Effect of Silica Nanoparticles on the Amyloid Fibrillation of Lysozyme

Mouli Konar, Ashwin Mathew, and Swagata Dasgupta*

Department of Chemistry, Indian Institute of Technology Kharagpur, Kharagpur 721302, India

ABSTRACT: Protein fibrils are regarded as undesired products as these are associated with numerous neuro- and non-neurodegenerative disorders. Increasing evidence suggests that the mechanism of fibrillation involves the formation of various oligomeric intermediates, which are known to be more toxic than mature fibrils. Here, we report the impact of synthesized silica nanoparticles (SiNPs) of diameters ~52 nm on the aggregation behavior of hen egg white lysozyme (HEWL) under heat and acidic conditions. Congo red as well as ThT binding assays and AFM imaging studies indicate that SiNPs trigger the amyloid formation of HEWL in a dose-dependent manner. ThT kinetic studies and FTIR studies suggest that the fibrillation kinetics does not involve the formation of toxic oligomeric intermediates at higher concentrations of SiNPs. By measuring fluorescence lifetime values of the bound ThT, SiNP-induced fibrillation of HEWL can easily be realized. CD spectroscopic studies indicate that native HEWL becomes unfolded upon incubation under the experimental conditions and is rapidly converted into the β-sheet-rich fibrillar aggregates in the presence of SiNPs with increasing concentrations. It has been further revealed that fibrillar aggregates formed at higher concentrations of SiNPs preferably adopt an antiparallel β-sheet configuration. The enhanced fibrillation in the presence of SiNPs is likely because of preferential adsorption of the non-amyloidogenic regions of HEWL, resulting in the exposure of the aggregation-prone regions of HEWL toward the solvent. The study will provide deeper insights into the evolution of oligomer-free fibrillation that can be useful to demonstrate the underlying mechanism of amyloid fibrillation.

1. INTRODUCTION

Amyloid fibrils are a special type of misfolded protein/peptide aggregates that form under stressful conditions both in vitro and in vivo.1-3 In vivo amyloid fibril formation is always considered as an undesired manifestation as it is linked with more than 20 different neuro- and non-neurodegenerative diseases including Alzheimer’s disease, Parkinson’s disease, and type II diabetes.4-8 Each amyloidosis process is associated with the aggregation of a particular amyloid-forming protein.9 Although all amyloid forming proteins are different from each other in terms of amino acid sequences and tertiary folds in their native state, they share a common structural morphology in the polymerized state, that is, after fibrillation, thus making amyloid fibrillation a generic property of all proteins.6,8 Fibrils are inherently stable, nonbranched protein aggregates of predominantly β-sheet structures with solvent-exposed grooves.3 The grooves run parallel with the β-strands, each of which is connected by loops and stabilized by intermolecular hydrogen bonds.10 Because of this structural aspect, fibrillar aggregates are typically stained by the dyes Congo red and thioflavin T that bind within the grooves.11,12 The formation of β-sheet-rich amyloid fibrils involves significant restructuring of the natively folded or intrinsically disordered protein.13 Increasing evidence has proven that the restructuring process proceeds either by a one-step or two-step assembly mechanism. In the one-step assembly mechanism, the energetically unfavorable oligomers generated during the course of fibrillation have a lifetime too short to be detected in the solution, and the long, straight fibrils are formed through an oligomer-free pathway. The two-step fibrillation mechanism proceeds via oligomerization at the nucleation phase followed by the conversion of the oligomers into long, cross-linked mature fibrils via the initial formation of curvilinear, small protofibrils. The oligomers produced during the fibrillation process are sufficiently stable and often detected along with the mature fibrils in the saturation phase.14-16 Even though the second pathway is well accepted and follows the most common fibrillation kinetics involving a lag phase, growth phase, and plateau or saturation phase, the one-step oligomer-free pathways are sometimes found to occur at elevated temperatures.17 It is still a matter of debate whether the restructuring of the native or intrinsically disordered state into a highly ordered fibrillar state occurs via oligomerization or an oligomer-free pathway. The cytotoxicity associated with the fibrils has been shown to be exerted to a greater extent from the oligomer intermediates rather than the mature fibrils.18 It
is therefore of particular interest to know if fibrillar aggregates are accompanied by the oligomer intermediates or not. It is important to establish and/or develop newer strategies/devices for the inhibition of protein oligomerization as the pathogenesis of amyloidosis is directly linked to the formation of toxic oligomeric intermediates during the fibrillation process.

Hen egg white lysozyme (HEWL), although not directly linked to any disease conditions, is widely studied as a model protein to explore many aspects related to protein structure, folding, and aggregation over the years. The use of HEWL in understanding the aggregation or fibrillation of proteins is relevant because it shares almost 70% structural resemblance with human lysoyme whose two specific mutants (I56T and D67H) are known to cause a kind of non-neuropathic amyloidosis through fibrillation.27 HEWL contains four disulfide linkages because of which it has high thermal stability and shows reluctance to become unfolded at elevated temperature and physiological pH. Unfolding of HEWL that can initiate the fibrillation process therefore requires some drastic denaturing conditions, such as the combination of high temperature and low pH or high temperature along with different denaturants if physiological pH is maintained.20-22 Most reports on the fibrillation of HEWL have used elevated temperature and acidic pH conditions for fibrillation because hydrolysis of HEWL resulting in the fragmentation into amyloidogenic peptide segments is facilitated under these conditions.23-25 It has already been documented that a few peptide fragments of HEWL such as 49−101 and 53−101 are more amyloidogenic than the full-length protein.26 In the present article, we have used the conditions of high temperature (65 °C) and low pH (pH 2) to monitor HEWL fibrillation in the presence of silica nanoparticles. Before discussion on the main objectives of this study, we would like to focus on the influence of nanomaterials on protein fibrillation. Recently, nanoscale materials have received significant attention because of their very small size, large surface area, and especially remarkable optical, electronic, and catalytic properties.28 Nanomaterials are also found to affect protein fibrillation in a positive sense, that is, the fibrillation process is enhanced in the presence of nanoparticles in most cases, although inhibition reports are also available.28-31 Silica nanoparticles (SiNPs) are one of such biologically relevant nanomaterials that have found use owing to its low toxicity, excellent biocompatibility, and high thermal stability.29 Moreover, the particle sizes of the SiNPs can be precisely manipulated for various applications. Facile preparation, large surface area, and high charge density make SiNPs ideal for specific protein adsorption and separation studies.33 The surface charge of functionalized SiNPs has profound implications in inducing or retarding protein fibrillation. For example, positively charged surface-modified mesoporous SiNPs are found to reduce the fibrillation process of α-synuclein, whereas the negatively charged SiNPs had the reverse effect.34 The presence of nanoparticles can thus either accelerate or inhibit the fibrillation process, and this depends on whether it is able to shorten the lag phase by favoring the nucleation or blocking the growing ends of the oligomer, thereby inhibiting the fibrillation process. This could mean that a nanoparticle-based approach could be considered to modulate the fibrillation process of proteins that can be useful to demonstrate the underlying mechanism of fibrillation. In the present study, we have synthesized silica nanoparticles (SiNPs) with a diameter of 52.5 ± 0.13 nm, and studied its influence on the secondary structure of HEWL under conditions of pH 2 and at a constant temperature of 65 °C for several days. These conditions were chosen because the amyloid fibrillation of HEWL takes place at elevated temperature and low pH in vitro conditions.35-38 Various biophysical techniques have been employed to analyze the effects of SiNPs with HEWL during the fibrillation process, and we have seen that SiNPs promote the progress of fibril formation from a disordered structure of HEWL at acidic pH and elevated temperature conditions. Using FTIR spectroscopy, we have demonstrated that long, cross-linked fibrils of an antiparallel β-sheet configuration are formed in an oligomer-free pathway in the presence of higher concentrations of SiNPs.

2. RESULTS AND DISCUSSION

During the formation of amyloid fibrils, a number of oligomeric species with varied morphology and stability have been identified.39-41 Although the function or the level of cytotoxicity associated with these intermediates is not exactly known, they are believed to be more cytotoxic than the mature fibrils.42 Typically, the in vitro fibrillation process follows a sigmoidal kinetic profile in which three distinct phases, such as nucleation or lag phase, growth phase, and plateau or saturation phase can be identified.7,43 Mounting evidence suggests that the conformationally altered oligomeric intermediates are predominantly formed in the nucleation or lag phase.4,44,45 Thus, monitoring the lag phase in the protein fibrillation process in the presence of an external additive is of great interest because this can directly predict how and to what extent protein fibrillation will proceed in the presence of that particular additive, in this case, silica nanoparticles, at low pH and high temperature conditions. The influence of silica nanoparticles on the lag phase of HEWL fibrillation kinetics is therefore of prime interest. This has been addressed by varying the concentration of silica nanoparticles and monitoring the extent of formation of the oligomeric species during the fibrillation process. Furthermore, we have also investigated the β-sheet configuration of HEWL fibrils in the presence of SiNPs.

2.1. Synthesis and Characterization of SiNPs. SiNPs were synthesized using a sol−gel method from TEOS, a precursor of silica.69 A typical sol−gel reaction is the hydrolysis and condensation of TEOS performed in ethanol medium in the presence of ammonium hydroxide serving as a catalyst of the reaction. The colloidal white nanoparticles precipitated were characterized for their size, surface charge, functional groups, and morphology using DLS, zeta potential, XRD, FTIR, TEM, and FESEM, respectively. DLS studies indicate that the majority of nanoparticles have 52.5 ± 0.13 nm diameter (Figure S1a). We have also determined the surface charge of the nanoparticles at two different pH values, under neutral (pH 7.4) and acidic (pH 2) conditions. At pH 7.4, the nanoparticles are found to have a high negative charge on their surface (ζ = −13.5 ± 0.4 mV), whereas at pH 2, they remain neutral or become slightly positively charged (ζ = 0.49 ± 0.02 mV) in the solution, indicating that the nanoparticles almost reach their isoelectric point at pH 2 (Figure S1b).46 The XRD pattern shows an intense sharp peak centered at 2θ = 24°, which indicates the high percentage of amorphous nanoparticles (Figure S1c). The FTIR spectrum of SiNPs shows four sharp bands at 1090, 967, 800, and 464 cm⁻¹ (Figure S1d). The absorption peaks at 1090, 967, and 800 cm⁻¹ are assigned to the Si−O−Si asymmetric stretching vibrations, Si−
OH asymmetric stretching vibrations, and Si–O symmetric stretching vibrations, respectively. The Si–O rocking vibrations are observed at 467 cm⁻¹, and the absorption band in the range of 3500–3600 cm⁻¹ is assigned to the OH stretching vibrations of hydroxyl groups in the nanoparticles. FESEM and TEM images of SiNPs as shown in Figure S1e,f revealed that the prepared nanoparticles were mostly monodisperse and spherical in shape with an average diameter of 50–60 nm, which is consistent with the DLS study.

2.2. Congo red binding studies. The extent of aggregation of HEWL into amyloid fibrils under heat and acidic pH conditions in the presence and absence of SiNPs has been evaluated using a Congo red binding assay after 12 days of incubation. Congo red is a histologic dye that can stain amyloid fibrils upon binding to the well-ordered β-sheets of fibrillar aggregates. In this assay, the formation of the fibrils in a solution are detected by a red shift in its absorption maxima from ~498 nm (in aqueous or native protein solutions) to ~540 nm (in fibrillar solution). Congo red molecules are thought to intercalate along the fibril axes in the grooves on the β-sheet surface of fibrillar aggregates, and the binding is facilitated by both hydrophobic as well as hydrophilic interactions. The red shift in the Congo red absorption maxima in the presence of amyloid fibrils is attributed to the stability of Congo red molecules within the fibrillar grooves. The Congo red spectra for different HEWL fibrillar solutions in the presence and absence SiNPs are represented in Figure 1. It is observed that the maximum absorbance peak of Congo red at 498 nm in aqueous solution is red-shifted to an extent of only 9–10 nm in the case of incubated HEWL without SiNPs. This points toward the lack of formation of mature fibrils in the solution of HEWL, although the formation of small oligomeric species from the native structure of HEWL during the incubation period cannot be ruled out. In the presence of increasing concentrations of SiNPs, the red shift gradually becomes more prominent, and the extent of red shifts is ~20, ~29, and ~40 nm in the presence of 20, 50, and 100 μg/mL SiNPs, respectively. Moreover, the peak of Congo red absorption maxima is broadened in the HEWL sample treated with SiNPs. This may be due to the contribution of both the free Congo red as well as fibril-bound Congo red. This clearly indicates that SiNPs induce the formation of mature fibrils in a dose-dependent manner under heat and acidic pH conditions.

2.3. Detection of HEWL Fibrillar Aggregates in the Presence of SiNPs using ThT Fluorescence and Lifetime Measurement Studies and AFM Imaging. A detailed analysis of the impact of SiNPs on the fibrillation process of HEWL has been carried out using ThT-based fluorescence studies. ThT is extensively used as a sensitive fluorescence active dye for probing the formation of fibrils. Although ThT is weakly fluorescent in water, it shows a several fold increase in fluorescence emission intensity when bound to highly ordered fibrillar aggregates where it inserts itself in the solvent-exposed “grooves” formed by aromatic side-chain amino acids containing crossed β-strands of amyloid fibrils and becomes stabilized. The steady state-fluorescence spectra of ThT for all the HEWL samples taken after 12 days of incubation are shown in Figure 2a. The ThT fluorescence intensity of the HEWL solution incubated without SiNPs is higher compared with that of ThT in water. This increase is however not to an extent (0.5 fold increase in ThT fluorescence intensity as compared to ThT in water) that can confirm the formation of mature HEWL fibrils. The lower extent of increase in the ThT fluorescence intensity for the incubated HEWL sample without SiNPs can account for the formation of non-fibrillar oligomeric protein aggregates or amorphous aggregates with which ThT does not show strong binding affinity. A significant enhancement in ThT fluorescence intensity observed for the HEWL solutions incubated with SiNPs is an indication of the formation of fibrillar aggregates. For 50 and 100 μg/mL SiNP-containing solutions, the increase in intensity is almost double in comparison to that of the 20 μg/mL SiNP-containing solution. This implies that the amount of fibril formation increases at higher concentrations of SiNPs.

ThT fluorescence-based fibrillation kinetic studies for different HEWL solutions have been monitored during the incubation period for up to 12 days, and the representative profiles are shown in Figure 2b. The kinetic profiles are analyzed using eq 1, and the resultant parameters such as lag time, rate constant of aggregation, and t½ are summarized in Table 1. Figure 2b indicates that in the presence of SiNPs, HEWL fibrillation proceeds through a nucleation-dependent polymerization process in which an initial lag phase is involved. From Table 1, it is evident that SiNPs shorten the lag phase of the fibrillation process in a dose-dependent fashion and at the same time increase the rate of the fibrillation process with respect to the control solution (where HEWL is incubated without nanoparticles). For example, the lag time of fibrillation for 20 μg/mL SiNPs is found to be 113.1 ± 2.0 h, whereas those for 50 and 100 μg/mL SiNPs are 78.1 ± 2.1 h and 62.3 ± 2.3 h, respectively. Because the oligomeric species appear in the lag phase, it can thus be inferred that oligomers are formed at all concentrations of SiNPs as in all cases, a lag phase of a certain period is involved. However, the stability of these oligomeric species varies depending upon the duration of the lag phase as well as the rate constant for the aggregation process for each sample. The lowest lag time and highest rate constant value (34.8 ± 0.7 h⁻¹) in the case of 100 μg/mL SiNPs results in the disappearance of the oligomer species soon after formation and promotes the aggregation into amyloid fibrils directly from the monomeric proteins. On the other hand, at lower concentrations of SiNPs (20 μg/mL), oligomers are likely to be stable for a finite time period before
participation in aggregation. An extended period of lag time with a much lower rate constant value for the incubated HEWL in the absence SiNPs indicates that oligomers produced at the lag phase are sufficiently stable and hence, they have high chances to be dissociated again into the monomer species.44,54

To further ensure the increasing aggregation propensity of HEWL in the presence of SiNPs, we have taken the AFM images of the samples after 12 days of incubation under the experimental conditions employed, and the images obtained are shown in Figure 3. In the absence of SiNPs, HEWL displays discrete globular aggregates that are distinctly different from the fiber-like species; meanwhile, the SiNP-treated samples show abundant unbranched as well as elongated fibril formation. The images of SiNP-treated samples also indicate that the extent of fibril formation as well as the extent of cross-linking among the aggregates increases with increasing nanoparticle concentrations. Moreover, the AFM images of SiNP-treated HEWL samples show the presence of SiNPs on the fibrillar structure. The microscopic results thus provide direct evidence for the increased propensity of the formation of mature fibrils of HEWL in the presence of SiNPs.

We further determined the average lifetime of ThT in all HEWL solutions incubated with and without SiNPs using time-correlated single-photon counting (TCSPC) spectroscopy. It is reported that ThT molecules can rotate freely in water for which they show a short fluorescence lifetime and low quantum yield, but upon binding to fibrillar aggregates, their rotation becomes restricted and hence, they show high fluorescence quantum yield along with a longer lifetime value.55 Figure 4 shows the fluorescence decay of ThT in the presence of different incubated HEWL solutions, and the average lifetime values obtained from the satisfactory fitting of the decay curves are given in Table 2. The decay of bound ThT in all HEWL solutions incubated with and without SiNPs is fitted by a multieponential function, whereas the unbound ThT in water and ThT mixed with native HEWL are fitted by a double-exponential function.55 From Figure 4, it is observed that the decay patterns of ThT in water and in the presence of native HEWL are almost similar indicating that ThT does not bind to the native state. The fluorescence decay of ThT in incubated HEWL solutions slows down and the associated average lifetime is found to increase. However, in the HEWL samples incubated with different concentrations of SiNPs, the

### Table 1. Calculated Lag Time, $t_{1/2}$, and Rate Constant of the HEWL Aggregation Process in the Presence and Absence of SiNPs

| Sample type                  | Lag phase (h) | $t_{1/2}$ (h) | Rate constant (h$^{-1}$) |
|------------------------------|---------------|---------------|--------------------------|
| Incubated HEWL              | 134.0 ± 0.9   | 195.5 ± 0.5   | 18.4 ± 0.3               |
| HEWL + 20 μg/mL SiNPs       | 113.1 ± 2.0   | 154.3 ± 0.7   | 26.5 ± 0.6               |
| HEWL + 50 μg/mL SiNPs       | 78.1 ± 2.1    | 116.8 ± 1.8   | 29.3 ± 0.7               |
| HEWL + 100 μg/mL SiNPs      | 62.3 ± 2.3    | 95.7 ± 1.4    | 34.8 ± 0.7               |

Figure 2. (a) ThT fluorescence profiles of HEWL–SiNP samples after incubation for 12 days. (b) ThT kinetic profiles for all incubated HEWL samples during the course of fibrillation.

Figure 3. AFM images of HEWL samples in the presence and absence of SiNPs: (a) incubated HEWL without SiNPs, (b) HEWL + 20 μg/mL SiNP, (c) HEWL + 50 μg/mL SiNP, and (d) HEWL + 100 μg/mL SiNP. The presence of SiNPs on the fibrillar structure is indicated by the circles.
decreasing effect in the decay rates of ThT becomes more pronounced with the concomitant increase of the average lifetime values. The longest average lifetime value of 0.790 ± 0.008 ns is observed for ThT in the incubated HEWL sample with 100 μg/mL SiNPs. This lifetime value is almost double with respect to the lifetime observed in incubated HEWL (0.410 ± 0.007 ns) without SiNPs. The data in Table 2 therefore supports the SiNP-induced fibrillation of HEWL.

### Table 2. Average Lifetime Values of ThT in the Presence of Native HEWL and HEWL after Incubation with Different Concentrations of SiNPs

| Sample type                  | Average lifetime (ns) |
|------------------------------|-----------------------|
| ThT                          | 0.020 ± 0.001         |
| Native HEWL                  | 0.020 ± 0.007         |
| Incubated HEWL               | 0.410 ± 0.007         |
| HEWL + 20 μg/mL SiNPs        | 0.520 ± 0.005         |
| HEWL + 50 μg/mL SiNPs        | 0.680 ± 0.001         |
| HEWL + 100 μg/mL SiNPs       | 0.790 ± 0.008         |

Figure 4. Fluorescence lifetime spectra of ThT of different HEWL samples in the presence and absence of SiNPs after incubation at 65°C and pH 2 for 12 days.

2.4. Assessment of Structural Changes of HEWL upon Fibrillation in the Presence of SiNPs: Trp and ANS Fluorescence Studies. HEWL has intrinsic fluorescence because of the presence of six Trp residues in its native structure; of these, Trp62 and Trp63 are placed at the hydrophobic cleft. The exposure of Trp residues affects the fluorescence quantum yield of Trp in the native state. Monitoring the Trp fluorescence of HEWL thus allows assessment of changes in the microenvironment of Trp after incubation in the presence and absence of SiNPs. Incubation of HEWL without SiNPs resulted in significant quenching of Trp fluorescence intensity and a red shift in the maximum emission wavelength from 348 to 360 nm (Figure 5a), indicating that the Trp moieties in HEWL are more accessible to the solvent that results in quenching. However, the Trp fluorescence intensity is found to increase gradually with increasing concentrations of SiNPs with no significant change of the red-shifted emission maxima. Moreover, the observed Trp fluorescence intensity in the case of the highest chosen concentration of SiNPs (100 μg/mL) is still lower as compared to the native HEWL (Figure 5a). The results suggest that Trp residues are buried in a hydrophobic environment in the presence of SiNPs, but the hydrophobic environment around the Trp residues in this case is different from that of native HEWL. The situation can be explained if we speculate that the Trp residues gradually become buried within the fibrillar network surrounded by the polar SiNPs and hence, a considerable extent of red shift in the emission maxima is still found in the Trp fluorescence profile of the solution with the highest concentration of SiNPs (100 μg/mL). In the AFM images, it is clearly observed that the fibrils formed in the presence of SiNPs are surrounded by nanoparticles.

Further information regarding the relative hydrophobic exposure of HEWL on treatment with SiNPs can be obtained using the hydrophobic fluorescent probe ANS. The intensity of ANS fluorescence emission is markedly enhanced upon binding to the hydrophobic surfaces of the protein, and consequently, the emission maxima show a blue shift. ANS fluorescence profiles for HEWL before and after treatment with SiNPs are shown in Figure 5b. It is observed that the fluorescence intensity of ANS in buffer medium and that in the presence of native HEWL are similar, but the intensity increases with a 14 nm blue shift when incubated in the

Figure 5. (a) Trp and (b) ANS fluorescence profiles of native HEWL and all incubated HEWL–SiNPs samples.
absence of nanoparticles. This suggests that the hydrophobicity around HEWL is increased upon incubation under the conditions employed. Increase in the ANS fluorescence intensity together with the shift of emission maxima further towards left (blue side) as compared to incubated HEWL is suggestive of the formation of fibrillar aggregates enriched with exposed hydrophobic residues in the presence of SiNPs. This study is in agreement with the observations from the Trp fluorescence studies.

2.5. Effect of SiNPs on the Secondary Structure of HEWL: Circular Dichroism (CD) Analyses. CD spectroscopy has been performed to measure the alteration of the secondary structure of HEWL after addition of SiNPs. Figure 6 shows the far-UV CD spectra of HEWL incubated in the presence and absence of SiNPs at different concentrations. Two negative minima, one at 208 nm and the other at 222 nm, are clearly visible in the spectrum of native HEWL (black line). This indicates the high percentage of helical conformation (47.6 ± 0.2% α-helix) in the native state of HEWL. The helical conformation of native HEWL is almost destroyed after incubation as indicated by the disappearance of the negative minima at 208 nm. A distinct negative band appears at 198 nm featuring the random coil conformation of HEWL. CD spectroscopy reveals that HEWL remains in the unfolded state even after incubation for 12 days under the conditions of high temperature and acidic pH. However, the addition of SiNPs triggers the aggregation process of HEWL under the same conditions resulting in a gradual shift of the negative minima from 198 to ~217 nm with increasing concentrations (Figure 6). In particular, 20 μg/mL SiNPs cannot change the structure of HEWL. The percentage of β-sheet-rich fibrillar aggregates increases in the solution increases with increasing concentrations of nanoparticles, and the maximum aggregation has been achieved at the highest concentration of SiNPs (100 μg/mL), and in this case, the minima are completely shifted from 198 nm to give rise to a new broad negative band near the 215–218 nm region, which is the characteristic β-sheet region. Tabulation using the online server DICHROWEB provides a quantitative estimation of the secondary structural content of HEWL before and after the treatment of SiNPs and is summarized in Table 3. Table 3 clearly indicates that native HEWL that contains the highest α-helical content shows maximum percentage of random coil conformation after incubation for 12 days in the absence of nanoparticles and renders the maximum extent of β-sheet formation in the presence of 100 μg/mL SiNPs.

2.6. Evaluation of the Structural Features of SiNP-Induced Fibrillar Aggregates Using FTIR Spectroscopy. Understanding the structural features of SiNP-treated fibrillar aggregates of HEWL can be obtained by FTIR spectroscopy. FTIR spectroscopy has an advantage over CD spectroscopy as it can discriminate parallel and antiparallel β-sheet configurations in the fibrillar aggregates of proteins. Fibrillar aggregates of antiparallel β-sheet structures show two positive peaks, one in the high-frequency region (above 1680 cm⁻¹) and the other in the low-frequency region (below 1640 cm⁻¹), and the absence of the high-frequency peak in the FTIR difference spectrum is a typical feature for the parallel β-sheet structure of fibrillar aggregates. Figure 7a displays the FTIR spectra for the protein samples. Here, we focus only on the amide I region (1700–1600 cm⁻¹) of HEWL to account for the alteration of the secondary structural components. There is no noticeable change except the shift of the higher-frequency band of native HEWL after incubation with SiNPs. This implies that alterations occur in the secondary structure of native HEWL during incubation. To obtain more detailed information about the secondary structural change, we have shown the FTIR difference spectra in Figure 7b where each spectrum is obtained after subtraction of the spectrum of native HEWL. In the difference spectra, the untreated HEWL does not show any high- or low-frequency peak. However, a peak in the higher-frequency region develops in the SiNP-treated samples, indicating the formation of fibrillar aggregates of an antiparallel β-sheet configuration. The peak gains prominence for higher concentrations of SiNPs, and in the case of 100 μg/mL concentration of SiNPs, this peak lies at 1686 cm⁻¹, which corresponds to the actual characteristic region for the antiparallel β-sheet structure aggregates. In addition, a peak developing in the lower-frequency region, that is, below 1630 cm⁻¹, is also observed at 100 μg/mL SiNPs. This indicates the formation of a large and extended β-sheet-rich fibrillar structure with a predominantly antiparallel configuration at the higher concentration of SiNPs.
The difference spectra also points toward the kinetics of the fibrillation in the presence of the SiNPs. It is reported that the fibrillation occurs following a one-step or two-step mechanism. The two-step mechanism is accompanied by the formation of toxic oligomers, whereas in the one-step mechanism, the oligomers formed in the nucleation phase are too unstable to exist in the solution for at least a measurable time period and dissociate instantaneously into the fibril precursor species, making the process free from oligomeric intermediates. Usually, the oligomer-free pathway is observed at elevated temperatures. The assembly process involving oligomer formation or without oligomer formation can be detected using FTIR spectroscopy. A low-frequency peak that develops below 1620 cm$^{-1}$ is indicative of oligomer assembly, whereas oligomer-free assembly induces a peak above 1620 cm$^{-1}$. From Figure 7b, it is evident that fibrillar aggregates formed in the 100 μg/mL SiNP-treated sample yield a distinct peak at 1623 cm$^{-1}$, implying that fibrillation proceeds through an oligomer-free pathway, especially at higher concentrations of SiNPs. The considerably lower lag time in the 100 μg/mL SiNP-treated sample (obtained from ThT-based kinetic studies) also supports this oligomer-free fibrillation kinetics. Because fibrillar toxicity is initiated from the oligomeric intermediates, we can say that the fibrillar aggregates developed at higher concentration of SiNPs should be apparently less toxic.

2.7. Measurement of Sizes of Fibrillar Aggregates Using Dynamic Light Scattering (DLS) Studies. The oligomer-free fibrillation process in the presence of SiNPs can also be probed using DLS measurements. The DLS measurements were conducted with the incubated samples to differentiate the sizes of the fibrillar aggregates in the presence of SiNPs. Figure 8 shows the histograms of the intensity of the scattered light for all samples, and the obtained average sizes are given in Table 4. HEWL in its native state has an average size of only 4.2 ± 0.1 nm, which upon incubation increases to 58.6 ± 0.7 nm. The increase in size after incubation can be accounted for by the formation of smaller globular aggregates from unfolded HEWL as observed in the AFM image (Figure 3). A significant size difference has been observed when HEWL is incubated with SiNPs, indicating the formation of larger fibrous aggregates. The average particle sizes of SiNP-treated HEWL samples are found to increase with concentration, that is, to 531.0 ± 3.0, 955.6 ± 3.0, and 1106.0 ± 7.0 nm in the presence of 20, 50, and 100 μg/mL SiNPs, respectively. This is consistent with the AFM images where the extent of cross-linking between the fibrillar aggregates was found to increase with increasing concentrations of SiNPs. Moreover, the broad size distribution pattern in the SiNP-
treated samples reveals the heterogeneity of the fibrillar aggregates. The broad distribution in sizes may also appear because of the presence of aggregated nanoparticles as SiNPs are reported to be aggregated upon interaction with HEWL.\textsuperscript{33,65} HEWL samples treated with 50 and 100 μg/mL SiNPs do not show any size distribution pattern at the lower ranges (<100 nm). This clearly ruled out the possibility of the formation of oligomeric intermediates during the fibrillation process, which is in agreement with the FTIR results.

2.8. Physical Explanation for the SiNP-Induced Fibrillation of HEWL. The zeta potential measurements of SiNPs suggest that nanoparticles are almost neutral at pH 2 as stated earlier, whereas HEWL (pI = 10.7) bears a high positive charge (\( \sim 15 \)−17 positive charges) at this pH.\textsuperscript{66} This implies that electrostatic interactions between HEWL molecules and SiNPs are expected to be weak. The high charge density on HEWL may lead to its adsorption on the surface of the SiNPs to get rid of the strong self-repulsion existing between HEWL molecules. The adsorption behavior of HEWL on SiNPs has been checked, and it was observed that the HEWL adsorption capacity of 1 mg/mL SiNPs is 0.05 mg at pH 2 (Figure S2 of Supporting Information). The adsorption most likely occurred because of the hydrogen bonding interactions between the silanol groups of SiNPs and the \(-\text{COOH}\) moieties present in the side-chain acidic amino acid residues (pK\(_a\) ≤ 3) or the regions containing the acidic amino acid residues of HEWL at pH 2. As the polypeptide chain is in an unfolded form under the conditions, the other regions are expected to be exposed to the solvent. If the solvent-exposed parts are really the “hot spots” of aggregation, the fibrillation process is likely to proceed at a faster rate. The hot spot areas of HEWL have been identified by the help of an online server “AGGRESCAN”,\textsuperscript{67} and the predicted hot spot areas against the amino acids sequence of HEWL are shown in Figure 9. Interestingly, the amino acid residues present in the hot spot regions show that almost all acidic amino acid residues are located outside the hot spot zones of HEWL (identified by a green color). Thus, we can say that the SiNPs are likely to adsorb the non-amylogenic regions of HEWL preferentially where most of the acidic amino acids are located, resulting in the exposure of the aggregation-prone or hot spot regions of HEWL toward the solvent. As a result, the fibrillation/aggregation process is triggered. Furthermore, earlier literature suggests that under heat and acidic conditions, HEWL is hydrolyzed into smaller fragments and the fragmentation occurs via the peptide bond cleavage of the Asp amino acid residues.\textsuperscript{23,68} Among the fragmented peptides, the 49−101, 53−101, and 57−107 residue fragments are identified as the most amyloid-prone fragments, whereas the 1−38 and 108−129 residue fragments correspond to the non-amyloidogenic regions of HEWL.\textsuperscript{23} Therefore, the adsorption of acidic amino acid residues, especially Asp residues by SiNPs, facilitates the cleavage of Asp-containing peptide bonds that results in the formation of amyloidogenic fragments at a greater rate.

Figure 9. “Hot spot” regions of HEWL obtained from AGGRESCAN are shown by the red color histogram plotted against the amino acid sequences of HEWL (all acidic amino acid residues are identified by a green color).
3. CONCLUSIONS

In the present study, we have demonstrated that SiNPs trigger the amyloid formation of HEWL in a concentration-dependent manner under heat and acidic conditions. Our results indicate that the fibrillation kinetics does not involve the formation of toxic oligomeric intermediates at the higher concentrations of SiNPs. We further reveal that the fibrillar aggregates formed at the higher concentrations of SiNPs preferably adopt an antiparallel β-sheet configuration. We believe that the present study will provide a deeper insight into the understanding of the evolution of nontoxic amyloid fibrils that could be useful to demonstrate the underlying mechanism of amyloid fibrillation.

4. MATERIALS AND METHODS

4.1. Materials. HEWL, Thioflavin T (ThT), Congo red, and 1-naphthalenesulfonic acid (ANS) were purchased from Sigma-Aldrich (St. Louis, U.S.A.). Tetraethoxysilane (purity, 98%) was purchased from Alfa Aesar, India. Other chemicals used for this study were obtained from SRL, India, and used as received. Spectroscopic measurements were performed using a UV−vis spectrophotometer (Shimadzu, Model 1800) and a Horiba Jobin Yvon Fluoromax-4 spectrofluorometer.

4.2. Preparation of Silica Nanoparticles. Colloidal solutions of SiNPs of diameters 50−60 nm were prepared in a nonaqueous system of tetraethoxysilane (TEOS) using a sol−gel method as described by Stöber et al. In brief, the required amount of ammonium hydroxide was mixed with half of the required volume of anhydrous ethanol. The alkaline solution was then added dropwise to a requisite volume of TEOS solution in ethanol with continuous stirring at room temperature (25 °C). The molar ratios of ethanol/NH₃/H₂O/TEOS are maintained at 15.40:0.11:2.40:1.00. The resulting reaction mixture was kept at room temperature for 3 days to allow the hydrolysis of TEOS as much as possible. Finally, the nanoparticles were separated from the reaction mixture using centrifugation followed by washing three to four times and stored at 4 °C for further use.

4.3. Preparation of HEWL Fibrillar Solutions. A stock solution of HEWL was prepared by weighing 50 mg of dry HEWL powder and dissolving in 1 mL double-distilled water and stored at 0 °C. The concentration of the stock solution was measured spectrophotometrically using HEWL (ε₂₃₀ = 37 646 M⁻¹cm⁻¹). HEW solutions in the presence and absence of various concentrations (20, 50, and 100 μg/mL) of SiNPs were prepared in 50 mM HCl−KCl buffer (pH 2) and incubated at 65 °C and pH 2 for 12 days. The pH of the protein solutions was checked before and after incubation and found to be stable. The final concentration of HEWL taken for incubation was 150 μM. All incubated solutions were left overnight at room temperature at the end of incubation for further use. The desired concentration for each experimental measurement was achieved by dilution with 20 mM phosphate buffer of pH 7.4.

4.4. Congo Red Binding Assay. The incubated HEWL solutions (10 μM) in the presence and absence of different concentrations of SiNPs were added to 7 μM Congo red in 20 mM phosphate buffer (pH 7.4), and the mixtures were kept at room temperature for 30 min in the dark before recording UV−vis absorption spectra. The spectra were recorded in the wavelength region 200−600 nm. The control experiments were also carried out in the absence of SiNPs.

4.5. Thioflavin T (ThT) Assay. For the ThT fluorescence assays, the samples (5 μM) were mixed with ThT solution (10 μM) and incubated at room temperature in the dark for 5 min to allow binding. The spectra were recorded in 20 mM phosphate buffer (pH 7.4) from 460 to 600 nm on excitation at 440 nm. The slit width and the integration time were set at 5 nm and 0.3 s, respectively.

Fibrillar growth was monitored by withdrawing aliquots at different time intervals up to 12 days, mixed with the solution of ThT, and the spectra were recorded using the same parameter. The kinetic profiles for all samples were then fitted by a sigmoidal curve and the lag phase, and the rate constant of the protein aggregation was determined from the following empirical eq 1:

\[ Y = Y_i + m_x x + \frac{m_x x}{1 + e^{-(x-x_0)/\tau}} \]

where, Y is the ThT fluorescence intensity and x is the incubation time. x₀ indicates the half maximal ThT fluorescence time (t₁/₂). The lag time and the rate of aggregation are defined by x₀ = τ and 1/τ, respectively. All other parameters present in the equation are determined by fitting.

4.6. Time-Resolved Fluorescence Measurement. For time-resolved measurements, a DeltaFlex modular fluorescence lifetime system from HORIBA Scientific was used. The samples were excited at 440 nm using a picosecond laser diode, and the signals were collected at the magic angle (55°) using a high-voltage microchannel plate photomultiplier tube (PPD-850) of HORIBA. The instrument response function of the setup was ∼183 ps. The analyses of the data were done using EZ Time and decay analysis software.

4.7. Trp and ANS Fluorescence Spectroscopic Studies. Trp and ANS fluorescence emission spectra for all fibrillar samples in the presence and absence of SiNPs were recorded at 315−450 and 400−600 nm wavelength ranges, respectively. For Trp fluorescence, samples were excited at 295 nm, whereas for ANS fluorescence spectroscopy, the excitation wavelength was set at 370 nm. The slit width as well as the integration time for both studies was kept at 5 nm and 0.3 s, respectively. Sample concentrations were kept at 10 μM in both cases. Before recording the ANS fluorescence spectroscopy, 2 μM ANS was mixed with the protein solutions and incubated at room temperature for 1 h in the dark. For each experiment, the spectra were corrected by blank subtraction.

4.8. Far-UV CD Measurements. Far-UV CD spectra of different fibrillar samples (10 μM) were taken in a quartz cuvette of 0.1 cm path length on a JASCO-815 spectropolarimeter at 25 °C in the wavelength range from 190−240 nm at a scan rate of 50 nm/min. The spectra were corrected by the respective controls. The secondary structural content was determined from these spectra using DICHROWEB software.

4.9. Atomic Force Microscopy (AFM) Studies. AFM images were obtained with 10 μL of fibrillar samples (10 μM) in the presence and absence of different concentrations of SiNPs applied on the freshly cleaved mica foils. They were then air-dried and scanned under an atomic force microscope (model no. S500, Agilent Technologies). Samples were imaged in a silicon probe cantilever of length 100 μm with a resonance frequency of 46−236 kHz and a force constant of 21−98 Nm⁻¹. The detailed instrumentation procedure and sample
preparation techniques for FESEM and TEM are given in the Supporting Information.

4.10. Fourier Transform Infrared Spectroscopy (FTIR) Studies. FTIR spectra of SiNPs (1 mg of solid SiNPs) and different SiNP-treated fibrillar samples (2 μL) were obtained from an IRTTracer-100 FTIR spectrometer (Shimadzu) at room temperature in the spectral range 4000–400 cm⁻¹. Diamond ATR was used for the acquisition of the spectra, and the number of acquisitions given for each measurement was 64. The spectral resolution of the FTIR instrument was kept at 8 cm⁻¹. A spectrum of air without samples was collected before scanning the samples. This background was used to correct the spectra collected for the samples.

4.11. Dynamic Light Scattering (DLS) and Zeta Potential Measurements. DLS measurements of SiNP solution (3 μg/mL) as well as different fibrillar samples (5 μM) were carried out on a Malvern Nano ZS instrument fitted with a 4 mW He–Ne laser beam (λ = 632.8 nm, scattering angle of 173°) and a thermostatic sample chamber at 25 °C. For zeta potential measurements of the samples (7 μM), a Malvern Nano ZS instrument equipped with the same accessories as mentioned for DLS studies was used.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b03169.

Characterization of the SiNPs by DLS and zeta potential measurements, XRD and FTIR studies, FESEM and TEM imaging techniques, and UV–vis spectra of native HEWL in the presence and absence of SiNPs (PDF)

AUTHOR INFORMATION

Corresponding Author
*E-mail: swagata@chem.iitkgp.ac.in. Phone: +91 3222 283306. Fax: +91 3222 255303.

ORCID
Swagata Dasgupta: 0000-0003-2074-1247

Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors would like to acknowledge the Ministry of Human Resource Development, Government of India for research grants (IIT/SRIC/ATDC/CDEM/2013-2014/118) and for financial support. M. K. is grateful to the University Grants Commission (UGC), India for her senior research fellowship. The authors express sincere thanks to Prof. S. DasGupta and his group, Department of Chemical Engineering, IIT Kharagpur, for DLS measurements, Central Research Facility, IIT Kharagpur, and Department of Chemistry, IIT Kharagpur for providing other experimental facilities.

REFERENCES

(1) Nelson, R.; Eisenberg, D. Structural Models of Amyloid-Like Fibris. Adv. Protein Chem. 2006, 73, 235–382.
(2) Eisenberg, D.; Jucker, M. The Amyloid State of Proteins in Human Diseases. Cell 2012, 148, 1188–1203.
(3) Sawaya, M. R.; Sambashivan, S.; Nelson, R.; Ivanova, M. I.; Sievers, S. A.; Apostol, M. I.; Thompson, M. J.; Balbirnie, M.; Wiltzius, J. J.; McFarlane, H. T.; Madsen, A. Ø.; Riekel, C.; Eisenberg, D. Atomic Structures of Amyloid Cross-β Spines Revealed Steric Zippers. Nature 2007, 447, 453–457.
(4) Agorogiannis, E. I.; Agorogiannis, G. I.; Papadimitriou, A.; Hadjigeorgiou, G. M. Protein misfolding in neurodegenerative diseases. Neuropathol. Appl. Neurobiol. 2004, 30, 215–224.
(5) Cohen, F. E.; Kelly, J. W. Therapeutic approaches to protein-misfolding diseases. Nature 2003, 426, 905–909.
(6) Dobson, C. M. Protein folding and misfolding. Nature 2003, 426, 884–890.
(7) Dobson, C. M. Protein misfolding, evolution and disease. Trends Biochem. Sci. 1999, 24, 329–332.
(8) Chiti, F.; Webster, P.; Talde, N.; Clark, A.; Stefani, M.; Ramponi, G.; Dobson, C. M. Designing Conditions for in Vitro Formation of Amyloid Protofilaments and Fibrils. Proc. Natl. Acad. Sci. U. S. A. 1999, 96, 3590–3594.
(9) Chiti, F.; Dobson, C. M. Protein Misfolding, Functional Amyloid, and Human Disease. Annu. Rev. Biochem. 2006, 75, 333–366.
(10) Jahn, T. R.; Makin, O. S.; Morris, K. L.; Marshall, K. E.; Tian, P.; Sikorski, P.; Serpell, L. C. The common architecture of cross-beta amyloid. J. Mol. Biol. 2010, 395, 717–727.
(11) Klunk, W. E.; Pettegrew, J. W.; Abraham, D. J. Two simple methods for quantifying low-affinity dye-substrate binding. J. Histochem. Cytochem. 1989, 37, 1293–1297.
(12) III LeVine, H. Thiolflavine T interaction with synthetic Alzheimer’s disease beta-amyloid peptides: detection of amyloid aggregation in solution. Protein Sci. 1993, 2, 404–410.
(13) Uversky, V. N.; Fink, A. L. Conformational constraints for amyloid fibrillation: the importance of being unfolded. Biochim. Biophys. Acta. 2004, 1698, 131–153.
(14) Jahn, T. R.; Tennent, G. A.; Radford, S. E. A Common β-Sheet Architecture Underlies in Vitro and in Vivo β2-Microglobulin Amyloid Fibrils. J. Biol. Chem. 2008, 283, 17279–17286.
(15) Gosal, W. S.; Morten, I. J.; Hewitt, E. W.; Smith, D. A.; Thompson, N. H.; Radford, S. E. Competing pathways determine fibril morphology in the self-assembly of beta2-microglobulin into amyloid. J. Mol. Biol. 2005, 351, 850–864.
(16) Goldsberry, C.; Frey, P.; Olivieri, V.; Aebi, U.; Müller, S. A. Multiple assembly pathways underlie amyloid-beta fibril polymorphisms. J. Mol. Biol. 2005, 352, 282–298.
(17) Lee, J.; Culyba, E. K.; Powers, E. T.; Kelly, J. W. Amyloid-β Fibrils by Nucleated Conformational Conversion of Oligomers. Nat. Chem. Biol. 2011, 7, 602–609.
(18) Vieira, M. N.; Forny-Germaino, L.; Saraiva, L. M.; Sebollera, A.; Martinez, A. M.; Houzel, J. C.; De Felice, F. G.; Ferreira, S. T. Soluble oligomers from a non-disease related protein mimic Aβ-induced tau hyperphosphorylation and neurodegeneration. J. Neurochem. 2007, 103, 736–748.
(19) Morozova-Roche, L. A.; Zurdo, J.; Spencer, A.; Norpe, W.; Receveur, V.; Archer, D. B.; Joniau, M.; Dobson, C. M. Amyloid fibril formation and seeding by wild-type human lysozyme and its disease-related mutational variants. J. Struct. Biol. 2000, 130, 339–351.
(20) Goda, S.; Takano, K.; Yamagata, Y.; Nagata, R.; Akutsu, H.; Maki, S.; Namba, K.; Yutani, K. Amyloid Protofilament Formation of Hen Egg Lysozyme in Highly Concentrated Ethanol Solution. Protein Sci. 2000, 9, 369–375.
(21) Vernaglia, B. A.; Huang, J.; Clark, E. D. Guanidine Hydrochloride Can Induce Amyloid Fibril Formation from Hen Egg-White Lysozyme. Biomacromolecules 2004, 5, 1362–1370.
(22) Khan, J. M.; Qadeer, A.; Chaturvedi, S. K.; Ahmad, E.; Rehman, S. A. A.; Gourinath, S.; Khan, R. H. SDS Can Be Utilized as an Amyloid Inducer: A Case Study on Diverse Proteins. PLoS One. 2012, 7, No. e29694.
(23) Chaudhary, A. P.; Vispute, N. H.; Shukla, V. K.; Ahmad, B. A comparative study of fibrillation kinetics of two homologous proteins under identical solution condition. Biochimie. 2017, 132, 75–84.
(24) Pramanik, S.; Ahmad, B. Exposure of Aggregation-Prone Segments is the Requirement for Amyloid Fibril Formation. Curr Protein Pept Sci. 2018, 19, 1024–1035.

DOI: 10.1021/acsomega.8b03169
ACS Omega 2019, 4, 1015–1026
(25) Frare, E.; Polverino De Laureto, P.; Zurdo, J.; Dobson, C. M.; Fontana, A. A Highly Amyloidogenic Region of Hen Lysozyme. J. Mol. Biol. 2004, 340, 1153–1165.

(26) Mishra, R.; Sörgierd, K.; Nyström, S.; Nordgärd, A.; Yu, Y. C.; Hammarström, P. Lysozyme Amyloidogenesis is Accelerated by Specific Nicking and Fragmentation but Decelerated by Intact Protein Binding and Conversion. J. Mol. Biol. 2007, 366, 1029–1044.

(27) Zaman, M.; Ahmad, E.; Qadeer, A.; Rabbani, G.; Khan, R. H. Nanoparticles in Relation to Peptide and Protein Aggregation. Int. J. Nanomedicine. 2014, 9, 899–912.

(28) Linse, S.; Cabaliero-Lago, C.; Xue, W. F.; Lynch, I.; Lindman, S.; Thulin, E.; Radford, S. E.; Dawson, K. A. Nucleation of protein fibrillation by nanoparticles. Proc. Natl. Acad. Sci. U. S. A. 2007, 104, 8691–8696.

(29) Zhang, J.; Zhou, X.; Yu, Q.; Yang, L.; Sun, D.; Zhou, Y.; Liu, J. Epigallocatechin-3-Gallate (EGCG)-Stabilized Selenium Nanoparticles Coated with Tet-1 Peptide To Reduce Amyloid-β Aggregation and Cytoxicity. ACS Appl. Mater. Interfaces. 2014, 6, 8475–8487.

(30) Deb Nath, K.; Shekhar, S.; Kumar, V.; Jana, N. R.; Jana, N. R. Efficient Inhibition of Protein Aggregation, Disintegration of Aggregates and Lowering of Cytoxicity by Green Tea Polyphenol-Based Self-Assembled Polymer Nanoparticle. ACS Appl. Mater. Interfaces. 2016, 8, 20309–20318.

(31) Palmal, S.; Maity, A. R.; Singh, B. K.; Basu, S.; Jana, N. R.; Jana, N. R. Inhibition of Amyloid Fibril Growth and Dissolution of Amyloid Fibrils by Curcumin–Gold Nanoparticles. Chem. Eur. J. 2014, 20, 6184–6191.

(32) Vallet-Regi, M. Nanostructured mesoporous silica matrices in Nanomedicine. J. Intern. Med. 2010, 267, 22–43.

(33) Lundqvist, M.; Stigler, J.; Elia, G.; Lynch, I.; Cedervall, T.; Dawson, K. A. Nanoparticle size and surface properties determine the protein corona with possible implications for biological impacts. Proc. Natl. Acad. Sci. U. S. A. 2008, 105, 14265–14270.

(34) Taebnia, N.; Morshedi, D.; Doostkam, M.; Yaghmaei, S.; Arpanaei, A. The effect of mesoporous silica nanoparticles on the protein corona with possible implications for biological impacts. J. Phys. Chem. B. 2012, 116, 8487–8496.

(35) Flory, P. J.; Huggins, C. M.; Jus, R.; Yu, Y. M.; Jus, R.; Yu, Y. M. Inhibition of Amyloid Fibril Growth and Dissolution of Amyloid Fibrils by Curcumin–Gold Nanoparticles. Chem. Eur. J. 2014, 20, 6184–6191.
(63) Serio, T. R.; Cashikar, A. G.; Kowal, A. S.; Sawicki, G. J.; Moslehi, J. J.; Serpell, L.; Arnsdorf, M. F.; Lindquist, S. L. Nucleated Conformational Conversion and the Replication of Conformational Information by a Prion Determinant. *Science* 2000, 289, 1317–1321.

(64) Foley, J.; Hill, S. E.; Mitl, T.; Mulaj, M.; Ciesla, M.; Robeel, R.; Perschilli, C.; Raynes, R.; Westerheide, S.; Muschol, M. Structural fingerprints and their evolution during oligomeric vs. oligomer-free amyloid fibril growth. *J. Chem. Phys.* 2013, 139, 121901.

(65) Bharti, B.; Meissner, J.; Findenegg, G. H. Aggregation of Silica Nanoparticles Directed by Adsorption of Lysozyme. *Langmuir* 2011, 27, 9823–9833.

(66) Kuehner, D. E.; Engann, J.; Fergg, F.; Wernick, M.; Blanch, H. W.; Prausnitz, J. M. Lysozyme Net Charge and Ion Binding in Concentrated Aqueous Electrolyte Solutions. *J. Phys. Chem. B* 1999, 103, 1368–1374.

(67) Conchillo-Solé, O.; de Groot, N. S.; Avilés, F. X.; Vendrell, J.; Daura, X.; Ventura, S. AGGRESCAN: a server for the prediction and evaluation of “hot spots” of aggregation in polypeptides. *BMC Bioinformatics* 2007, 8, 65.

(68) Mishra, R.; Sorgjerd, K.; Nystrom, S.; Nordigarden, A.; Yu, Y.-C.; Hammarstrom, P. Lysozyme Amyloidogenesis is Accelerated by Specific Nicking and Fragmentation but Decelerated by Intact Protein Binding and Conversion. *J. Mol. Biol.* 2007, 366, 1029–1044.

(69) Stober, W.; Fink, A.; Bohn, E. Controlled Growth of Monodisperse Silica Spheres in the Micron Size Range. *J. Colloid Interface Sci.* 1968, 26, 62–69.

(70) Pace, C. N.; Vajdos, F.; Fee, L.; Grimsley, G.; Gray, T. How to measure and predict the molar absorption coefficient of a protein. *Protein Sci.* 1995, 4, 2411–2423.

(71) Uversky, V. N.; Li, J.; Fink, A. L. Evidence for a partially folded intermediate in alpha-synuclein fibril formation. *J. Biol. Chem.* 2001, 276, 10737–10744.

(72) Whitemore, L.; Wallace, B. A. DICHROWEB, an online server for protein secondary structure analyses from circular dichroism spectroscopic data. *Nucleic. Acids. Res.* 2004, 32, 668–673.