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A method of predicting the *in vitro* fibril formation propensity of A β 40 mutants based on their inclusion body levels in *E. coli*

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Overexpression of recombinant proteins in bacteria may lead to their aggregation and deposition in inclusion bodies. Since the conformational properties of proteins in inclusion bodies exhibit many of the characteristics typical of amyloid fibrils. Based on these findings, we hypothesize that the rate at which proteins form amyloid fibrils may be predicted from their propensity to form inclusion bodies. To establish a method based on this concept, we first measured by SDS-PAGE and confocal microscopy the level of inclusion bodies in *E. coli* cells overexpressing the 40-residue amyloid-beta peptide, A β 40, wild-type and 24 charge mutants. We then compared these results with a number of existing computational aggregation propensity predictors as well as the rates of aggregation measured *in vitro* for selected mutants. Our results show a strong correlation between the level of inclusion body formation and aggregation propensity, thus demonstrating the power of this approach and its value in identifying factors modulating aggregation kinetics.

Alzheimer's disease (AD) is associated with abnormalities in protein folding, resulting in the misfolding and aggregation of a characteristic set of proteins^{1–4}. In particular, the major components of the amyloid plaques found in the brain tissue of AD patients are A β peptides, which are derived by proteolysis from amyloid precursor protein (APP)^{5–8}. A β peptides of different lengths are formed in the brain by proteolytic cleavage of APP by the β - and γ -secretases^{9–13}. Since the concentration of the A β 40 peptide is higher than that of any other amyloid peptides in the aggregates found in human brains¹⁴, it is important to understand the molecular driving forces for aggregation of this major A β form.

A powerful approach to obtain such understanding is to perform the systematic kinetic analysis of a series of designed mutations of the A β sequence combined with changes in the solution composition^{15–17}. The results of such studies indicate that the aggregation of unstructured A β monomers into highly ordered amyloid fibrils is a complex process that is influenced by a vast number of factors. This includes intrinsic factors such as charge, hydrophobicity and other amino acid properties¹⁸, as well as extrinsic factors like pH, temperature and ionic strength^{16,19–22}.

A related strategy for understanding the driving forces for A β 40 aggregation is to carry out random mutagenesis coupled to an *in vivo* assay, the readout of which may correlate with aggregation propensity. This strategy can be implemented by performing overexpression of recombinant A β 40 in *E. coli*, which is widely used as an affordable source of human proteins. The recombinant proteins can be found either in the soluble or insoluble fraction, the latter usually appears in the form of inclusion bodies. Inclusion bodies contain misfolded proteins of β -sheet structure^{23,24} and can typically interact with β -sheet binding dyes such as Congo red and thioflavin-T

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K K H K K K E K K E
 N Q Q R N Q R R E Q Q N D Q
 M D A E F R H D S G Y E V H H Q K L V F F A E D V G S N K G A I I G L M V G G V V

Figure 1. Amino acid sequence of wild-type A β 40 (colored until residue number 30 based on amino acid properties). The 24 single amino acid mutations analyzed in this work are also shown (in black).

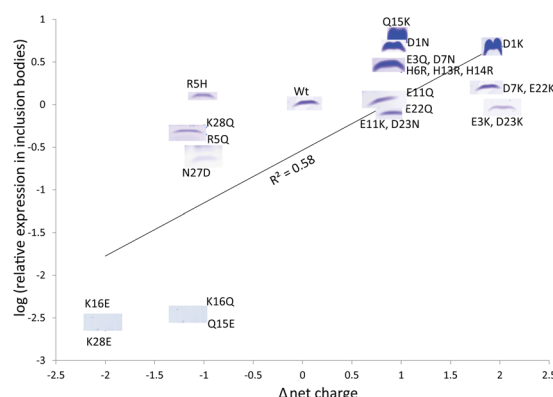


Figure 2. Expression profile of A β 40 charge mutants in inclusion bodies (y-axis has log values of relative score obtained from imageJ analysis of SDS-PAGE gel bands of mutants) versus the change in net charge relative to the wild-type peptide. Supplementary Fig. S3 shows uncropped SDS-PAGE gels of the A β 40 charge mutant bands included here.

(ThT), which are also used to identify and characterize amyloid fibrils^{25,26}. The presence of aggregates with amyloid fibril morphology has been observed for inclusion bodies of *E. coli* expressing A β peptide shown by electron microscopy and AFM²⁷. Inclusion body formation has been exploited for purification of several recombinant proteins, because of the ease of isolation from the majority of soluble *E. coli* proteins by repeated sonication and centrifugation²⁸. While expression of soluble short unstructured peptides and proteins may lead to rapid proteolysis and poor yield, the formation of inclusion bodies protects peptides from degradation and enables the tag-free expression of A β peptides²⁹.

Several studies have demonstrated a connection between formation of inclusion bodies and the propensity to form amyloid fibrils^{25,27,30–32}. Here we ask whether such a correlation can be utilized to build a screening method for identification of A β 40 peptide variants with increased or decreased aggregation propensity. An earlier method using green fluorescent protein fused to A β as a reporter of A β misfolding made it possible to screen for variants with decreased aggregation propensity only³³. We also investigate if there is a correlation between the amount of inclusion bodies formed and measures of overall aggregation rate *in vitro*, such as the time it takes until the reaction is half completed, the steepness of the transition or the concentration dependence of the reaction. We use an A β 40 mutant library of the wild-type protein and 24 single mutation variants (Fig. 1 and see Supplementary Table S1), in which we have changed all charged positions to oppositely charged or neutral hydrophilic amino acid residues, and all His and Asn residues to charged ones. The rationale for this design comes from earlier studies that indicated that electrostatic interactions strongly affect the aggregation kinetics of A β peptides^{16,18,20,22,34}. Using a charge mutant library should thus provide a benchmark for an inclusion-body-based screening method.

Results

Analysis of A β 40 charge mutants expression profiles. Based on the amino acid sequence, the wild-type A β 40 peptide has a net charge between -3 and -4 at neutral pH. After successful expression and cell disruption of several mutants, the soluble fraction and inclusion bodies of each mutant were analyzed by SDS-PAGE to evaluate the A β 40 expression profile (monomeric band at 4.5 kDa). A clear variability in the amount of inclusion bodies was observed between the mutants and wild-type A β 40. No expression in the soluble fraction could be detected by SDS-PAGE analysis, this is likely a combination of rapid formation of inclusion bodies by the aggregation-prone A β 40 and because the peptide is relatively small and unstructured and thus gets degraded in *E. coli* if not protected in the form of inclusion bodies. As shown in Fig. 2, there is a clear correlation between the amount of inclusion bodies formed and the peptide net charge. The mutants that form a high amount of inclusion bodies have a positive change of the net charge towards neutral (top right corner of Fig. 2). In contrast, the mutants that form a low amount of inclusion bodies have an increased net negative charge (bottom left corner of Fig. 2). A few mutants with inclusion body levels similar to the wild-type are seen close to the center of the plot (Fig. 2).

Of the mutants with one unit less negative charge (i.e. substitution of a negatively charged amino acid by a neutral one or a neutral amino acid by a positively charged one) D1N, Q15K, H6R, D7N, H13R, and H14R formed more inclusion bodies than wild-type. Especially Q15K, but also D1N, stand out as mutants with massive inclusion body formation. Of the mutants with two units less negative net charge, especially D1K but also D7K, form much more inclusion bodies than the wild-type A β 40. However, E3K, E11K, E11Q and D23K do not; E3K shows lower amounts of inclusion bodies while the other three form similar amounts of inclusion bodies as the

Parameter	All	Less negative	More negative
charge	0.76	−0.21	0.59
α -helix propensity	0.49	0.23	0.90
β -sheet propensity	−0.33	−0.08	0.26
hydrophobicity	−0.04	0.29	−0.52
CamSol score	0.75	−0.41	0.64
linear component	0.81	−0.11	0.82

Table 1. Correlation(R) between the logarithmized inclusion body levels and several quantitative descriptors for all 24 charge mutants and wild-type A β 40, and, separately, for mutants that are less (16) and more (7) negative than the wild-type.

wild-type peptide. These results imply that net charge is not the only determinant of inclusion body formation, but that also the position of the charge change and other properties matter. For most of the A β 40 mutants for which the net charge is decreased (i.e. becomes more negative), a decrease in the level of inclusion body formation is observed. The mutants that showed lower amounts of inclusion bodies include N27D and K28Q, both of which are one unit more negatively charged. Furthermore, some of the more negative mutants failed to form any inclusion bodies using the overnight expression protocol. This includes K16Q and Q15E, both of which are one unit more negatively charged and K16E which is two units more negatively charged.

Taken together, these results show that the level of inclusion body formation depends strongly on peptide net charge, but that it is also affected by the position of the substitution and changes in other properties that accompany the charge change.

To explore the influence of other parameters, we calculated the correlation coefficients between amount of inclusion bodies as judged on a score from imageJ and the mutation induced change in a number of parameters, including charge, helix propensity, β -sheet propensity, hydrophobicity and a computationally predicted aggregation propensity (CamSol score)^{35,36} (Table 1) CamSol is a general algorithm that can be used for any peptide variant and we are testing if it provides a correlation with observed aggregation propensity.

We found a weak correlation between inclusion body formation level and charge when considering all mutants ($R^2 = 0.57$), but less so when looking at the less or more negatively charged mutants separately, especially for the less negative mutants that show a slightly negative correlation with charge. Thus, it seems like the direction of the change relative to wild-type is more important than the amplitude of the change. Furthermore, the correlation with changes in helix propensity is very strong for the more negatively charged mutants. This doesn't imply that the mutants adopt a helical conformation but reflects the properties of the amino acids. We note that the substitutions Q to E, K to Q and K to E reduce the helical propensity while N to D increases it.

In order to obtain a more detailed overview of the dependence of the inclusion body formation level on the different sequence dependent properties affected by the mutations, a principal component analysis (PCA) was performed on the mutation-induced changes in charge, α -helix propensity, β -sheet propensity, hydrophobicity and CamSol score. The four resulting principal components, describing 99.8% of the variation in these five parameters, were rotated in order to find the component that showed the most linear relationship with inclusion body formation level (Fig. 3). This linear component (Fig. 3A) explains 48% of the variation of the 5 properties. The overall correlation of the linear component is higher than for any of the 5 individual properties and it shows a strong correlation also if the more negatively charged mutants are considered separately (Table 1).

The plots of the loading (Fig. 3B) show to what extent the different parameters contribute to the linear component. Notably, all properties except β -sheet propensity give a positive contribution and charge has the most important contribution.

These results show that the sign of the change in charge is the most important factor for determining the level of inclusion body formation, that it is also dependent on other factors, and that the less negative and more negative mutants may be sensitive to different factors.

Confocal laser scanning microscopy (CLSM) imaging. We used CLSM to test whether the variations in inclusion body formation of different mutants in bacterial cells is observable at the single-cell level by comparing images obtained from samples prepared in an identical manner (Fig. 4). The mutants selected for aggregation kinetic studies (see below) were imaged. The cell-permeable dye thioflavin S (ThS) was used as a reporter on inclusion bodies, which were observed in the fluorescent channel (green color) with bright field images showing the outline of bacterial cells and slightly darker inclusion bodies (Fig. 4). Live bacterial cells expressing the mutants showed variations in the intensity of fluorescence and can be broadly classified in three classes relative to the wild-type in inclusion body level - higher (D1K, D1N and D7N), similar (E3Q, H6R, H13R and H14R) and lower (K28Q and N27D). The observed pattern agrees with the levels determined by SDS-page analysis (Fig. 2). Detailed classification is limited with this technique as intensities can depend on minor details like sample depth due to scattering effects, laser instability or optics oscillation, slight changes in the angle of coverslip on the sample, etc. While many factors contribute to the recorded intensity, a striking trend is observed that underlines the results found in our other experiments.

In vitro aggregation kinetics of selected mutants. The propensities of A β 40 mutants to form fibrils were assessed by monitoring changes in ThT fluorescence intensity for purified and initially monomeric peptides. This approach relies on the specific property of ThT to undergo an intensity increase of its fluorescence emission

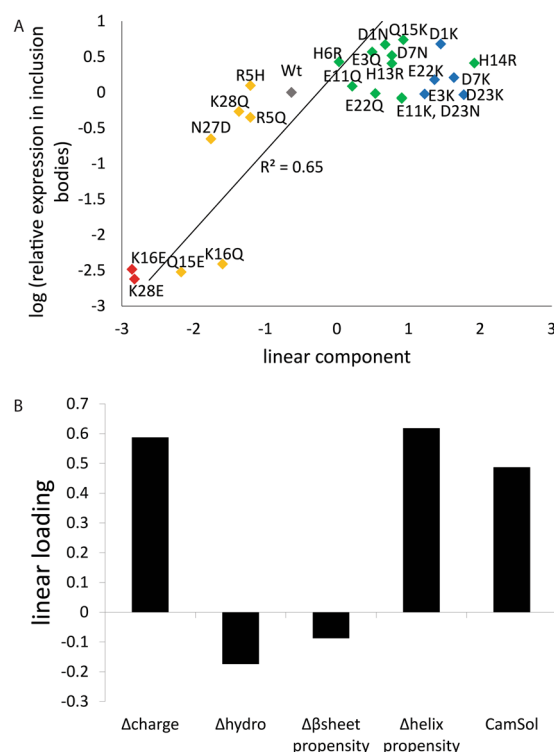


Figure 3. Linear principal component including all quantitative parameters. Scores vs inclusion body levels (A) and loadings (B). The symbols are colored as per the net charge difference relative to wild-type peptide (blue: 2 units, green: 1 unit, grey: 0, yellow: -1 unit, red: -2 unit).

due to appearance of a new excitation peak close to where we excite upon binding to amyloid fibrils³⁷. *In vitro* aggregation kinetics was studied for eleven of the 24 peptide variants, which were chosen to be representative of those showing higher and lower amounts of inclusion bodies compared to the wild-type Aβ₄₀, as well as mutants having similar levels of inclusion bodies to wild-type Aβ₄₀. The aggregation kinetics of these mutants was monitored at a set of different monomer concentrations the lowest being 0.1 μM in each case and the highest in the range 30–89 μM depending on what concentration could be achieved after purification. To facilitate the comparison of the rate of fibril formation of each mutant, the raw data for all mutants were plotted over a fixed range of monomer concentrations (Fig. 5), from 0.1 to 32 μM. However, half-time plots over the whole concentration range sampled are shown in Fig. 6. All mutants except N27D showed sigmoidal-like ThT fluorescence curves with a clear lag phase, a transition and a plateau over most of the concentration range studied (above ca. 2–3.5 μM), indicating that fibrils were formed in most of the samples during the time-frame of the experiment (up to 27 h). The N27D mutant seems to have reached the end of the lag phase after ca. 40–100 h, at concentrations of 32–71 μM, whereas at lower concentrations no increase in fluorescence was observed.

To verify fibril formation, samples from the plateau phase of a reaction starting from 10 μM monomer were monitored using AFM (Fig. 7). Fibrils were observed for wild-type and all nine mutants examined. The fibrils of wild-type and eight of the mutants are between 0.2 and 1.7 μm (Table 2). Distinct periodic twists were observed for fibrils of two mutants (D1N: 0.05 μm and H14R: 0.25 μm) and the wild-type (0.1 μm). The N27D peptide barely showed any fibrils and contained spherical objects that appeared to be of two different sizes. The larger spherical particles resemble those seen in samples containing buffer and ThT only, suggesting that the smaller clusters were detected.

To quantify the aggregation propensity over a broad concentration range, the aggregation half-time ($t_{1/2}$) was extracted from the ThT data and plotted *versus* monomer concentration for each mutant (Fig. 6). The half-time will be shorter for the more aggregation prone variants. The trend of each of the mutant is compared to the wild-type on double logarithmic plots, $\log(t_{1/2})$ vs $\log(\text{monomer concentration})$ (Fig. 6). The half-time plot of wild-type and all mutants except K28Q at higher concentration showed a change in scaling exponent indicating the saturation of aggregation kinetics within the range studied. D1K, D1N and D7N showed half-time curves below the wild-type at all concentrations, while for H13R it crossed the wild-type curve at higher concentrations indicating an increased level of saturation at higher concentrations. H6R, H14R and E3Q curves overlap with wild-type although E3Q flattens above the wild-type curve at higher concentrations. For K28Q and N27D, the half time is above that of the wild-type at all peptide concentrations. Clearly, mutants D1K, D1N, D7N and H13R tend to aggregate faster than wild-type over the whole or most of the concentration range. N27D, K28Q aggregate more slowly while H6R, H14R, E3Q show similar aggregation kinetics as the Aβ₄₀ wild-type peptide. In Supplementary Fig. S1, the aggregation half-times of all mutants and wild-type are plotted in a single frame.

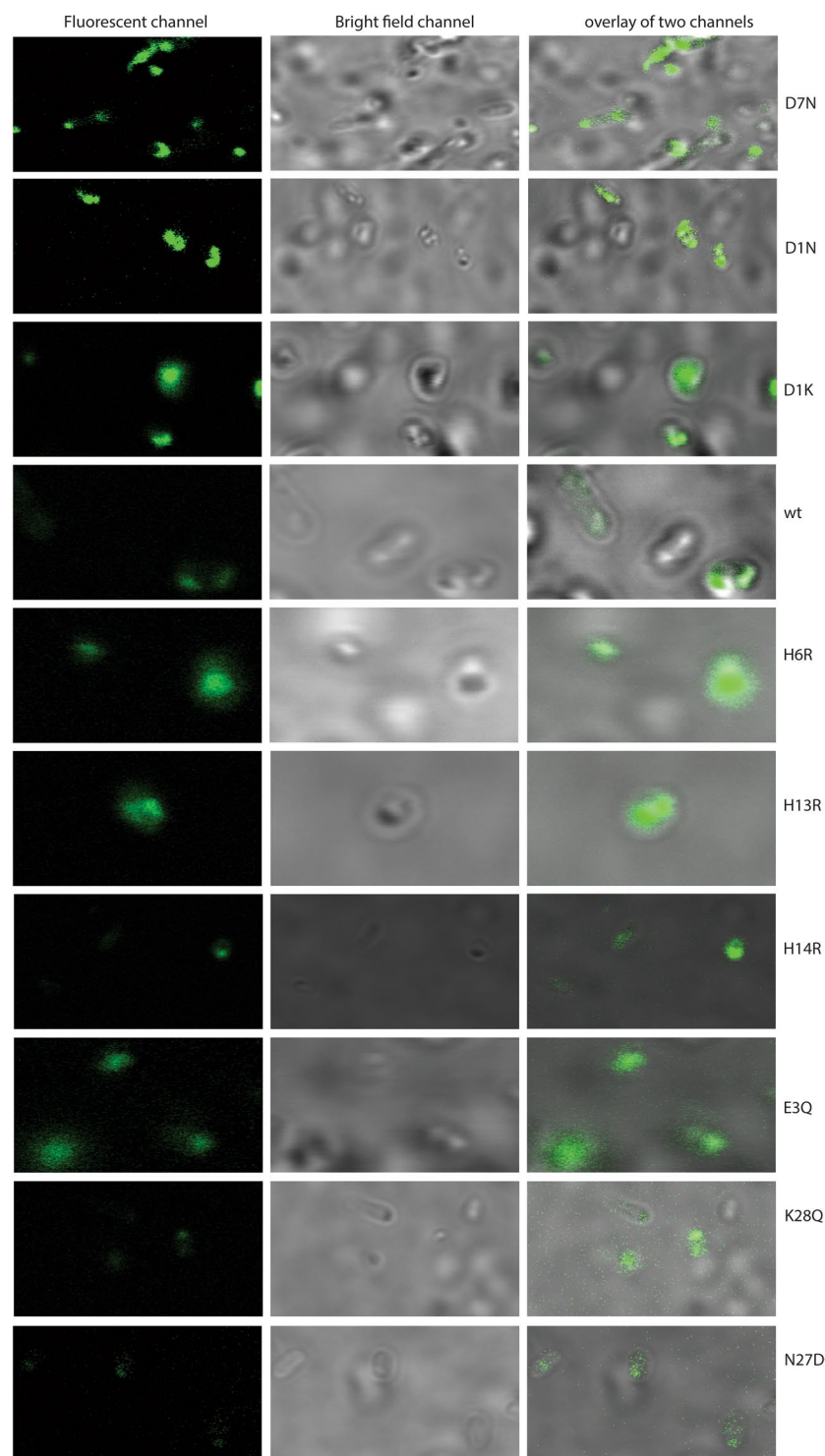


Figure 4. Confocal microscopy images of ThS stained inclusion bodies localized in live *E. coli* cells expressing A β 40 charge mutants and the corresponding bright field images.

Correlation between expression level and measure of aggregation rate. The strong correlation ($R^2 = 0.81$) between the aggregation propensities (as inversely reported on by the half-time) of selected mutants and their expression in inclusion bodies is demonstrated in Fig. 8A where the half time of aggregation is plotted against the inclusion body formation keeping in mind that the reduced half-time reports on increased aggregation propensity. As a comparison the correlation with charge change is plotted in Fig. 8B ($R^2 = 0.58$). To see if a stronger correlation could be obtained by including more parameters, the linear principal component calculated from all parameters (as described above for expression) without and with expression included (Fig. 8C,D). Notably the inclusion body expression together with other parameters increases the correlation

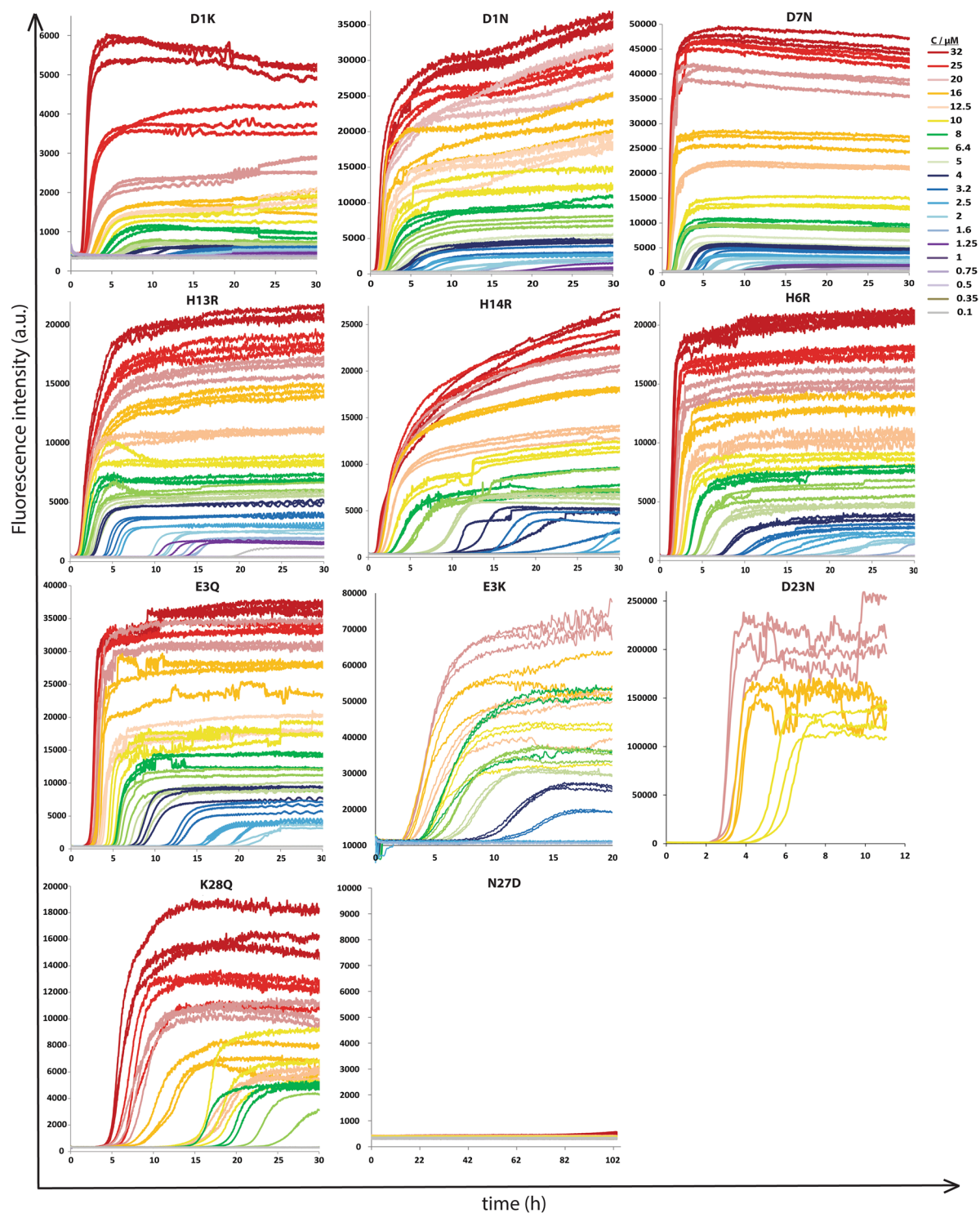


Figure 5. Aggregation kinetic curves of A β 40 charge mutants (D1K, D1N, D7N, H13R, H14R, H6R, E3Q, E3K, D23N, K28Q and N27D) at a concentration range of 0.1 μ M to 32 μ M. Aggregation was followed for 30 h except for N27D where it was followed for 102 h. Note: Y-axis scale is not the same because of variation in ThT intensity.

further ($R^2 = 0.86$) (Fig. 8C) illustrating that expression efficiency is the most important factor for predicting aggregation propensity (Fig. 8). Parameter loadings for 8C and 8D are shown in Supplementary Fig. S2. Other than expression and charge, β -sheet propensity shows the largest loading.

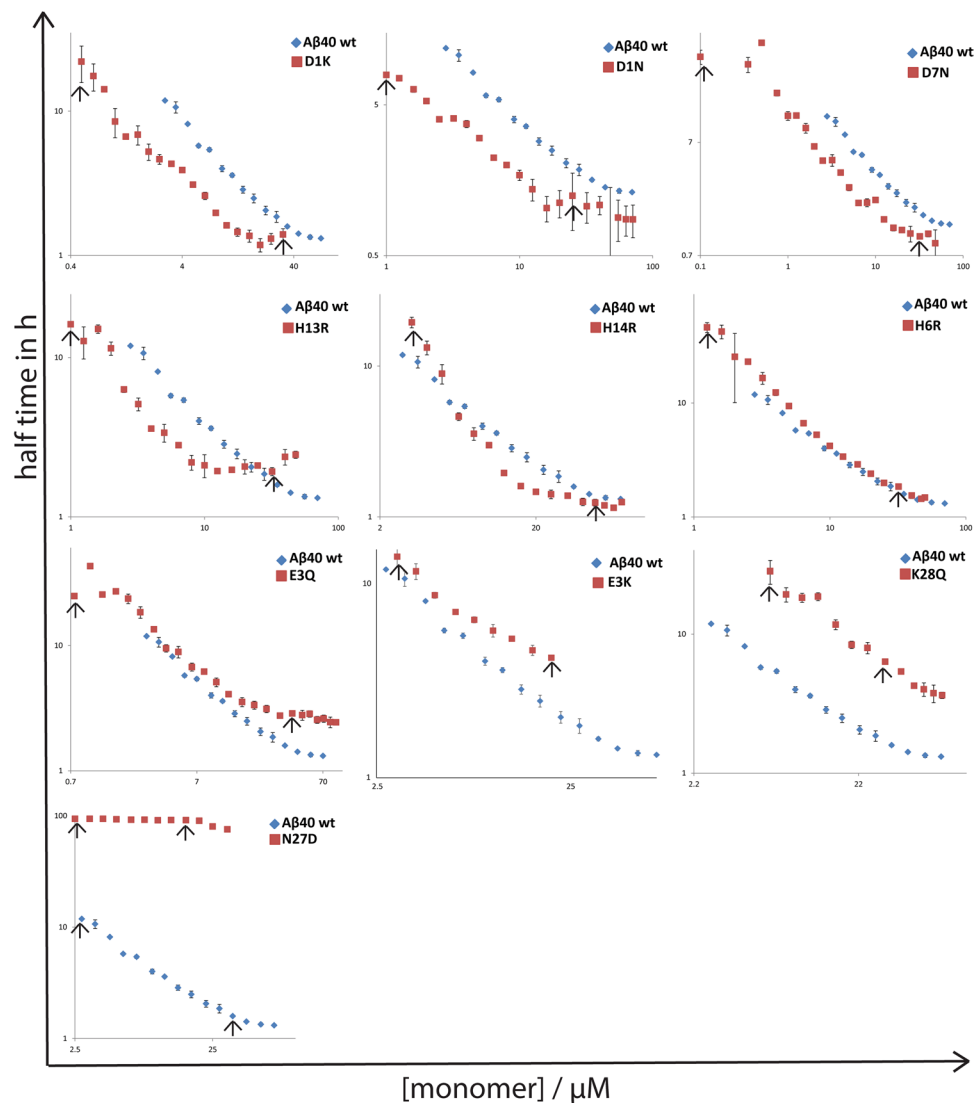


Figure 6. Comparison of $t_{1/2}$ of aggregation for different variants [A β 40 wild-type, D1K, D1N, D7N, H13R, H14R, H6R, E3Q, E3K, K28Q and N27D] vs. concentration. Log-log plots of faster fibril forming mutants (D1K, D1N, H13R, H14R and D7N) lie below the A β 40 wild-type curve while the slower fibril forming mutant (E3K, K28Q and N27D) show its curve above the wild-type. H6R and E3Q almost merge with the A β 40 wild-type. Data points between the arrows represent the half time points of concentrations 32 μ M (E3K – 20 μ M) to lowest possible. D23N is not included as we have half times available only for three different concentrations.

Discussion

Several studies have shown that mutations in A β may affect its rate of aggregation^{8,17,22,38–46}. The aim of the present study was to examine whether the aggregation propensity of A β 30 mutants correlates with the formation of inclusion bodies in *E. coli*. This hypothesis was based on the notion that inclusion-body formation may be considered to be an aggregation process.

Soluble unstructured peptides and small proteins may suffer from fast degradation in *E. coli*, preventing efficient recombinant production unless a protective tag is added. The high aggregation propensity of amyloid peptides rescues them from proteolysis and their formation of inclusion bodies in *E. coli* can be exploited as an advantage to enable tag-free expression of A β peptides²⁹. Moreover, the low solubility of inclusion bodies allows for repeated sonication and removal of the majority of soluble *E. coli* proteins to obtain a suitable starting material for ion-exchange and size-exclusion isolation of A β with wild-type as well as mutant sequences²⁹. Expression of amyloid proteins in *E. coli* is therefore fast, robust and inexpensive.

Although several factors influence the quality of expression, wild-type A β 40 as well as most of the mutants were expressed at reproducible levels at the conditions used in this study. The amount of inclusion bodies did vary in a reproducible manner between different mutants as classified by SDS-PAGE analysis (Fig. 3). The amount of inclusion bodies is likely a reflection of the aggregation propensity relative to the degradation rate of soluble species rather than the expression level. Increased inclusion body formation is found for a set of mutants with a net charge that is less negative than for wild-type: D1K, D1N, E3Q, H6R, D7N, D7K, H13R, H14R and Q15K.

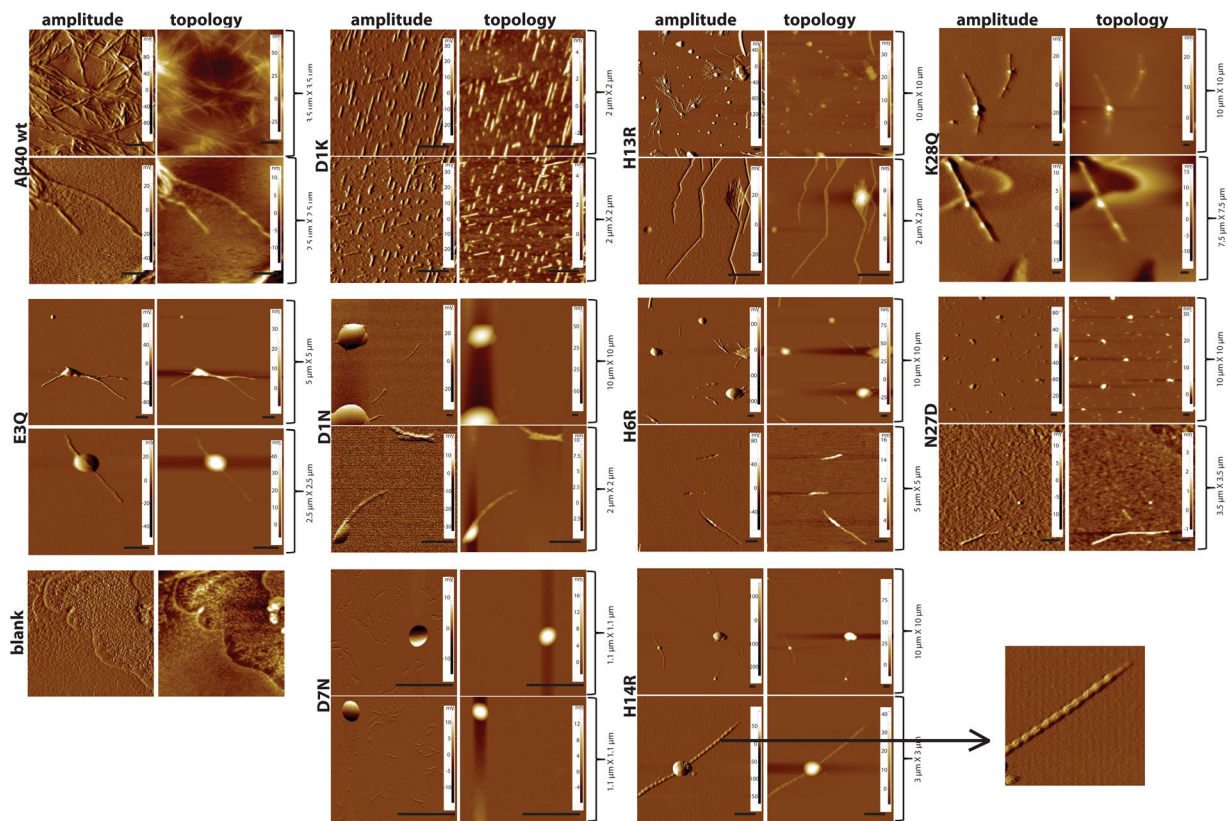


Figure 7. AFM images of fibrils formed by Aβ40 wild-type and mutants formed in 20 mM sodium phosphate buffer with 0.2 mM EDTA pH 7.4. Images of 256 × 256 pixels are shown and the scale bar corresponds to 0.5 μm. Fibril images are taken at different sizes as the length of fibrils varied between mutants. Amplitude and topology frames of each image are included.

Mutant	Length (μm)	Width (nm)
Wild-type	1.1 ± 0.4	55 ± 10
D1N	0.95 ± 0.3	70 ± 12.5
D1K	0.35 ± 0.1	70 ± 12.5
E3Q	3.3 ± 0.5	100 ± 30
D7N	0.3 ± 0.2	30 ± 10
H6R	1.3 ± 0.3	80 ± 20
H13R	1.3 ± 0.5	60 ± 25
H14R	3.3 ± 0.7	80 ± 20
K28Q	3.3 ± 1.05	200 ± 50
N27D*	0.75 ± 0.3	65 ± 10

Table 2. Length of the amyloid fibrils formed by Aβ40 and selected mutants.

Following this trend, we observe reduced inclusion body formation for all mutants with a net charge that is more negative: R5Q, Q15E, K16E, K16Q, N27D, and K28Q, some of which did not show any detectable peptide in inclusion bodies after overnight expression. Exceptions from the above patterns are the E3K and D23N mutants that show a reduced amount of inclusion bodies (which are less negatively charged than the wild-type) but also show slower aggregation than wild-type, suggesting that other factors than charge attenuate their inclusion body formation. In contrast to Aβ42, for which several labs have converged on the same structure^{47–49}, a wide variety of Aβ40 fibril structures have been observed. However, in all Aβ40 fibril structures, both the E3 and D23 side chains are exposed to solvent, and in most cases D23 side chain is found on the surface of the ordered part of the fibril and E3 is in a more disordered region^{50–55}.

The localization by CLSM of ThS-stained inclusion bodies in single bacterial cells showed a variation in the fluorescence intensities over the expressed mutants, which correlates with the amount of inclusion bodies as derived from the SDS-PAGE analysis. These results suggest the possibility to expand this system into a high-throughput technique for screening of a random library of Aβ peptide variants using techniques like fluorescent activated cell sorting (FACS). Earlier studies showed applications of FACS to screen for hydrolytic enzymes producing

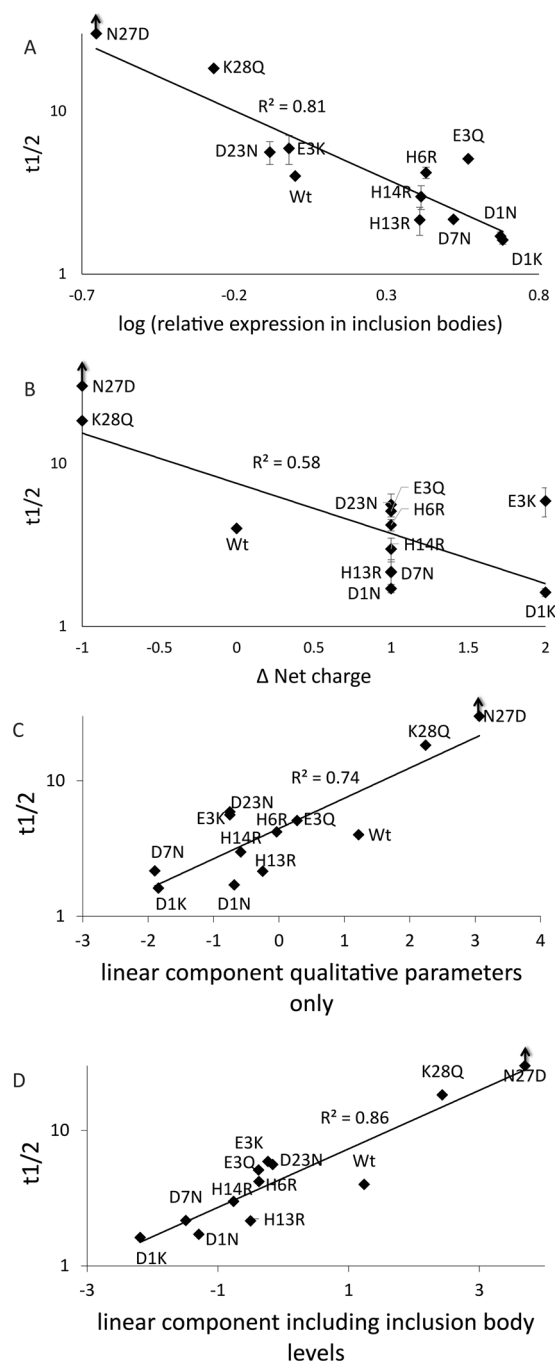


Figure 8. Plots showing the trends of: (A) half time vs relative expression of various A β mutant peptides in inclusion bodies (B) half time vs net charge of various A β mutant peptides, (C) half time vs linear principal component score based on all quantitative parameters, except inclusion body levels (D) half time vs linear principal component score based on all quantitative parameters including inclusion body levels. Note that logarithm y-axis is used and for x-axis log values of relative expression in inclusion bodies are used.

hydrogel-forming peptides in *E. coli* cells⁵⁶. In the past, FACS has also been used in purification of amyloid proteins from brain tissues⁵⁷. A low-throughput but detailed flow cytometry analysis has been reported for amyloid proteins expression in yeast cells and wild-type amyloid- β peptides in bacterial cells^{58,59}. However, to our knowledge, screening the level of inclusion bodies of amyloid mutant libraries in bacterial cells, as an approach to find variants with altered aggregation propensity, has not been presented.

To test if the similar characteristics of proteins in inclusion bodies and in amyloid fibrils can be used to predict the rate at which proteins form amyloid fibrils we looked at the correlation between the rate at which proteins form amyloid fibrils and the amounts of inclusion bodies formed as characterized by SDS-PAGE and CLSM (Fig. 8). Our results show a correlation between the level of inclusion body formation and aggregation propensity, R^2 of =0.81, is higher than obtained with any other parameters and further increased by inclusion of other

parameters, suggesting the possibility of an approach to use inclusion body formation as proxy for aggregation propensity and another probe to further the understanding of factors modulating aggregation kinetics.

Conclusions

In conclusion, we discovered a correlation between the charge of the A β 40 mutants and the amount of inclusion bodies formed. The direction of the charge change relative to wild-type is more important than the amplitude of the change, i.e. less negatively charged mutants form higher levels of inclusion bodies, and more negatively charged mutants form lower amounts of inclusion bodies. Furthermore, we see a strong correlation between inclusion bodies in *E. coli* and aggregation propensity in an *in vitro* aggregation kinetics assay for A β 40 mutants. Thus mutants that form more inclusion bodies in *E. coli* also tend to aggregate faster and A β 40 mutants that form less inclusion bodies tend to aggregate more slowly. Therefore, we propose that the method described in this study can be used as a preliminary screening technique to identify potentially more or less aggregation prone variants from screening of directed or random libraries for more in-depth kinetic analysis.

Materials and Methods

Expression of A β (M1-40) charge mutants. To be able to analyze the expression profile of all the charge mutants under typical growth conditions, four replicates of 500 mL cultures for each mutant as well as the wild-type was set up for overnight expression in auto inducing medium⁶⁰. The A β (M1-40) peptides carry an N-terminal methionine residue to provide a start codon and are elsewhere referred to as A β 40. The mutant plasmids (Pet3a with ampicillin resistance) were designed to each have a codon change leading to a single mutation at a hydrophilic positions of the A β 40 peptide, and together the library members cover all such positions (Table S1). Acidic residues were changed to basic ones and *vice versa*. All plasmids were produced by GenScript (Piscataway, New Jersey). Chemically competent *E. coli* BL21* plysS cells were used for transformation of the mutant plasmids and plated on LB agar plates with 50 μ g/mL ampicillin and 30 μ g/mL chloramphenicol. Single colonies were picked and each released into 50 mL LB medium containing 50 μ g/mL ampicillin and 30 μ g/mL chloramphenicol and allowed to grow to an OD between 0.6–1.8 at 125 rpm orbital shaking. Several pre-cultures were set up for each mutant from separate colonies, and 500 μ L of each pre-culture was added to 500 mL of pre-warmed auto-inducing medium with the same antibiotics, which was grown for 15 h at 37 °C in 2.5 L baffled flasks at 125 rpm orbital shaking. 1 mL of each overnight culture was harvested and used for analysis of the expression profile of mutants by SDS-PAGE. The rest of each overnight culture was centrifuged (6000 g for 10 minutes) and stored at –20 °C for A β 40 peptide purification.

Cell disruption. Harvested cells from 1 mL of culture were suspended in 100 μ L distilled water with 0.1% benzonase and incubated for 10 min at 37 °C. Cells were frozen at –80 °C for half an hour and thawed at room temperature for another half an hour. Freezing and thawing of cells was repeated five times and the samples were then centrifuged for 2 min at 13000 rpm. Each time, the supernatant was collected and the pellet was re-suspended in 100 μ L of distilled water with 0.1% benzonase. The re-suspended solution was frozen at –80 °C overnight, thawed for half an hour and centrifuged for 2 min at 13000 rpm. Pellet was re-suspended in 50 μ L of 8 M urea, incubated at room temperature for 30 minutes and further analyzed by SDS-PAGE.

SDS-PAGE analysis. To compare the expression profiles and inclusion body formation of various mutants, the supernatant and the re-suspended pellet samples obtained after cell disruption were loaded on to the SDS-PAGE gel (see Supplementary Fig. S3). Samples were mixed in 1:1 ratio with sample buffer (0.5 M Tris HCl pH 6.8, 10% glycerol, 5% DTT, 0.05% bromophenol blue), incubated for overnight at room temperature and loaded on to a 10–20% gradient tricine gel (Invitrogen). The gel was run for 3 h at 80 V in 100 mM Tris, 100 mM tricine, 0.1% SDS, pH 8.3, stained in staining solution (0.25% Coomassie brilliant blue, 40% ethanol, 10% acetic acid, 50% water) for at least 4 h, destained in destaining solution (30% ethanol, 7% acetic acid) and finally scanned using imagescanner III, GE healthcare. BenchMark™ Low-range prestained protein standard from Novagen was used.

Purification of the A β 40 mutants. Frozen cell pellet from 2 L overnight culture was thawed on ice and re-suspended in 40 mL of 10 mM Tris/HCl pH 8.0, 1 mM EDTA (buffer A). The solution was sonicated twice for 1 min (50% duty cycle, strength 9.5, pulsed, ½ horn) on ice. Between sonications, the solution was cooled down on ice for 45 seconds. The solution was centrifuged for 10 min at 18000 \times g, 4 °C in a JA 25.50 rotor, Avanti J-26 XP centrifuge. Supernatant was removed and pellet was re-suspended in 40 mL buffer A, sonicated two more times with centrifugation in between to remove supernatants. The final pellet was re-suspended in 20 mL ice cold 8 M urea, 10 mM Tris/HCl pH 8.0, 1 mM EDTA and sonicated to obtain a clear solution. The urea solubilized inclusion bodies were then 4-fold diluted with buffer A to obtain a final concentration of 2 M urea. Approximately 20 mL of DEAE-cellulose equilibrated in buffer A was added for purification via ion exchange chromatography. The slurry was carefully stirred with regular intervals for 20–30 minutes on ice. The slurry was poured in to a Büchner funnel with filter paper and placed on vacuum glass bottle to remove the filtrate. The resin was washed twice with 20 mL buffer A and followed by 20 mL portions of buffer A with 5 or 10 or 25 mM NaCl to remove weakly bound impurities. Low negative charge A β 40 mutant peptides were eluted with buffer A with 100 mM NaCl and high negative charge A β 40 mutants were eluted with buffer A with 150 mM NaCl. After eluting the protein, the resin was washed with 8 M urea 500 mM NaCl to elute all other proteins bound to the resin as a pre-cleaning before regeneration of the resin. Samples were collected at every filtration step and small aliquots were withdrawn for SDS-PAGE analysis (Fig. 9). Eluted A β 40 peptides were filtered through a 30 kDa spin filter (Vivaspin 15 R, Sartorius Stedim Lab Ltd, UK) to remove proteases and other proteins that may digest or perturb the aggregation of amyloid beta peptide. An optional step of concentrating the peptide using 5 kDa spin filter was performed in some cases where the expression level of mutant peptide was low. Aliquot of 3–5 mL of peptide

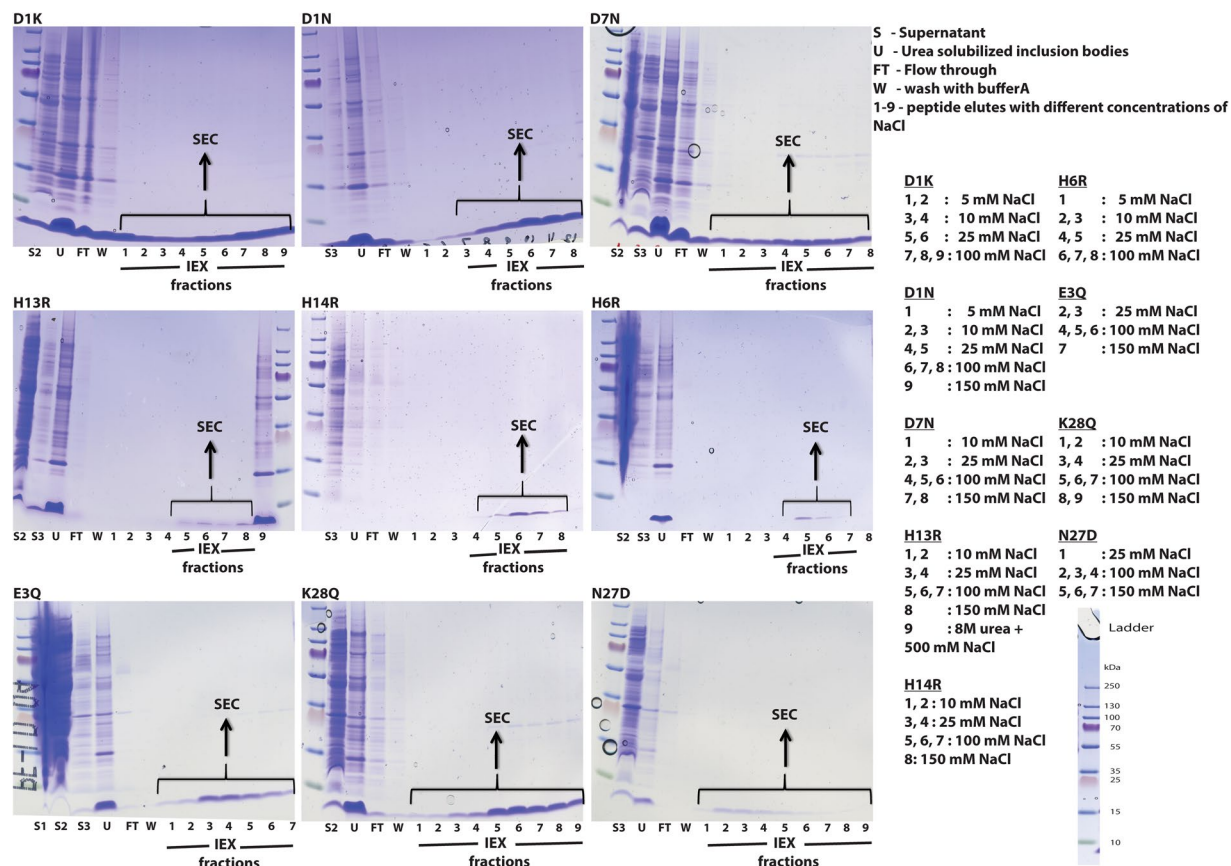


Figure 9. Ion exchange chromatography (IEX) of A β 40 charge mutants. IEX fractions of each mutant are run on separate SDS-PAGE gel and labelled with respective mutation. The uncropped SDS-PAGE gels shown in Supplementary Fig. S4.

sample was lyophilized and stored at -20°C until needed. A fresh gel filtration step was performed and center of monomer peak was collected and used immediately after collection to study aggregation kinetics of the peptide using ThT fluorescence as a reporter of fibril formation.

In vitro aggregation kinetics of selected mutants. The freshly prepared monomer solution in 20 mM sodium phosphate buffer with 0.2 mM EDTA pH 7.4, as well as the same buffer without peptide, were supplemented with ThT at a final concentration of 20 μM and kept on ice. Dilution series of A β 40 mutant peptides with concentrations ranging between 0.1–30, 0.1–50, 0.1–71 or 0.1–89 μM were prepared in low binding Eppendorf tubes (Genuine Axygen Quality, Microtubes, MCT-200-L-C). A 96-well PEG coated plate with a clear bottom (Corning 3881) was used to read the ThT fluorescence emitted from each mutant peptide sample. Each well was loaded with 100 μL of sample. Each mutant was studied in two different plates with triplicate samples for each concentration. (i.e. six replicates of each condition) The plate was sealed with a plastic film (Corning 3095). The plate was placed in a Polarstar Omega plate reader (BMG Labtech, Offenburg, Germany) and incubated at 37°C without shaking. ThT fluorescence was measured every 2 s up to 120 h through the bottom of the plate, with the excitation and emission wavelengths at 440 and 480 nm, respectively. The half time ($t_{1/2}$) was estimated by taking the values half-way in between start and end baseline.

Analysis of the relationship between mutation-induced changes in physical properties and protein expression levels in inclusion bodies. Principal component analysis (PCA) was performed to assess the overall dependence of the amount of inclusion bodies, measured in SDS-PAGE bands using imageJ, or log ($t_{1/2}$) for fibril formation on the mutation induced change in charge, helix propensity, β -sheet propensity, hydrophobicity and CamSol score using SIMCA15 (Umetrics, Malmö, Sweden). The resulting principal components were rotated to find the component that showed the strongest linear relationship with log inclusion body formation or with log ($t_{1/2}$). In the latter case models were made both without and with log inclusion body formation included in the PCA.

Mica surface preparation for AFM. AFM-grade mica in 11 mm \times 11 mm pieces (agar scientific) and magnetized-stainless steel coin-like sample holders (12 mm diameter) were used. The mica pieces were glued on the top of the holder using double-sided sticky paper. Fibril samples were prepared from 10 μM or 20 μM monomer solutions. Identical protocol was followed for preparing A β 40 mutant peptide samples as followed for

sample preparation in kinetic analysis. Approximately 5 μL of sample was placed on each mica sheet and let to dry overnight by keeping it in a clean Petri dish.

Atomic force microscopy. Images were obtained using an AFM instrument (Park systems, model 82) with the following settings: XY-scanner: 5 μm , 50 μm , or 100 μm ; working distance of Z-scanner: 12 μm or 25 μm ; optics of the AFM: direct on-axis vision of sample surface and cantilever; focus range: 20 mm; motorized magnification: 780x (optional 160x, 390x, or 1500x); field of view: 480 μm \times 360 μm and optical resolution: 1 μm ; AFM probes: non-contact mode, intermittent. Specifications of AFM probes: length- 125 μm , mean width- 30 μm , thickness- 4 μm , frequency- 330 kHz and force constant- 42 N/m.

Confocal microscopy. Approximately 1 mL of bacterial cells expressing charge mutants were pelleted by centrifugation. Pellets were re-suspended and incubated for 1 h on ice in 0.3% Thioflavin S (ThS) solubilized in PBS and pelleted by centrifugation. Pellets were washed with PBS, centrifuged and re-suspended in PBS and placed on glass slide. Sample cells were mounted on an inverted CLSM (Leica TCS-SP5 tandem scanner), and imaged using a 100x oil immersion objective while in a thermostatic enclosure. Dye was excited with a 488 nm argon ion laser.

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Author Contributions

S.L., C.C.L., K.S. and I.N. designed the project. K.S. performed expression of, purification and kinetic experiments of all mutants. A preliminary test of expression, purification and kinetics of few mutants was performed by E.N. I.N. worked with SDS-PAGE expression analysis of three mutants and participated in the discussion of work with E.N. K.S. prepared samples and performed A.F.M. K.S. prepared samples for C.F.S.M. and J.I. performed C.F.S.M. P.S. calculated CamSol scores of mutants. G.M. analyzed the kinetic curves. A.M. performed P.C.A. of all mutants. K.S. prepared the first draft of manuscript and all the authors (S.L., A.M., T.P.J.K., G.M., M.V., I.N., E.N., C.C.L., J.I., P.S.) helped to improve the manuscript with their feedback.

Additional Information

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