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Role of *N*-methyl-8-(alkoxy)quinolinium iodide in suppression of protein–protein interactions

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Abstract. There is a great deal of interest in developing small molecule inhibitors of protein misfolding and aggregation due to a growing number of pathologic states known as amyloid disorders. In searching for alternative ways to reduce protein–protein interactions or to inhibit the amyloid formation, the inhibitory effects of cationic amphiphile viz. *N*-methyl-8-(alkoxy)quinolinium iodide on aggregation behaviour of hen egg white lysozyme (HEWL) at alkaline pH has been studied. Even though the compounds did not protect native HEWL from conformational changes, they were effective in diminishing HEWL amyloid formation, delaying both nucleation and elongation phases. It is likely that strong binding in the HEWL compound complex, raises the activation energy barrier for protein misfolding and subsequent aggregation, thereby retarding the aggregation kinetics substantially.

Keywords. Inhibitors; amyloid; protein; aggregation; conformation; HEWL.

1. Introduction

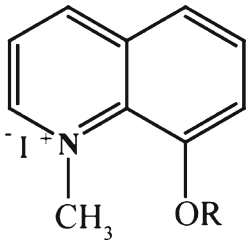
Self-assembly of proteins into their native conformation has been one of the seminal topics in biochemistry for more than half a century.[1–3] Proper conformations of native protein play an essential role in normal cell functioning and if somehow, the correct folding of protein does not occur, appropriate cellular repair machinery fails to function, protein aggregation and amyloid formation may occur, a process evident in several human diseases.[4,5] Accumulation of insoluble protein aggregates or plaques is responsible for nearly twenty different types of amyloidoses which are either neuropathic like Alzheimer's disease (AD), Parkinson's disease (PD) or non-neuropathic such as diabetes type II and hereditary systemic amyloidoses.[6,7]

Although the detailed mechanism of protein misfolding and aggregation remains to be elucidated, inhibiting the disease-prone alteration in protein structure is urgently required as therapy against a wide range of diseases.[8,9] Aggregation of proteins is a multi-step process with several intermediates; hence varied approaches at different stages are being used to block the growth of protein oligomers either *in vitro* or *in vivo*.[8,9] One of the most promising strategies to prevent protein–protein interactions is to stabilize their native state by small molecules.[10,11] As the native structure of protein undergoes partial unfolding before aggregation, stabilization of the native state may increase the activation energy barrier, thereby slowing down the aggregation and moving away from amyloidogenic state.

Hen egg white lysozyme (HEWL) has been used as a model protein to study fibril formation *in vitro*[12–15] as its structure and folding mechanisms have been extensively characterized.[16–18] These features make lysozyme an interesting model to study the amyloid aggregation. HEWL can be converted to amyloid fibrils under environmental conditions of high pH[19] or low pH and elevated temperatures.[12,20] The fibrils of HEWL share similar characteristics to other amyloids and are toxic to the cultured cells.

Thus, inhibition or reversion of amyloid formation may represent a possible therapeutic strategy for the prevention and treatment of amyloid diseases.[21–24] A number of small molecules like ligands, substrates or enzyme inhibitors, can selectively bind to native protein conformer and prevent its structural fluctuation.[8,25,26] In our quest to study the interaction between synthetic amphiphiles and proteins,[27–32] here, we report the suppression of protein–protein interactions at alkaline pH by the cationic amphiphiles viz. *N*-methyl-8-(alkoxy)quinolinium iodide (scheme 1).[32] Where, R = C_4H_9(**1**), C_8H_{17}(**2**) and $C_{12}H_{25}$(**3**).

*For correspondence



Scheme 1. Structure of the compounds (**1**, **2** and **3**).

2. Experimental

2.1 *Materials*

Hen egg white lysozyme (HEWL), 8-hydroxyquinoline, alkyl halides, congo red (CR) and thioflavin T (ThT) were procured from Sigma USA. All other chemicals employed were of analytical grade and used without further purification.

2.2 *Synthesis of the compounds*

Initially, 8-(alkoxy)quinolines were prepared followed by *N*-methylation using methyl iodide to get the *N*-methyl-8-(alkoxy)quinolinium iodide.[32,33] Briefly, to a solution of 8-(alkoxy)quinoline in dry acetonitrile methyl iodide was added and refluxed for 6 h to complete the reaction. The products (scheme 1) obtained were re-crystallized from methanol and characterized by NMR, IR and melting point (supporting information).

2.3 *Sample preparation*

Stocks of HEWL and *N*-methyl-8-(alkoxy)quinolinium iodide were freshly prepared in deionized water and DMSO. Aggregation was induced by dissolving the HEWL solutions of 1 mg/mL (~70 μM) in a 50 mM phosphate buffer of pH 12.2, while the concentrations of the compounds used to check their inhibitory effect on HEWL aggregation was 60 μM. To assess the effect of inhibitors, control samples which contained no inhibitors but were identical otherwise were also run in parallel. All experiments were performed at room temperature (RT) and repeated 4–5 times to obtain the reproducible results for each set.

2.4 *Congo red binding assay*

After overnight incubation (~12 h) of HEWL either alone or with the compounds (60 μM) in a 50 mM phosphate buffer of pH 12.2 was mixed with a freshly prepared aqueous solution of CR (20 μM). Absorbance of CR under different experimental conditions was recorded from 400 to 800 nm on a Perkin Elmer Lambda-25 UV-visible spectrophotometer, while the emission spectrum of CR with HEWL under different experimental conditions was measured using Jobin-Yvon Fluoromax-4 and Carry Eclipse spectrofluorometer. All samples were excited at 490 nm, using slit widths of 5 nm in 540 to 750 nm range.

2.5 *Thioflavin T binding assay*

Time dependent kinetics of HEWL amyloid formation was also monitored by thioflavin T (ThT) binding assay. After overnight incubation (~12 h) of HEWL in a 50 mM phosphate buffer of pH 12.2, either alone or with the compounds (60 μM) were transferred to a freshly prepared aqueous solution of ThT diluted with 20 mM, glycine–glycine buffer of pH 8.5, in such a way so that the molar concentration of ThT was two-fold excess over HEWL in the assay medium.[8,9] The protein was typically ~10 μM in the assay medium after dilution. The samples were excited at 450 nm and subsequent fluorescence emission between 470 and 550 nm was recorded on Jobin-Yvon Fluoromax-4 and Carry Eclipse spectrofluorometer at different time intervals.

2.6 *Circular dichroism*

CD spectra of HEWL under different experimental conditions were recorded from 190 to 250 nm (far-UV) using a Jasco J815 spectropolarimeter. The spectra were collected at room temperature using a quartz cell of 10 mm path length with a bandwidth of 1 nm and at a scan speed of 100 nm per minute. The spectra were recorded by diluting the concentrated samples by ~10 fold in a 50 mM phosphate buffer of pH 7.0. The spectra could not be measured at pH 12.2 due to the large absorption by the buffer alone below 230 nm wavelengths.

2.7 *Atomic force microscopy*

HEWL samples incubated under different experimental conditions were diluted 5-fold in incubating buffer and added to freshly cleaved mica. After a few minutes, these were rinsed with deionized water to remove unabsorbed sample and dried at room temperature. AFM imaging was performed on Picoplus microscope (Molecular Imaging, USA) under non-contact or MAC MODE.

3. Results and discussion

3.1 *Binding of the compounds with HEWL*

Proteins fluorescence is a sensitive tool to investigate the interaction with ligands or other compounds.

Fluorescence of protein is mainly due to the presence of tryptophan (Trp) residues and is widely used as a tool to monitor changes in protein structure and to make inferences regarding local structure and dynamics.[34] Hence, in order to investigate the interactions between the amphiphilic compounds and HEWL we used fluorescence spectroscopy. Binding of the compounds with HEWL after overnight incubation were ascertained by comparing the tryptophan emission of HEWL bound compounds with unbound one. The fluorescence spectrum of HEWL presents strong emission at 345 nm, when excited at 295 nm in an aqueous buffer solution of pH 7.0. Excitation wavelength of 295 nm was chosen to avoid the contribution from the tyrosine residues in proteins and the compounds (**1**–**3**) were non-fluorescent under the present experimental conditions. The overnight incubation of HEWL with compounds leads to a significant change in its fluorescence spectrum, indicating interaction of the compounds with HEWL (figure 1). Figure 1 depicts that the overnight incubation of HEWL with the compounds at pH 7.0 results in diminished fluorescence intensity accompanied by a slight blue shift in λ_{max} of HEWL by ~5 nm upon complexation with the compounds. The blue shift in HEWL compound composites arises due to the change in the environment of tryptophan from a solvent exposed region to a relatively more non-polar environment in the presence of compounds,[35] confirmed the binding of the compounds with HEWL.

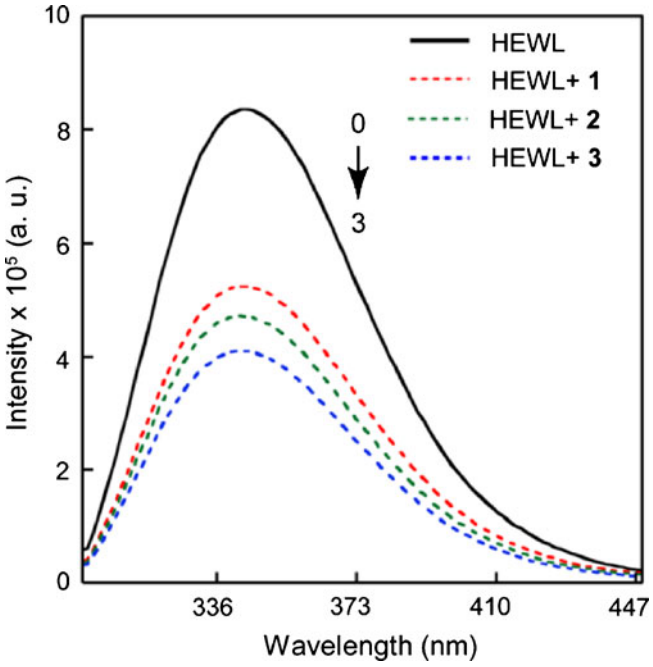


Figure 1. Emission spectra of HEWL in the absence (solid line) and presence of compounds (dotted line) in phosphate buffer of pH 7.0. Where trace 0: HEWL only, trace 1–3: HEWL in the presence of compound **1**, **2** and **3** after overnight (~12 h) incubation.

3.2 *Congo red absorbance assay*

As protein starts to aggregate, the spectral properties of congo red (CR) exhibits a considerable red shift along with an increase in absorbance maxima.[36,37] Hence, we have used CR absorbance property to investigate the inhibitory effect of the compounds on HEWL aggregation process (figure 2). The absorbance of CR at ~515 nm in the presence of HEWL incubated with and without ccompounds have been plotted as a function of incubation time (figure 2b) along with the change in wavelength maxima as a function of time (supporting information). The HEWL samples alone exhibited a greater absorbance difference and wavelength maxima, which is indicative of a strong binding affinity between CR dye and HEWL,[36,37] thus signifying a higher degree of aggregate formation. The control samples of HEWL without compounds showed a shift of CR wavelength maxima from 490 to 515 nm, while in the presence of the compounds **1**, **2** and **3** the shift was from 490 to 514, 512 and 510 nm, respectively (supporting information). The absorbance spectra of CR in the presence of the compounds have been also recorded and found that compounds by themselves did not alter the CR spectral property significantly.

3.3 *Congo red fluorescence assay*

The inhibitory effect of the compounds, on the aggregation of HEWL was also examined via CR fluorescence measurements (figure 3). As shown in figure 3b, the CR fluorescence intensity in the presence of HEWL at pH 12.2 by itself increased dramatically in the first 50 h and then reached a plateau. In contrast, the pH 7.0 samples revealed a relatively low fluorescence intensity that remained fairly invariant with time (supporting information).

The analysis of results indicate that the percentage of maximum CR fluorescence intensity in the presence of HEWL samples incubated with compounds **1**, **2** and **3** were 83, 51 and 21, respectively, implying that considerably smaller amount of aggregates were present in HEWL samples incubated with compounds **3** and **2** as compared with that of compound **1**. Notably, we have also found that none of the compounds by itself quenched CR fluorescence significantly at the concentrations examined in this study (supporting information). As it is evident from the control experiment between dyes

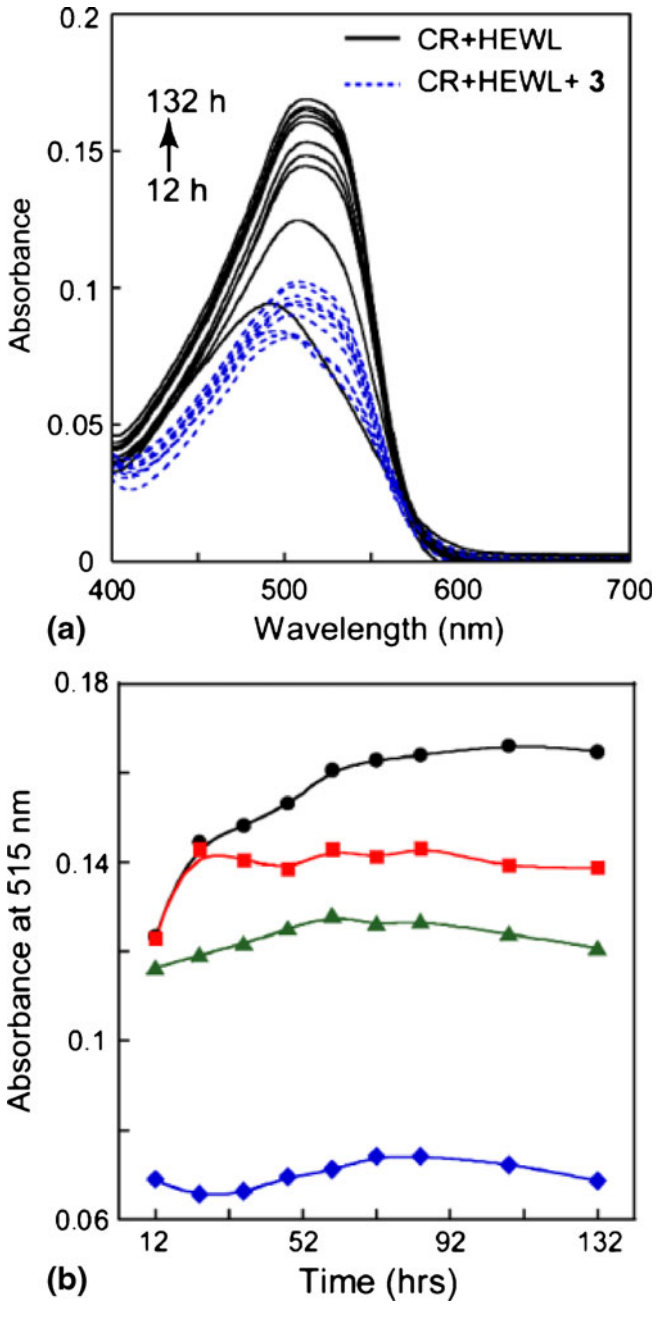


Figure 2. CR absorbance in the presence of HEWL under different experimental conditions: (**a**) CR with HEWL in the absence (solid line) and in presence of the compound **3** (dotted line) and (**b**) Absorbance of CR at 515 nm with HEWL in the presence of the compounds (**1**–**3**) as a function of incubation time. Lysozyme without compound at pH 12.2 (●), lysozyme at pH 12.2 with compound **1** (■), compound **2** (▲) and compound **3** (◆).

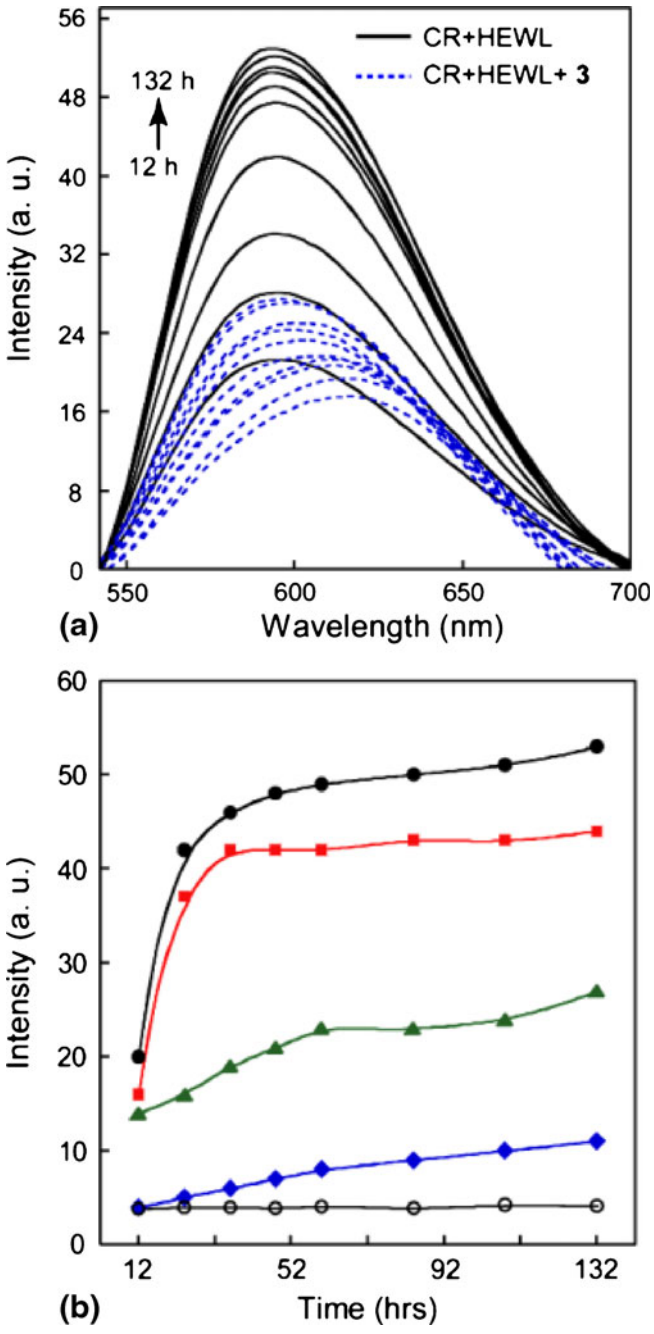


Figure 3. CR fluorescence in the presence of HEWL under different experimental conditions: (**a**) CR with HEWL in the absence (solid line) and in the presence of the compound **3** (dotted line) and (**b**) CR fluorescence intensity with HEWL in the presence of the compounds (**1**–**3**) as a function of incubation time. Lysozyme without compound at pH 12.2 (●) and pH 7.0 (○), lysozyme at pH 12.2 with compound **1** (■), compound **2** (▲) and compound **3** (◆).

and the compounds that there is an enhancement in the intensity of dyes due to the change in their environment, further indicates that the reduction in CR fluorescence intensity observed in the samples is due to the arrest of HEWL aggregation in the presence of the compounds.

3.4 *Thioflavin T fluorescence assay*

ThT fluorescence has been widely used to determine the amyloid fibrillization[38] and in order to ascertain the amyloid characteristics of the aggregates formed, we have also examined the binding ThT to HEWL as

a function of incubation time for a period of ~132 h (figure 4). A higher intensity and a gradual rise in ThT fluorescence for the pH 12.2 samples have been observed with the progress of time as expected due to the amyloidogenic conditions. It is therefore likely that amyloid-like aggregates are being formed on prolonged exposure to alkaline pH. The analysis of results revealed that the percentage of maximum ThT fluorescence intensity of HEWL samples after 132 h of incubation period in the presence of the compounds **1**, **2** and **3** were 97, 63 and 53, respectively which leads to the conclusion that the compound **3** is much more effective to reduce the aggregation kinetics of HEWL when compared to compounds **2** and **1**. Notably, it has been also found that neither the compounds are able to quenched the ThT fluorescence at the concentrations examined (supporting information), indicating the fact that reduction in ThT fluorescence observed in HEWL samples incubated with the compounds could be attributed to attenuation of fibrillation.

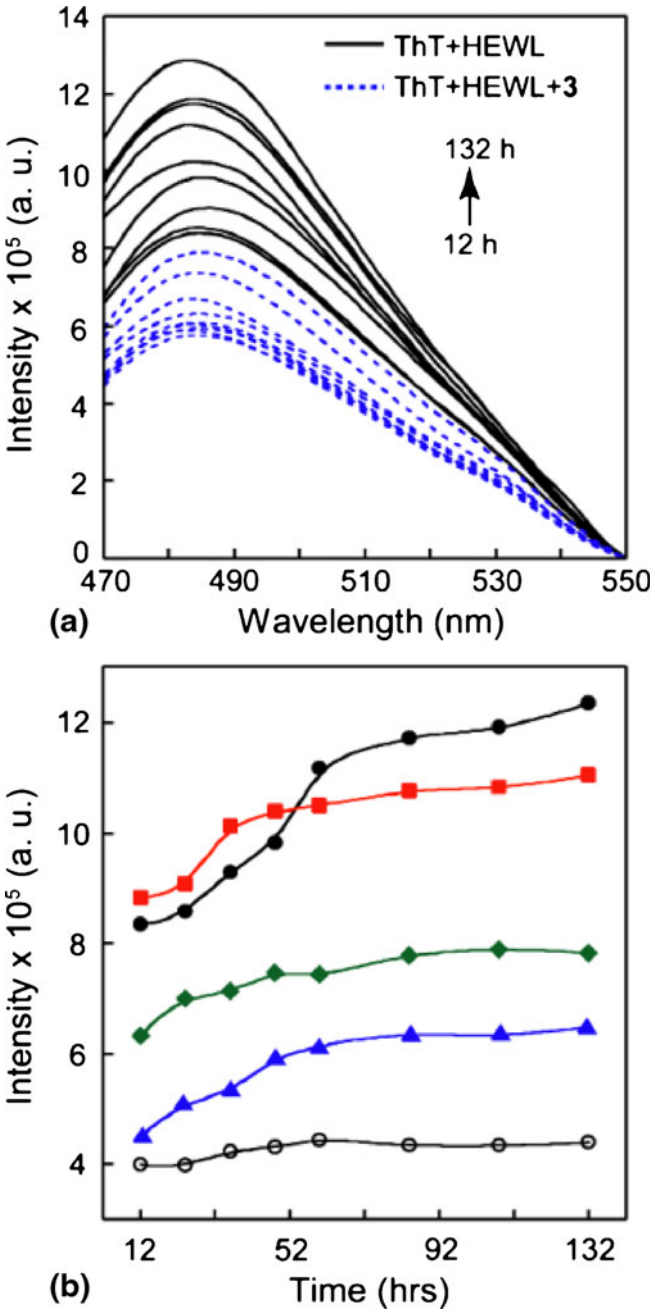


Figure 4. ThT fluorescence in the presence of HEWL under different experimental conditions: (**a**) ThT with HEWL in the absence (solid line) and in the presence of the compound **3** (dotted line) and (**b**) ThT fluorescence intensity with HEWL in the presence of the compounds (**1**–**3**) as a function of incubation time. Lysozyme without compound at pH 12.2 (•) and pH 7.0 (○), lysozyme at pH 12.2 with compound **1** (▪), compound **2** (♦) and compound **3** (▲).

3.5 *Circular dichroism spectra*

Circular dichroism (CD) is a powerful technique for studying the structures of proteins in solution, as well as structural changes that may occur when proteins bind to ligands hence it has been employed to gather some more molecular evidence about the aggregation of HEWL. The CD spectra of HEWL samples under different experimental conditions are shown in figure 5. The CD spectrum of HEWL at pH 7.0 showed the strong negative ellipticity at 208 and 222 nm and a positive ellipticity at 191 nm, typical of proteins with high helical content, while the spectrum of HEWL at pH 12.2 in the absence of any additives showed prominent decrease in

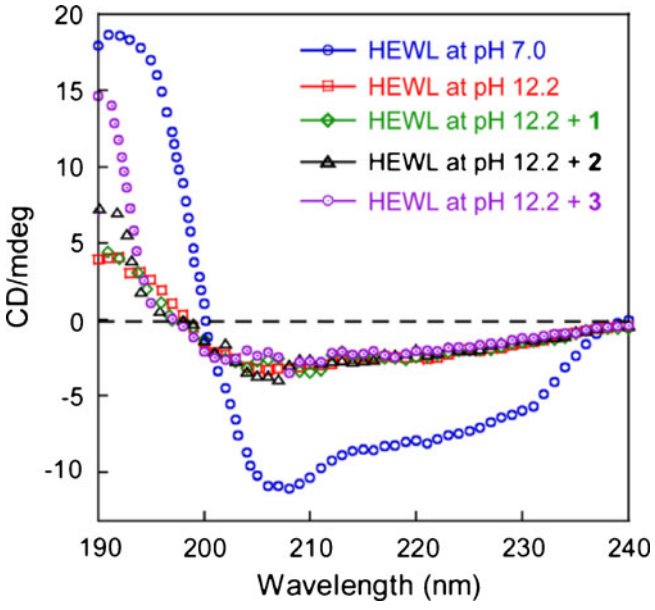


Figure 5. Circular dichroism spectra of HEWL samples under different experimental conditions after 132 h of incubation.

helical content relative to pH 7.0, based on the decrease in molar ellipticity over a wide range of wavelengths. However, the HEWL samples treated with the compounds **3** and **2**, showed a positive peak at 190 nm along with a minimum near 208 nm, compared to the HEWL sample without any additives, which has reduced positive ellipticity at 190 nm. Thus it can be concluded that HEWL samples in the presence of the compounds **2** and **3** showed less helical content than that in the presence of compound **1** and in control sample i.e., without any additives after incubation period of 132 h at alkaline pH.

3.6 *Atomic force microscopy imaging*

In order to confirm the presence of amyloid fibrils and to observe the morphology of aggregated protein under different experimental conditions, atomic force microscopy (AFM) imaging was used. The panel A, B, D, E and F of figure 6 displays the representative micrographs of HEWL alone at pH 7.0 and at pH 12.2, HEWL at pH 12.2 with the compounds **1**, **2** and **3**. In panel A, the monomeric HEWL appears at neutral pH as expected,[39] while in panel B the amorphous aggregates of HEWL with heterogeneity in size and shape appears at alkaline pH after 132 h, along with some ordered aggregates (30 nm width) resembling amyloid fibrils (panel C) after 252 h of incubation in the absence of any additives. Aggregation of protein is a well-known nucleation process, and the formation of amyloid fibrils occurs through different intermediate steps that can be divided into two distinct phases, initially through the deposition of small spherical oligomers to large oligomers that changes the height and width of the aggregates followed by their bidirectional extensions into a fibril.[40,41] These differences in morphology of aggregates also suggest that protein can exist as a mixture of amorphous or fibrillar forms under similar experimental conditions. Similarly, in the presence of compound **1** at pH 12.2, HEWL showed a heterogeneous mixture of amorphous aggregates in panel D similar to panel B, indicating that HEWL oligomerization is not completely arrested before the presence of compound **1**, which is also consistent with the enhanced fluorescence intensity observed with CR and ThT samples having HEWL alone and with compound **1**. Neither the ordered fibrillar nor large amorphous aggregates of HEWL were observed in the presence of compounds **2** and **3** (panel E and F), resembled with low fluorescence intensity of CR and ThT samples having compounds **2** and **3**, clearly demonstrating the inhibitory effect of the compounds on HEWL aggregation.

3.7 *Plausible mechanism of inhibition*

Comparison of dye binding with HEWL at pH 12.2 and 7.0 (supporting information) suggests a significant

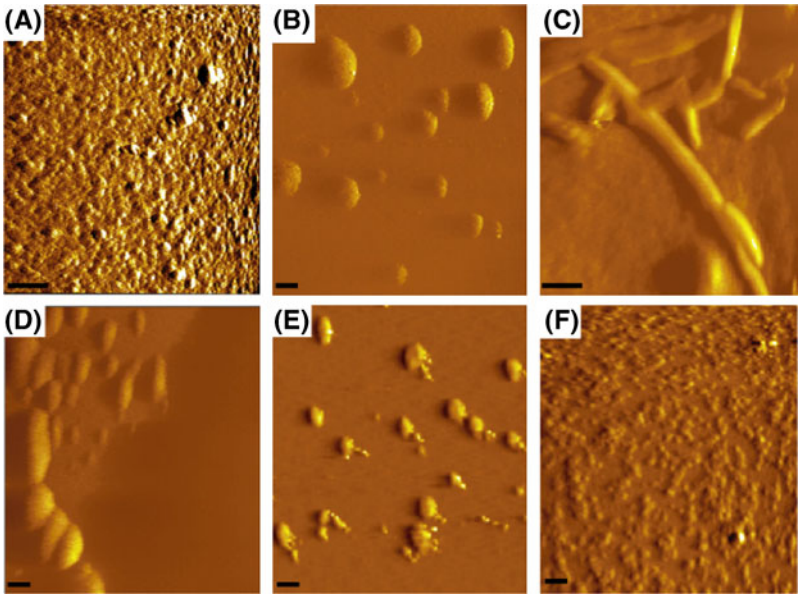


Figure 6. AFM images of HEWL samples under different experimental conditions after 132 h: (**A**) Native lysozyme at pH 7.0, (**B**) lysozyme incubated at pH 12.2, (**C**) lysozyme incubated at pH 12.2, (**D**) lysozyme incubated with compound **1** at pH 12.2, (**E**) lysozyme incubated with compound **2** at pH 12.2, (**F**) lysozyme incubated with compound **3** at pH 12.2. AFM images are in amplitude mode and the scale bar denotes 100 nm.

unfolding and aggregation of the protein at alkaline pH. CR and ThT are known to show an enhanced fluorescent activity with the growth of aggregates as the time progress. Hydrophobic side-chains of proteins exposed to water, and acts as an important driving force for the aggregation process.[42] It is apparent that increasing the exposure of hydrophobic surfaces mirrors the growth of HEWL aggregates by lowering the energy barrier for nucleation. Therefore, it is quite likely that amphiphilic compounds will favourably bind to these available hydrophobic regions of HEWL, thereby not only diminishing their exposure to polar solvent but also halted the aggregation. It could be presumed from our results that the compounds could directly bind to the partially unfolded protein due to their hydrophobicity is well-supported by electrostatic interactions between anionic HEWL (at pH 12.2) and oppositely charged cationic compounds and these binding can assist in stabilizing the protein by shielding off their hydrophobic residues from water, as its functional groups are more exposed to the environment. Amphiphilic molecules offer an apolar environment and thus will minimize the exposure of hydrophobic regions of HEWL from water. In order to elucidate the role of cationic head group and aliphatic tail of the amphiphilic compounds, in the inhibition of HEWL aggregation, we pursued control experiments with the neutral counterpart (8-alkoxy quinoline) and the parent non-amphiphilic (8-hydroxy quinoline) compound independently (supporting information). The control compounds were unable to arrest the growth of HEWL aggregates under identical experimental conditions, depicting the fact that the cationic head group as well as the aliphatic tail group is indispensable for inhibition of aggregation process. The lower potency of the compound **1** (least hydrophobic) relative to **2** and **3** suggests the significant contribution of hydrophobic interactions in the anti-aggregation process. A proposed mechanism of compound mediated inhibition of HEWL aggregation is shown in scheme 2. Another type of non-covalent interactions through which the compounds may affect the stability of HEWL would be through aromatic-π interactions with the aromatic amino acids present in HEWL.[43–45]

4. Conclusions

Here, we have demonstrated that quinolinium-based cationic amphiphiles were able to inhibit HEWL aggregation via CR and ThT binding assay experiments and CD along with AFM techniques. It could be deduced from our results that the compounds could directly bind to the partially unfolded protein and the binding can assist in stabilizing of protein. The control experiments depict the fact that the cationic head group as well as the aliphatic tail group of the compound is indispensable for inhibition of aggregation process. Compounds **2** and **3** appear more potent in halting the growth of aggregates compared with **1**. We believe that the current results are informative and can provide an aid in designing potential targets for molecular therapeutics in the prevention or retardation of amyloid formation implicated in pathological diseases.

Supplementary materials

Figures S1–S8 as supplementary information can be seen in www.ias.ac.in/chemsci.

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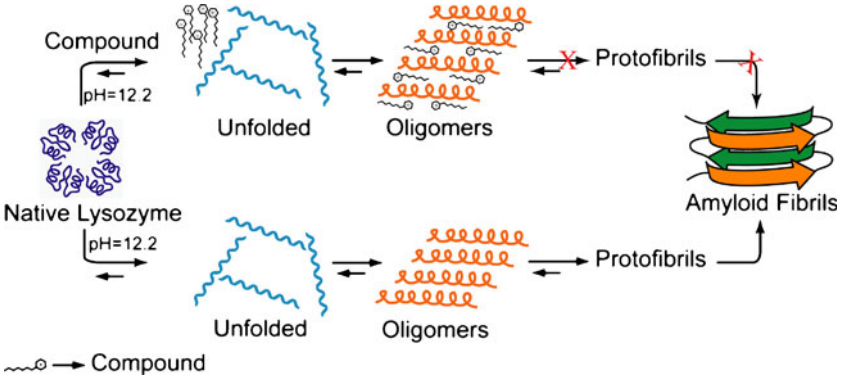

Scheme 2. Plausible schematic illustration showing the role of the compounds in arresting the growth of HEWL aggregates.

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