringence. To do that, pellets were stained with CR solution for 20 min while continuous vortexing. The mixtures were again centrifuged at 90,000 rpm for 1 hr and pellets were washed twice with 500 µL of 20% ethanol. The pellets were re-suspended in MQ water and then spread evenly onto glass slides and air-dried at room temperature. The slides were analyzed using a microscope (Olympus SZ61 stereo zoom) equipped with two polarizer equipped with a CCD camera.

Amyloid formation by poly aminoacids- The preparation of poly amino acid (PAA) amyloids were reported previously (45-47). Briefly, the 1 mg of lyophilized powder of positively charged poly-L-Lysine (PLL) (MW 70,000-150000, Sigma, USA) and negatively charged poly glutamic acid (PE) (MW 2000-15000, Sigma, USA) were separately dissolved in 1 ml of sterile MQ. The pH of PLL solution was adjusted to 11.1 by adding few drops of 10 mM NaOH and pH of PE was adjusted to pH 4.1 with dilute HCl. These freshly prepared PAA were then incubated at 65°C for 48 hrs. For preparing poly-L-Alanine (PLA) (MW 1000-5000, MP Biomedicals, USA) amyloid, 2 mg of PLA was dissolved in 100 µL of trifluoroacetic acid (TFA) as it does not dissolve in most of the other solvents studied due to the highly hydrophobic nature of PLA. 10 µL of PLA solution was then gradually added into 190 µL of sterile MQ casted on a clean coverslip and the entire set up was dried in steam. The resultant dried aggregates were collected and was used for the study. The secondary structure of PAA aggregates were analyzed using FTIR and morphological analysis was done using electron microscope. Further amyloid formation was confirmed using CR binding by birefringence study and X-ray diffraction studies.

For secondary structural analysis of the aggregated PAA, FTIR spectroscopy was performed. FTIR spectra of the PAA were recorded using Vertex-80 FTIR instrument (Bruker, Germany) equipped with DTGS detector. 10 µL of each aggregated PAA was spotted and dried on translucent KBr pellet that was made prior to the experiment by compressing ground KBr powder. 10 µL of sterile MQ was spotted on another KBr pellet and used for background spectrum. FTIR spectra (1500-1800 cm⁻¹) were recorded as an average of 32 scans and raw data corresponding to amide-I region (1600-1700 cm⁻¹) was deconvoluted by Fourier self deconvolution (FSD) method. The deconvoluted spectra were then subjected to Lorenzian curve fitting procedure using opus-65 software. Two independent experiments were performed for each sample.

For X-ray diffraction studies, sample solutions were either dried in glass capillary tubes or at the center of two glass rods arranged end to end. The resulting film was mounted parallel to X ray beam. The images were obtained using Rigaku R Axis IV++ detector (Rigaku, Japan) mounted on a rotating anode. The sample to detector distance was 300 mm and the image files were analyzed using Adxv software.

Amyloid formation by Aβ fibrils- Lyophilized powder of Aβ42 was a kind gift from Prof. Sudipta Maiti, TIFR, India. 1 mg of Aβ42 fibril was suspended in 50 mM sodium phosphate buffer, pH 7.4 and the peptide was dissolved by adding few drops of 10 mM NaOH into it. After which the pH of the solution was brought back to pH 7.4. The final concentration of this solution was 300 µM.
Aβ(25-35) was purchased from BACHEM. For amyloid fibril preparation, 0.5 mg of this peptide was dissolved in 500 µL of sterile MQ to obtain 1mM solution. Both of the Aβ solutions were incubated at 37°C and the aggregation and amyloid formation was monitored using ThT binding studies. The secondary structural changes were determined by CD spectroscopy and morphological analysis was done using electron microscope.

**Biocompatibility Assay** - The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was carried out to evaluate the toxicity of all amyloid fibrils formed in presence and absence of heparin using neuronal cell line SH-SY5Y. Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) (HiMedia, India) medium supplemented with 10% FBS (Invitrogen), 100 units/ml penicillin and 100 µg/ml streptomycin in a 5% CO2 humidified environment at 37°C. Cells were seeded at a density of 1x10⁴ cells per well on a 96-well plate. After 24 hours of incubation, the old culture media was replaced with fresh media along with the addition of various amyloid fibrils of 10 µM concentrations and cells were further incubated for 24 hours at 37°C. After incubation, 10 µl of a 5 mg/ml MTT stock in PBS was added to each well and the incubation was continued for 4 hours. Finally, 100 µl of a solution containing 50% dimethylformamide and 20% SDS (pH 4.7) was added to each well and incubated. After overnight incubation in a 5% CO2 humidified environment at 37°C, absorption values at 560 nm were determined with an automatic micro plate reader (Spectramax M2, Molecular Devices).

**Surface functionalization** - For evaluation of cell adhesion on amyloid fibrils, the fibrils were coated on glass cover slips. To do so, round glass coverslips of 12 mm diameter were first washed with 0.1 N NaOH for 30 minutes, then rinsed with PBS and subsequently sterilized. All coverslips were kept in 24 well plates and coated subsequently with different substrates. Glass coverslips were coated with 10 µg/cm² of collagen and each amyloid fibril. For coating, each fibril solution was diluted in PBS, pH 7.4 and applied to the surface of the coverslip and kept at 4°C overnight. Next day, excess solution was removed and the surfaces were washed with PBS, before plating the cells.

**Cell Culture** - Mouse fibroblasts (L929 and NIH 3T3), neuroblastoma cell SH-SY5Y and PC12 cells were obtained from NCCS (Pune, India) and maintained as per established protocols. All cells were maintained at 37°C with 5% CO₂ and plated at a density of 4 x 10⁴ per well.

For density dependent experiments, glass coverslips were coated with kassinin at concentrations ranging from 0.5 to 7.5 µM at 37°C for 2 hours, blocked with 2% F127 Pluronic (Sigma) for 15 min at room temperature to prevent non-specific cell attachment and UV sterilized. NIH 3T3 fibroblasts were cultured for 24 hours and plated at a seeding density of 4500 cells/cm² on the coated substrates. For drug studies, cells were cultured for 24 hours and then exposed to 10 µM (for NIH 3T3) and 1 µM (for SH-SY5Y) Y27632 or ML7 (Calbiochem) for 1 hour. For integrin blocking experiments, ~80 cells/µl of media was treated with anti-integrin β1 antibody (1:100, clone P4C10, Millipore) for 15 min at 37°C, and subsequently plated on fibril-coated substrates. Cells were allowed to settle for 1 hour at 37°C and processed for imaging.

For RGD mediated integrin blocking experiments, the 48-well plates were coated with fibronectin (5 µg/cm²), collagen, monomer and fibril of Kassinin at a density of 10 µg/cm² and was incubated for overnight at 4°C. On removing the solution, these wells were blocked with 2% pluronic for 15 minutes and UV sterilized. NIH 3T3 cells were harvested using 0.5% trypsin, seeded at a density of 4000 cells/cm² and incubated for 2.5 hrs in DMEM containing 10% FBS at 37⁰C with 5% CO₂. After incubation, the cells were washed with 1x PBS and incubated with 0.2 mg/ml GRGDSP (Sigma, USA) in DMEM for 2.5 hrs at 37⁰C after which the floating cells were removed using a PBS wash. All the wells were imaged at 10x magnification and cells from 10 random fields were counted and the cell spread area for each condition was analyzed.

**Quantification of cell shape and cell area** - For evaluation of cell adhesion, the glass coverslips in
24 well plates were gently rinsed with PBS to remove unattached cells. Subsequently the coverslips were imaged in phase contrast (Olympus XL51/IX71) at 10 X magnifications. The cell-shape analysis was performed using Image J software (NIH). Cell morphology (spread area and circularity) was quantified by analyzing approximately 100 cells per condition from two independent experiments.

For cell migration studies, cells were cultured on 35 mm petri dishes with culture media supplemented with 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.2. Petri dishes were placed on a custom-made temperature controller maintained at 37°C. Phase contrast images were captured at 10 min intervals under 20 X magnification for 3 hours using Nikon Ti Eclipse inverted microscope coupled with NIS Elements BR (Ver 4.20.00) image acquisition software. All the frames were analyzed using the manual tracking plugin in Image J (NIH). For quantifying cell speeds, at least 30 cells were analyzed per condition across two independent experiments.

**Immunocytochemistry**- To study the focal adhesion complex formation on adhered cells, immunocytochemistry was performed. To do that, cells were cultured on different substrates and after 24 hour, cells were washed with PBS and fixed with 4% paraformaldehyde for 15 minutes at 37°C. After rinsing with PBS, cells were permeabilized by 0.2% Triton X-100, blocked with 5% BSA, and incubated with rabbit anti-FAK (Sigma, 1:350 dilution) / mouse anti-β1 integrin (Santa curz, 1:250 dilution) antibody at 4°C overnight for visualizing focal adhesions. After washing off the primary antibody, cells were incubated either with donkey anti-rabbit secondary antibody or donkey anti-mouse secondary antibody (Molecular Probes, 1:500 dilution) and Phallodin (Molecular Probes, 1:300 dilution) for 1 hour at room temperature. Subsequently, samples were washed with PBS and stained with DAPI for nuclear staining (Molecular probes, Invitrogen). Finally, the coverslips were mounted using Cytoseal (Sigma) and observed under fluorescence microscope (Zeiss Axiocam MRM). The distributions of focal adhesions/integrins were processed and calculated using Image J software (NIH).

**RT-PCR**- For studying the integrin expression profile of cells grown on various substrates, RT-PCR experiments were performed. For this, 35 mm petri dishes were coated with 10 µg/cm² of each amyloid /ECM substrate and kept for overnight incubation at 4°C. Next day, excess solution was removed and the surfaces were washed with PBS before plating the cells. One million cells per dish were plated on each condition and cells were harvested for RNA extraction after 24 hrs in culture. RNA was extracted by the Trizol (Ambion, US) method according to the manufacture’s protocol. cDNA preparation was carried out using first strand cDNA synthesis kit from Fermentas (Thermo Scientific, US) and PCR was performed using kit from Novagen ( Millipore, US) according to manufacturer’s instructions. GAPDH primers were used from the same kit. Integrin primers were custom manufactured by Sigma with the following sequences:

β1 integrin Forward 5’- ACG CCG CGC GGA AAA GAT GA - 3’
Reverse 5’- GCA CCA CCC ACA ATT TGG CCC T - 3’

β3 integrin Forward 5’- GGG GAC TGC CTG TGT GAC TC - 3’
Reverse 5’- CTT TTC GGT CGT GGA TGG TG - 3’

**RBC adhesion study**- For experiments with red blood cells (RBCs) (48), 1 µl packed blood was collected from healthy individual and added to 1.5 ml of 1 X phosphate buffer saline (PBS, pH 7.4) and seeded on pre-coated wells at a seeding density of 30000 cells/well in a 96 well plate. The wells that coated with 0.01% poly-L-lysine and 2% pluronic served as the positive and negative controls, respectively. After seeding the wells with desired number of cells, the plates were incubated at RT for 30 min and taken for imaging. RBC adhesion was quantified by imaging 10-12 fields per well at 40 X magnifications. The morphology of the cells was analyzed and representative images were used for calculating spreading area.
The image analysis was done by Image J software (NIH).

De-adhesion experiments- De-adhesion experiments were conducted as reported previously (49). Briefly, cells were mounted on the microscope stage (Olympus), washed with 1X PBS, incubated with 0.25% trypsin-EDTA (HiMedia, India) and images were captured at 2 second interval at 10 X magnification until the cells rounded up but remained attached to the substrate. Images were processed in Image J (NIH) to obtain the experimental de-adhesion time. For quantifying the influence of fibril density on de-adhesion time, at least 30 cells across two independent experiments were analyzed.

Statistical analysis- The statistical significance was either determined by student’s t test or one-way ANOVA followed by Newman-Keuls Multiple Comparison post hoc test; *P and **P value for each plot is mentioned in the corresponding legend.

Results

Amyloids support mammalian cell adhesion- To address whether amyloid fibrils, irrespective of their sequence and composition, can support cell adhesion, more than 20 proteins/peptides possessing different sequences and length were chosen. These peptides were also previously shown to form non-toxic amyloids in vitro, many of which is suggested to be the storage state in secretory granules (14,50,51). Since most of these proteins/peptides were non-toxic, we chose these peptides for our cell adhesion study. All the peptides were found to be unstructured in solution immediately after dissolution (day 0) as observed by CD. However, after 15 days of incubation, all of them underwent structural transition predominantly to β-sheet-rich structure except for Sub P and somatostatin (Fig. 1). Sub P and somatostatin were previously reported to form unusual structures after amyloid formation (14,50,52). Amyloid formation was probed using thioflavin (ThT) fluorescence study. ThT is an amyloid detecting dye, which mostly binds to cross-β-sheet rich amyloids but not their monomeric counterparts (41). An increase in ThT binding of two weeks incubated protein/peptides suggests amyloid formation by these protein/peptides (Fig. 2). Only kassinin did not show any significant ThT binding even after 15 days of incubation (almost similar ThT fluorescence at day 15 as of day 0 sample) (Fig. 2). This observation is consistent with previous study (52). The negligible ThT binding even after amyloid formation was also evident previously for many other proteins/peptides (53-55). The amyloid morphology of the aggregates was confirmed using electron microscopy, wherein all the peptide/protein aggregates showed fibrillar morphology (Fig. 3). Thus the fibril formation was verified by monitoring aggregation using circular dichroism (CD) spectroscopy, binding to amyloid specific dye Thioflavin T (ThT) (except for kassinin) and TEM imaging (Table 1). Finally, the toxicity of different fibrils was evaluated using MTT assay. Most of the fibrils were found to be nontoxic to cells consistent with the previous study (14) (Fig. 4). Only few peptide fibrils showed higher than 30% cellular toxicity. The adhesiveness of the different fibrils was probed by plating equal number of SH-SY5Y cells on coverslips coated with fibrils at identical density, and quantifying cell attachment and spreading. Coverslips coated with the ECM protein collagen I at identical density served as control. After 24 hours in culture, while cell attachment was observed across all the conditions (Fig. 5), higher attachment was observed on many of the amyloid fibrils compared to that on collagen (Fig. 6A). In particular, cell attachment was observed nearly 1.5 fold higher on kassinin fibrils compared to that on collagen. Further the cell spreading area was calculated using Image J software to evaluate the cell adhesion on various surfaces. The study showed that on most of the amyloid fibrils, cell spreading was as efficient as that on collagen, with maximal spreading observed on β-endorphin (β-end) and bombesin fibril-coated surface (Fig. 6B). When we compared the cell spreading on monomer and fibril surfaces, we observed cell spreading was significantly higher on fibrils than their corresponding monomeric counterpart (Fig. 6B). Moreover, we also found that adhesion to amyloid fibrils was not specific to SH-SY5Y neuronal cells, but was also observed with other cell types including L929 fibrosarcoma cells,
NIH 3T3 fibroblasts and PC12 neuronal cells (Fig 7A-B). Though these cells were able to adhere and spread on kassinin amyloid fibrils similar to collagen, differences in spreading area was present among the different cell types (Fig 7B). This difference is could be due to differences in cell lineage and their different substrate requirement. However, the study indicates that the favorable response of cells to the amyloid surface and suggests that adhesion to amyloid fibrils is not cell type specific, rather generic for most mammalian cell types.

While the above studies were done at constant coating density, to understand the influence of fibril density on cell spreading and motility, NIH 3T3 fibroblast cells were seeded on varying coating density of fibril created by dilution of the kassinin fibril stock. The study suggests that similar to the effect of ECM density on cell spreading, 3T3 fibroblasts exhibited a biphasic spreading response on coverslips coated with kassinin at varying fibril density with maximal spreading was observed at a coating density of 1.5 μM (Fig. 8A upper panel and Fig. 8B).

Further, to probe the role of serum protein in cell adhesion on amyloids, glass coverslips were coated with varying density of kassinin fibrils and 3T3 fibroblasts were cultured on them in serum free media. In serum free media, cells attached but remained rounded, suggesting that while the amyloid nano-topography is sufficient for initial cell adhesion (36), the serum proteins are required for cell spreading (Fig. 8A lower panel and Fig. 8B). Further, random cell motility on these substrates closely mirrored the cell spreading response with maximum migration speeds observed on the same coating density corresponding to maximal spreading (Fig. 8C).

Lipid-fibril interactions and integrin-based adhesions contribute to cell spreading on amyloid fibrils- To obtain further insights into the attachment and spreading of cells on amyloid surfaces, cytoskeletal organization and focal adhesion structures (56) were tracked in SH-SY5Y cells cultured on collagen and kassinin-coated substrates at identical coating density. Although cytoskeleton is important for cell shape and their spreading on ECM matrix (57), the extent and size of the focal adhesion complex will dictate the robustness in adhesion of cell on the surfaces (58). Our data showed that while no drastic differences were observed in the pattern of stress fibers visualized by F-actin staining, a difference in focal adhesion kinase (FAK) staining was observed in cells attached on collagen and amyloid surfaces (Fig. 9A). FAK is known to be involved as a primary integrin effector and is considered as a measure of cell spreading and migration (59-61). FAK and other focal adhesion proteins show a well distributed cytoplasmic staining pattern in adhered cells (62-64). Similar distribution of FAK was also observed in our study for the cells cultured on amyloid surfaces indicating strong cell adhesion. In contrast, a strong perinuclear FAK staining was observed in cells cultured on monomers of these peptides (Fig. 9B). Further, quantitative analysis of FAK-positive focal adhesions of cells on collagen and amyloid surfaces using Image J software revealed the prevalence of larger adhesion complexes on selected amyloid fibrils of kassinin and Sub-P compared to their corresponding monomers and on collagen (Fig. 9C). The presence of more and larger FAK positive adhesions and higher cell spreading area in cells cultured on amyloid fibrils, suggest the attachment of cells is integrin mediated.

To directly study the role of integrin in cell adhesions on amyloid fibrils, expression levels of β1 and β3 integrins were studied. The expression of β1 and β3 integrins and the localization of β1 integrin at focal adhesions in SH-SY5Y cells were found to be higher on kassinin amyloid surfaces (Fig. 9D-F). To further probe if integrin engagement is a consequence of attachment to amyloid fibrils or is required for cell spreading on amyloids, integrin blocking experiments were performed wherein fibroblasts were incubated for 30 mins with integrin blocking antibody P4C10 and then plated on collagen and kassinin amyloid substrates. After 1 hour of incubation, cell spreading was reduced by ~50% in P4C10-treated cells compared to untreated controls on both collagen and kassinin substrates (Fig. 10A, B).
The data indicate that β1 integrins at least partly modulate cell spreading on amyloid fibrils similar to collagen.

To further confirm the role of integrins in cell adhesion, we studied the effect of RGD, an integrin ligand to block cell adhesion of adhered cells (65). RGD has a high affinity towards several integrin receptors and plays a major role in mediating cell adhesion (66-68). Treating adherent cells with RGD can temporarily disengage integrins and cause cells to de-adhere (Fig 10C) (69). In these experiments, 3T3 fibroblasts were first allowed to adhere and spread on monomeric and amyloid forms of kassinin coated surfaces for 2.5 hours. Cells cultured on coated surface of collagen and fibronectin was used as positive controls. Subsequently, the adhered cells were treated with 200 µg/mL GRGDSP peptide for another 2.5 hours. We found that more than half of cells de-adhered after this treatment when compared to the control where no RGD was added (Fig 10 D-F). Additionally, for the cells that remained attached to the substrate, cell spread area was also reduced considerably when compared to untreated cells. The mean cell spreading area of RGD treated cells adhered on kassinin amyloid fibrils was less than that of cell adhered on kassinin monomers (Fig 10 D-F). Together, these results demonstrate the role of integrins in mediating cell adhesion and spreading on amyloid surfaces.

Further, to test if amyloids can facilitate any mode of adhesion other than integrin based cell adhesion, experiments were performed with human red blood cells (RBCs), which lack any integrin machinery (70,71). Kassinin fibril coated substrates were incubated with freshly isolated RBCs for 30 mins, given a brief wash, and processed for imaging to check for the attachment (Fig. 10G). Pluronic (Plu) and poly-L-lysine (PLL) coated substrates were used as negative and positive controls, respectively. While attachment on pluronic-coated substrates was negligible, cells attached robustly on PLL-coated substrates as expected, through charge-based interactions (71). Interestingly, RBCs attached to kassinin substrates in a fibril density-dependent manner (Fig. 10H), with attachment increasing with fibril density (Fig. 10I). Since RBCs are devoid of integrins, we propose that attachment of RBCs on amyloid could occur through cell membrane-amyloid interactions. Collectively, these data suggest that amyloids are capable of supporting the attachment of both adherent and non-adherent cells through a combination of membrane-fibril interactions and integrin based focal adhesions.

Rho kinase signaling modulates cell contractility on amyloids- Given that the focal adhesions were observed in adherent cells both on collagen and on amyloid fibrils, we next probed if the downstream signaling pathways that modulate focal adhesion dynamics were similar on collagen and amyloid fibrils. Specifically, we focused our attention on Rho-associated kinase (ROCK) and myosin light chain kinase (MLCK), both of which regulate cellular processes by modulating the activity of myosin II (72). To delineate the contributions of ROCK and MLCK pathways, experiments were performed with NIH 3T3 fibroblasts and SH-SY5Y cells cultured on collagen and kassinin amyloid fibrils in the presence of the ROCK inhibitor Y-27632 and the MLCK inhibitor ML7. For NIH 3T3 fibroblasts, ML7 and Y-27632 treatment led to significantly reduced cell spreading on kassinin fibrils, but not on collagen (Fig. 11A-B). However, drastic alterations in cell shape were observed in Y-27632-treated cells on both kassinin fibril and collagen substrates.

Next, to test the relative contributions of MLCK and ROCK pathways to cell contractility, trypsin-induced de-adhesion experiments were performed with SH-SY5Y and NIH 3T3 cells, where both cells were incubated with warm trypsin and imaged under a phase contrast microscope till cells become rounded up but remained attached to the substrate (Fig. 11C), with the rounding off times directly set by actomyosin contractility (49). Interestingly, while both Y-27632 and ML7 treatment led to delayed de-adhesion on collagen substrates, only Y-27632 treatment led to delayed de-adhesion on kassinin substrates, with ML7 treatment causing no difference (Fig. 11D-E). In SH-SY5Y neuronal cells, similar to NIH 3T3 fibroblasts, Y-27632 treatment also inhibited spreading of cells on collagen and kassinin substrates. However, ML7 treatment had no effect on cell spreading on both collagen and kassinin substrates (Fig. 12A-B). Moreover, the cell
contractility experiments on SH-SY5Y cells adhered on amyloid fibrils and collagen also showed similar results to that of NIH 3T3 cells (Fig. 12C) suggesting cell adhesion on amyloid fibrils might be modulated by common signaling pathways in different cell lines. Together, these results indicate that while relative contributions of ROCK and MLCK pathways to cell contractility differ on collagen and amyloids, both are involved in cell spreading on collagen and amyloids.

**Amyloid version of a non-adhesive protein is also capable of supporting cell adhesion** - Since most of the peptides used in our studies do not possess direct integrin binding sites, then how do amyloid fibrils modulate these complex cellular processes in such a robust manner? We hypothesize that the amyloid form of any peptide/protein and higher order cross-β-sheet organization is sufficient to support cell adhesion. This may be achieved through both membrane-fibril interactions and integrin signaling. To probe this directly, cell studies were performed on monomer and amyloid forms of bovine serum albumin (BSA), which in its monomeric form, is routinely used to prevent non-specific adhesion (73). To prepare BSA amyloids, 300 µM of BSA was freshly prepared in PBS pH 7.4 and the solution was further diluted to 100 µM in 20 mM glycine-HCl buffer pH 3.0. The solution was treated with 300 mM NaCl and incubated at 65°C for 1 hr for BSA fibril formation. As before, formation of BSA fibrils was monitored using CD, ThT and CR binding and electron microscopy (Fig. 13). The freshly dissolved BSA monomer showed CD spectrum characteristics of helical structure with two distinct minima one at 220 nm and other at 208 nm. Whereas BSA incubated with 300 mM NaCl for 1 hr at 65°C showed spectrum with a single minimum at ~218 nm indicating the presence of β-sheet (Fig. 13A). The amyloid formation by BSA was further analyzed by ThT and Congo red binding studies. Both ThT and CR are known to bind mostly to the amyloid form of the protein not to the corresponding monomeric counterpart (14). BSA aggregate formed in 20 mM glycine-HCl, pH 3.0 in presence of NaCl showed higher ThT fluorescence at 480 nm compared to its monomer (Fig. 13B). Moreover, the BSA aggregates formed in presence of 300 mM NaCl at pH 3.0 showed an increase in CR absorbance (Fig. 13C) and these aggregates showed green-gold birefringence when observed under cross-polarized light, which is typically observed in β-sheet rich amyloid aggregates (Fig. 13D). Finally, the morphology of these BSA aggregate was analysed using electron microscopic studies, which showed long fibrillar aggregates. However, BSA monomer showed only small amorphous aggregates (Fig.14A, upper panel). Thus the CD spectra, ThT and CR binding study and the fibrillar morphology of the incubated BSA aggregates under TEM are indicative of amyloid formation by BSA in the presence of salt at low pH.

Cell studies performed with SH-SY5Y cells showed 3-fold higher cell adhesion on BSA fibril surfaces compared to that on the monomeric surfaces (Fig. 14A-B). On BSA coated surfaces, cell spreading was also greater on fibril surfaces compared to that on their corresponding monomers (Fig. 14C). In line with increased attachment and spreading on BSA fibrils, mRNA expression analysis also revealed higher expression of β1 integrins on BSA fibrils (Fig. 14D). Further, the co-staining of SH-SY5Y cells with FAK and β1 integrin revealed greater number of co-localized clusters on the fibril surfaces compared to the monomeric surfaces (Fig. 14E) suggesting the formation of active focal adhesion complexes. These results suggest that amyloid form of a non-adhesive protein can also support cell adhesion.

**Relative contribution of charge and amyloid form on cell adhesion** – Further, to probe the role of cross-β structure in cell adhesion and also to decouple structure dependence and charge on cell adhesion, we expanded our study to three different charged poly amino acids (PAAs) and their amyloid counter parts. To do so, we used positively charged poly-L-Lysine (PLL), negatively charged poly glutamic acid (PE) and neutral poly-L-Alanine (PLA). All of these PAAs are previously reported to form amyloid in vitro (45-47).

The formation of PAA fibrils was characterized using FTIR (for secondary structure), electron microscopy (morphology), CR birefringence and X-ray diffraction. The freshly dissolved PLL and PE showed random coil structure in CD (data not shown). To examine the secondary structure of the
incubated PAAs, FTIR spectroscopy of these aggregates were performed. (74). For the analysis of peptide secondary structure, FTIR spectra in the range of 1600 cm\(^{-1}\) to 1700 cm\(^{-1}\) (amide-I band) were deconvoluted and curve fitted. The FTIR spectra of incubated PAAs showed peaks in the range of 1640 cm\(^{-1}\) to 1650 cm\(^{-1}\) (amide-I band) corresponding to random coil conformation. In addition, the incubated PAAs also showed peaks at 1630 and 1617 cm\(^{-1}\) for PLL, 1633 and 1623 cm\(^{-1}\) for PE and 1631 and 1615 cm\(^{-1}\) for PLA indicating β-sheet structure of incubated PAA (Fig. 15A). Further, the morphology of the aggregates was analyzed using TEM, which revealed fibrillar morphology for all the aggregated PAAs. However, the fibril morphologies were different for each of the PAA (Fig. 15B). To confirm the amyloid nature of these aggregates, CR birefringence was performed. Each of the PAA fibrils showed a greenish yellow birefringence under cross-polarized light further confirming the amyloid nature of the PAA fibrils (Fig 15C). In each of these experiments α-Syn is associated with amyloid fibrils, which reveals that integrins are expressed more in cells attached to PLL amyloid fibrils (Fig 16E) compared to monomer. These findings clearly indicates that integrins play a significant role in amyloid mediated cellular adhesion and suggests that even though charge of the peptide/proteins helps in adhesion, the cross-β structured state further promotes stronger adhesion.

**Cell adhesion on amyloids derived from the disease-associated protein/peptide-** While the above experiments illustrate the cell adhesive property of a range of different non-toxic amyloid fibrils, it remains unclear if amyloids associated with human diseases also possess this cell adhesive property. To study cell adhesion on disease amyloids, adhesivity of α-synuclein (α-Syn), Aβ42 and Aβ(25-35) fibrils was assessed (Fig. 17A). While α-Syn is associated with Parkinson’s disease (77), Aβ42 is associated with Alzheimer’s disease (78). Aβ(25-35) is a short peptide derived from Aβ42 that shows similar toxicity to the full length Aβ42, and hence used as a model peptide to study Aβ toxicity in cells (79). When SH-SY5Y cells were cultured on α-Syn, Aβ42 and Aβ(25-35) fibril coated surface for 24
hours, cell survival was found to be more on α-Syn and Aβ42 fibrils compared to Aβ(25-35) fibrils (Fig. 17B). Moreover, SH-SY5Y cells (that survived) exhibited greater spreading on α-Syn fibrils than that on collagen, Aβ42 and Aβ(25-35) fibrils (Fig. 17C). While β1 integrin expression and localization in cells cultured on α-Syn fibrils was similar to that on collagen (Fig. 17D, E), in cells that survived on Aβ(25-35) fibrils, integrin staining was found to be very diffused.

Our cell adhesion data show that cells were able to adhere and spread on Aβ42 fibrils coated surfaces. This result is in contrast with cell adhesion on Aβ(25-35) fibril coated surface, where very less cells were found to adhere. We used 10 µg/cm² Aβ42 fibrils for our study, which is ~ 4.5 µM of Aβ42 fibrils per well. Interestingly, when 4.5 µM of Aβ42 fibrils were added directly to the media of attached cells, cell death was observed (Fig 16F). Such difference in cell viability when fibrils are either precoated or added in solution phase in media has also been reported for other amyloids (80). The reason for this phenomenon could be that the toxic epitopes such as fibril ends are exposed when Aβ42 fibrils are added to the media and could result in membrane damage and cell death (80,81). Alternatively, the precoating of Aβ42 fibrils on glass surfaces might also immobilize the fibrils and thereby restricting them from freely interacting with cell membrane and inducing toxicity. The Aβ(25-35) fibrils are short peptides derived from full length Aβ42 and forms short amyloid fibrils. Due to this, the toxic epitope might be more available even after coating on glass surface leading to the resultant toxicity. These results suggest that disease-associated amyloids also possess the cell adhesive property. Moreover, the data further indicates that nanotopography of amyloid fibrils (both disease and functional) could support cell adhesion.

Discussion

Adhesion to the ECM is required by cells not only for their proliferation, differentiation and survival but also for their organization into functional tissues (82). The ECM, an amalgamation of proteins secreted by cells, is composed primarily of a network of fibrils and components of the basement membrane (83). One of the common features shared by many ECM proteins with amyloids is their fibrillar nature. Moreover, amyloids possess a highly repetitive cross-β-sheet structure and a unique combination of charge and hydrophobic surfaces, which makes them sticky (22,84,85). It is perhaps these combinatorial features that enable amyloids to bind to membrane with high affinity (86). These properties of amyloids are utilized by lower organisms for their ECM formation (87). The fact that amyloid fibrils can behave analogous to ECM raises the exciting possibility that amyloids may have served as primitive cell adhesive substrates. Therefore, we hypothesize that amyloid fibrils, irrespective of the primary structure, can support cell adhesion.

We show here that irrespective of sequence, composition and absence of integrin binding sites, ~ 20 different amyloid fibrils support mammalian cell adhesion (Fig. 5). Interestingly, BSA, a cell repulsive protein (73), when converted into amyloid is also able to support cell adhesion (Fig 14). Moreover, the cell adhesion on PAA amyloids was higher than their corresponding soluble counterpart. Interestingly though, the neutral PLA was mostly incapable of supporting cell adhesion, its amyloid counterpart showed robust adhesion of cells (Fig 16A-C). Further, the increased expression of FAK and β1 integrin in cells cultured on PLL amyloid fibrils than the PLL monomer further decouple the role of amyloid fibril structure vs charge on cell adhesion (Fig 16D and E). Taken together, these studies demonstrate the universal cell adhesive capacity of amyloid fibrils. We therefore surmised that, similar to lower organisms, mammalian cells can also utilize the stickiness of amyloids for their adhesion. Both adherent and non-adherent cells can attach to amyloids through passive (i.e., lipid-fibril interactions) as well as active (i.e., integrin based) modes (Fig. 18). The initial adhesion of cells to amyloids could be spontaneous, consistent with their membrane binding property (86), and as observed here with adhesion of RBCs to kassinin amyloid fibrils. Subsequent to the initial immobilization of cells on amyloid surfaces, cell spreading and motility require integrin engagement and formation/turnover of focal adhesions (88,89). We have demonstrated this directly by quantifying integrin expression and

Discussion

Adhesion to the ECM is required by cells not only for their proliferation, differentiation and survival but also for their organization into functional tissues (82). The ECM, an amalgamation of proteins secreted by cells, is composed primarily of a network of fibrils and components of the basement membrane (83). One of the common
Localization at adhesion sites on amyloid fibrils similar to that on native ECM proteins (Fig. 9D-F). Further, in line with this, integrin-blocking experiment showed a marked reduction in cell spreading (Fig. 10A-F). Interestingly, in SH-SY5Y cells, focal adhesion formation was more robust on amyloid fibrils compared to collagen (Fig. 9A and C), suggesting that for some cell types, amyloid fibrils may be even more efficient than native ECM proteins in supporting cell adhesion.

It has been suggested previously that amyloids may mediate cell adhesion by accelerating and stabilizing the deposition of serum proteins (33,34). Though we observed a decrease in cell adhesion on amyloid surfaces in serum-free media, complete abolition of the same was not observed (Fig. 8A-B), suggesting that serum protein deposition is important but not necessary for cell adhesion on amyloid surfaces. Further, this implies that similar to ECM, amyloid fibrils can also entrap proteins and small molecules that can facilitate spreading and growth of the cell. In addition, we and others have recently demonstrated that 3D cell culture systems derived from amyloid networks can be used for long-term cell culture and stem cell differentiation (35,37).

Cell adhesion to the underlying matrix and to neighboring cells is mediated by transmembrane integrins (focal adhesions) (90), cadherins (cell-cell adhesions) (91,92), and various other proteins. These proteins enable cells to sense and respond to external stimuli. Rho-based signaling is one of the prominent signaling cascades associated with reorganization of adhesion structures and the cytoskeleton following such stimuli (56). In neuronal cells, the ROCK pathway is involved in synaptic plasticity and activates MLCK for stimulating retraction of axonal and dendritic growth cones (93-95). Our results suggest, that similar to that on ECM proteins (72,96), cell adhesion and spreading are mediated by both ROCK and MLCK pathways (Fig. 11-12).

Interestingly, we found that the disease related amyloids fibrils, Aβ42 (78,97) Aβ(25-35) (79) and α-Syn (77) also support cell adhesion (Fig. 15). However, this was found to be less on Aβ(25-35) as compared to α-Syn and Aβ42. Although both Aβ42 (78,97) and Aβ(25-35) fibrils are known to be neurotoxic (79,98-100), the differences in the cell adhesion on both of these coated surfaces (Fig 17) could be due to the availability of toxic epitopes (80). Moreover, both nanotopography and roughness of the coated fibrils can also play an important role for death/apoptosis of adherent cells on amyloid surfaces (80,81). Specifically, increased roughness of amyloid plaques was shown to directly influence the cell survival (101).

The discovery of non-toxic functional amyloids, and the ability of all proteins to form amyloids under specific conditions, suggests that amyloids possess a much broader function than only being associated with diseases (7). Further, functional amyloid formation in organisms is tightly regulated thus avoiding their possible toxicity (8,102). Together, these results suggest that while cell adhesivity of amyloid fibrils is a generic property, the physio-chemical properties of the amyloid fibrils and their tight regulation may dictate the extent of toxicity that they may elicit, and adhesiveness and toxicity may represent two independent aspects of amyloid fibrils.

In conclusion, our study demonstrates several similarities between natural ECMs and amyloid fibrils including their nano-fibrillar morphology, integrin engagement and signaling, and density-dependent responses. These properties make amyloids a promising candidate for developing biomaterials (32,35,103). Cells can adhere to amyloids through a combination of interactions with the lipid membrane and activation of cell adhesion machinery. The present data suggest that cell adhesion could be a generic property of amyloids irrespective of amino acid sequences and thus may explain its association with diseases and utilization as an extracellular matrix by wide variety of organisms.
Acknowledgments:
Authors wish to acknowledge IRCC (IIT Bombay), Cell Biology lab, BSBE, IITB for fluorescence imaging and CRNTS and IRCC (IIT Bombay) for electron microscopy, Protein Crystallography, IRCC (IIT Bombay) respectively. Authors also wish to acknowledge DBT (BT/PR9797/NNT/28/774/2014), Government of India for financial support. R.S.J acknowledges UGC (Govt. of India) for her fellowship.

Conflict of interest:
The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions:
R.S.J., S.K.M., E.G and S.Sen designed the experiments. R.S.J., E.G., P.K.S.,A.A., and S.Salot performed experiments. All authors analyzed the data. S.K.M., R.S.J., E.G., S.S. wrote the manuscript. All authors approved the final version of the manuscript.

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**FOOTNOTES**

*This work was supported by grants from DBT (BT/PR9797/NNT/28/774/2014), Government of India

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2R.S.J and E.G are equally contributed authors.

3The Abbreviations used are: CD, circular dichroism; TEM, transmission electron microscopy; CR Congo Red; ThT Thioflavin T; AD, Alzheimer's disease; PD, Parkinson's disease; Hep, Heparin; FAK-Focal adhesion kinase.

**FIGURE LEGENDS**

**FIGURE 1.** Secondary structural transition of peptides/proteins during amyloid formation. CD spectra of freshly solubilized peptide/protein showing secondary structure at day 0 (red) and conversion to mostly β-sheet rich structure after 15 days of incubation (blue).

**FIGURE 2.** Amyloid formation by peptide/proteins using ThT fluorescence. ThT binding of peptide/proteins showing increased ThT fluorescence after 15 days incubation. The error bars indicate standard deviation of two independent experiments.

**FIGURE 3.** Morphology of peptide/protein aggregates. TEM images of protein/peptide aggregates formed after 15 days of incubation showing fibrillar morphology. Scale bars are 200 nm.

**FIGURE 4.** Cytotoxicity of the amyloid fibrils. MTT assay showing cytotoxicity of amyloid fibrils for SH-SY5Y cells. Most of the amyloids fibrils were mostly nontoxic to the cells except for Aβ(25-35) and glucagon fibrils.

**FIGURE 5.** Cell Adhesion on amyloid fibrils. Representative phase contrast images of SH-SY5Y cells that cultured on various protein/peptide amyloid fibrils showing robust cell adhesion on the substrate. Scale bar is 100 µm.
FIGURE 6. Amyloid fibrils support cell adhesion and spreading. (A) Higher cell attachment of SH-SY5Y cells was observed on most of the amyloid fibril surfaces compared to that on collagen. Error bar represents SEM. (B) Cell spreading analysis showing statistically higher spreading on different amyloid surfaces than on collagen and its corresponding monomers. Statistical significance **p < 0.005 and *p<0.05.

FIGURE 7. Adhesion of various cell types on collagen and kassinin amyloid fibrils. Phase contrast image of PC12, NIH3T3 and L929 cells adhered on kassinin amyloid and collagen after 24 hrs of culture. Scale bar are 100 µm for both upper and bottom panels. (B) Spreading of PC12 cells shown to be unchanged across the different conditions, whereas the other cell types spread more on kassinin substrates compared to collagen. Stars indicate statistical significance (*p < 0.01).

FIGURE 8. Adhesion and motility of NIH 3T3 fibroblasts on kassinin at different fibril densities. (A) Phase contrast image of NIH3T3 cells adhered on kassinin amyloid at different fibril densities, in the presence and absence of serum after 24 hrs in culture. Scale bar = 100 µm. (B) Quantification of cell spreading on kassinin substrates in the presence (+S) and absence (-S) of serum. While cells were found to spread extensively in the presence of serum across the different conditions, cells remained rounded when serum was absent, suggesting though serum proteins are needed for cell spreading, amyloid topography by itself is sufficient for cell adhesion. (C) Random cell motility of fibroblasts on glass coverslips coated with kassinin at varying fibril densities. Cell speed was maximum on 1.5 µM kassinin-coated substrates.

FIGURE 9. Attachment and cytoarchitecture of SH-SY5Y cells on collagen and amyloid fibrils. (A) Cytoskeletal organization and focal adhesion formation in SH-SY5Y cells on collagen and amyloid fibrils. Phalloidin (green), FAK (red) and DAPI (blue) were used to visualize F-actin, focal adhesions and nuclei, respectively. Scale bar = 20 µm. (B) Cytoskeletal organization and focal adhesion formation in SH-SY5Y cells on kassinin and Sub P monomers. Phalloidin (green), FAK (red) and DAPI (blue) staining showing organization of F-actin, focal adhesions and nuclei in immunofluorescence study, respectively. Scale bar = 20 µm. Perinuclear staining of FAK was observed in cells grown on both monomers of kassinin and Sub P. (C) Quantitative analysis of size and number of FAK-positive focal adhesions. The higher number and larger size focal adhesion complex are shown in case of kassinin and Sub P fibrils relative to collagen. Statistical significance (*p < 0.05) was determined by student’s t test. (D) β1 and β3 integrin expression profile in SH-SY5Y cells cultured on uncoated, collagen coated and kassinin fibril coated glass coverslips. The mRNA expression of β1 integrin was highest in cells cultured on kassinin fibrils. (E) Localization of β1 integrin of cells grown on uncoated glass, collagen and kassinin amyloid surfaces. Scale bar = 20 µm. Increased β1 integrin staining and clustering (white arrows) was found in cells cultured on amyloid surfaces. (F) Clustering of β1 integrins in SH-SY5Y cells (quantified from fluorescence intensity) is shown to be higher on kassinin substrates compared to collagen. (**p < 0.005).

FIGURE 10. Lipid-fibril interactions and integrin-based adhesions contribute to cell spreading on amyloid fibrils. (A, B) Spreading of NIH 3T3 fibroblasts on collagen and kassinin substrates after incubation with β1 integrin-blocking antibody (P4C10). Cell spreading was reduced in integrin-blocked samples on both collagen and kassinin. Statistical significance *p < 0.05. Error bar represents SEM. (C) Schematic of integrin blocking experiment using GRGDSP peptide on amyloid coated substrates. (D) Phase contrast images of cells with and without RGD treated cells. (E) Quantification of number of cells adhered and (F) spread area of RGD treated and untreated cells grown on various substrates. (G) Schematic of experiment testing spreading of red blood cells (RBCs) on kassinin-coated substrates. RBCs were allowed to attach and spread on kassinin-coated substrates for 1 hour and then washed to remove the loosely attached cells. (H) Phase contrast images of RBCs attached to kassinin-coated substrates. Scale
bar = 50 μm. (I) Quantification of RBC attachment on coverslips coated with pluronic (Plu), poly-L-lysine and kassinin. RBCs spread on kassinin substrates in a density-dependent manner. Error bar represents SEM.

**FIGURE 11. Modulation of spreading and contractility by ROCK and MLCK signaling pathways in NIH 3T3 cells.** (A) Phase contrast images of NIH 3T3 fibroblasts cells cultured on collagen and kassinin substrates for 24 hrs and subsequently treated with and without 10 μM ML7 (MLCK inhibitor) or 10 μM Y27632 (ROCK inhibitor) for 1 hr. Scale bar = 40 μm. (B) Quantification of cell spreading area in drug-treated cells compared to controls. Statistical significance *p < 0.05. Error bar represents SEM. (C) Schematic of trypsin de-adhesion assay. Cells were washed with PBS, incubated with warm trypsin and imaged in time-lapse microscopy till cells become rounded (but remain attached to the substrate). De-adhesion time is an indirect measure of cell contractility. (D) Representative phase contrast images of cells rounding up upon incubation with warm trypsin. Scale bar = 50 μm. (E) De-adhesion times of control and drug-treated cells cultured on collagen and kassinin. Both ML7 and Y-27632 treatment shown to delay de-adhesion on collagen substrates, whereas on kassinin substrates, cells were sensitive to Y-27632 treatment only. Statistical significance *p < 0.05. Error bar represents SEM.

**FIGURE 12. Modulation of spreading and contractility by ROCK and MLCK signaling pathways in SH-SY5Y cells.** (A) Phase contrast images of SH-SY5Y cells after 24 hours of culture on collagen and kassinin substrates after treatment with 1 μM ML7 (MLCK inhibitor) or 1 μM Y27632 (ROCK inhibitor) for 1 hour duration. Scale bar = 20 μm. (B) Quantification of cell spreading area in drug-treated cells compared to controls. Bars indicate statistical significance (**p < 0.005). (C) De-adhesion times of control and drug-treated cells cultured on collagen and kassinin (**p < 0.005). While both ML7 and Y-27632 treatment led to delay de-adhesion on collagen substrates, cells were sensitive to ML7 treatment only on kassinin substrates.

**FIGURE 13. Biophysical characterization of BSA amyloid.** (A) Far-UV CD spectrum of BSA monomer and BSA aggregates (formed in presence of 300 mM NaCl at pH 3.0) showing helical and β-sheet rich secondary structure, respectively. (B) ThT fluorescence showing higher ThT binding for BSA incubated in presence of salt and low pH. BSA monomer showed negligible ThT binding. (C) CR absorbance spectrum showing higher CR absorbance of BSA aggregate. CR alone was used as a control. (D) BSA aggregate formed in presence of salt and low pH showed greenish-yellow CR birefringence under crossed polarized light indicating the amyloidogenic nature of this aggregate. The corresponding bright-field image is also shown in the left panel.

**FIGURE 14. Amyloid versions of BSA support cell attachment.** (A) TEM images of soluble and fibrillar BSA (upper panel). Scale bar = 200 nm. Phase contrast images showing attachment of SH-SY5Y cells on BSA monomer and amyloid surfaces (lower panel). Scale bar = 100 μm. (B) Quantification of SH-SY5Y attachment on BSA monomer/fibril surfaces showing cell attachment was higher on the fibril surfaces compared to the soluble protein. Statistical significance *p < 0.05. Error bar represents SEM. (C) Analysis of cell spreading area of SH-SY5Y cells on different substrates. Statistical significance (*p < 0.05). (D) Integrin expression profile of cells cultured on soluble and fibrillar BSA showing higher β1 integrin expression on BSA fibrils compared to BSA monomers. (E) Co-localization of β1 integrin and FAK in SH-SY5Y cells on different substrates. FAK (red), β1 integrin (green), and DAPI (blue) were used to visualize focal adhesion complex and nuclei, respectively. Scale bar = 20 μm.

**FIGURE 15. Amyloid formation by PAAs.** (A) FTIR spectra of PLL, PE and PLA showing peaks at 1630 and 1617 cm⁻¹, 1633 and 1623 cm⁻¹ and 1631 and 1615 cm⁻¹, respectively indicative of β-sheet rich structure in incubated PAAs. (B) TEM images of PAA aggregates showing fibrillar morphology. Scale
bars are 200 nm. (C) The PAA fibrils showing greenish-yellow CR birefringence under crossed polarized light revealing their amyloidogenic nature. (D) X-ray diffraction pictures of PAA fibrils showing meridional reflection at ~ 4.7 Å and equatorial at ~ 8 to 15 Å. α-Syn fibrils was used as a positive control.

**FIGURE 16. Cell adhesion on PAAs and its corresponding amyloid fibrils.** (A) Phase contrast images of SH-SY5Y cells after 24 hours of culture on freshly dissolved PAAs and their corresponding amyloid fibrils. Scale bars are 100 μm. (B) Quantification of cell adhered on PAA and their fibrils. (C) The morphology of cells adhered on PAAs and their fibrils are quantified by calculating the spreading area of the cells. Increased cell number and spreading area on PAA fibrils suggest stronger cell adhesion on PAA amyloid fibril coated surface. (D) Cytoskeletal organization and focal adhesion formation in SH-SY5Y cells on PLL and its corresponding amyloid fibril. Phalloidin (green), FAK (red) and DAPI (blue) were used to visualize F-actin, focal adhesions and nuclei, respectively. Scale bar = 20 μm. (E) β1 integrin expression profile in SH-SY5Y cells cultured on PLL and PLL fibril coated glass coverslips. The higher integrin expression and large FAK clusters were observed in cells adhered on PLL fibrils indicating stronger cell adhesion on amyloid fibrils than its soluble counterpart.

**FIGURE 17. Cell adhesion on disease associated amyloids.** (A) TEM images of α-Syn, Aβ42 and Aβ(25-35) amyloid fibrils used for cell adhesion (upper panel). Scale bar = 200 nm. Cell adhesion of SH-SY5Y neuroblastoma cells on the amyloid surface (lower panel). Scale bar = 100 μm. (B) Quantification of SH-SY5Y cell attachment on α-Syn, Aβ42 and Aβ(25-35) amyloid fibrils. Error bar represents SEM. (C) Cell spreading area on different amyloid surfaces showing higher spreading on α-Syn amyloids compared to Aβ fibrils and collagen. Statistical significance *p < 0.05. (D) Localization of β1 integrin of cells grown on α-Syn and Aβ(25-35) amyloid fibrils. Scale bar = 20 μm. (E) β1 integrin expression profile in SH-SY5Y cells cultured on α-Syn amyloid fibrils and collagen. The mRNA expression of β1 integrin was highest in cells cultured on collagen. (F) Phase contrast image showing cell death of attached cells after addition of 4.5 μM of Aβ42 fibrils in the cell culture media.

**FIGURE 18. Schematic of cell spreading on amyloid fibrils.** Proposed cell adhesion on amyloid fibrils mediated by lipid-fibril interactions. This is followed by integrin clustering and activation of downstream signaling cascades that leading to formation of focal adhesions and cell spreading.

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Table 1. Sequence and physical property of amyloid fibril under study

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<table>
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<tr>
<th>S. No</th>
<th>Peptide/Protein</th>
<th>Amino Acid Sequence</th>
<th>Secondary structure</th>
<th>ThT Binding (D15)</th>
<th>ThT Fibrillating (D0)</th>
<th>Toxicity</th>
<th>#Cell adhesion ((Cell spreading area) (µm²))</th>
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<td>Monomers</td>
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<tr>
<td>1</td>
<td>GLP 1</td>
<td>HDEFERHAEGTFDVSVE</td>
<td>RC</td>
<td>β-sheet</td>
<td>+</td>
<td>23</td>
<td>*Non toxic</td>
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<td>EGQAAKEFIWLV</td>
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<tr>
<td>2</td>
<td>Kassinin</td>
<td>DVPKSDQFVGLM</td>
<td>RC</td>
<td>β-sheet</td>
<td>+</td>
<td>16</td>
<td>Non toxic</td>
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*100-85% viability, ** 85-70% viability, ***< 70% viability
*D0 and *D15 are Day 0 and Day 15, respectively.
** Averaged spreading area
*** Averaged fibril diameter
^ Not Done

For Sequence see reference (104)
FIGURES

Figure 1.
Figure 2.
Figure 3.
Figure 5
Figure 7

A

Collagen

PC12  NIH 3T3  L929

Kassinin

B

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Area (μm²)
Figure 8

(A) Images showing cell morphology at different concentrations of a compound in the presence (+Serum) and absence (-Serum) of serum.

(B) Graph showing cell spreading area vs. concentration for different compounds and conditions.

(C) Graph showing cell speed vs. concentration for different compounds and conditions.
Figure 9
Figure 10
Figure 11
Figure 14
Figure 16

A

B

C

D

E

PLL Monomer
PLL Fib
PE Mono
PE Fib
PLA Mono
PLA Fib

β1 integrin
GAPDH
Figure 17

A

Fibrils

SH-SY5Y

D

Collagen

α-Syn

Aβ(25-35)

Integrin β1

F-Actin+Nuclei

B

Cell count/Frame

C

Cell Spreading Area (µm²)

Collagen

α-Syn

Aβ 42

Aβ(25-35)

E

α-Syn

Collagen

β1 integrin

GAPDH

F

Untreated

Aβ 42 treated

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1. Plating cells
Plate cells

2. Immobilization through lipid-fibril interactions
Lipid bilayer
Amyloid fibril

3. Integrins clustering on fibrils
Integrins cluster

4. Focal adhesion formation

Amyloid fibril network
Cell Adhesion on Amyloid Fibrils Lacking Integrin Recognition Motif
Reeba S. Jacob, Edna George, Pradeep K. Singh, Shimul Salot, Arunagiri Anoop, Narendra Nath Jha, Shamik Sen and Samir K. Maji

J. Biol. Chem.  published online January 7, 2016

Access the most updated version of this article at doi: 10.1074/jbc.M115.678177

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