Electronic Supplementary Material (ESI) for Analyst.

Using amyloid autofluorescence as a biomarker for lysozyme aggregation inhibition

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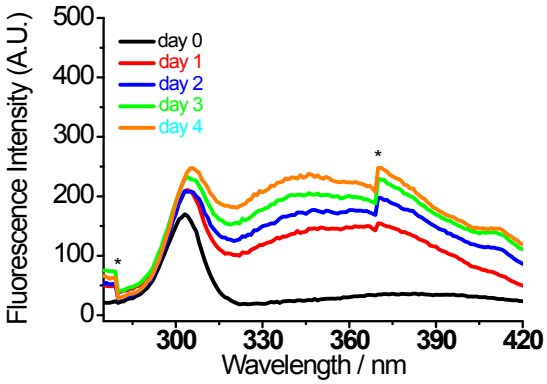
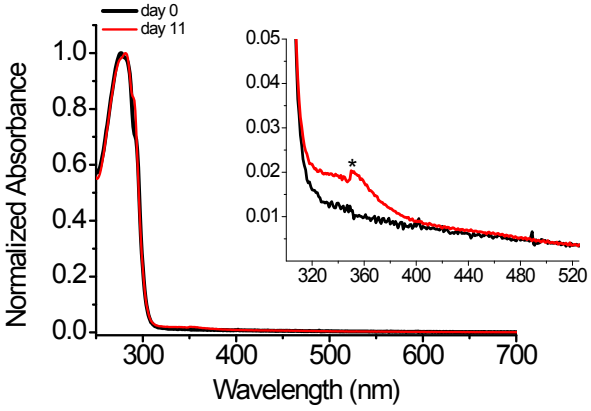
Supplementary Information

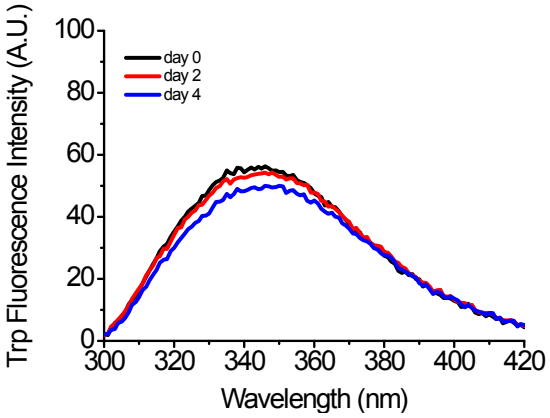


Supplementary Figure S1. Excitation spectra of the HEWL amyloid formation along the incubation time course. A new peak with a maximum at approximately 350 nm occurs with the HEWL aggregation, increasing gradually over time. The emission wavelength was 470 nm. *Asterisk marks instrumental artifact.

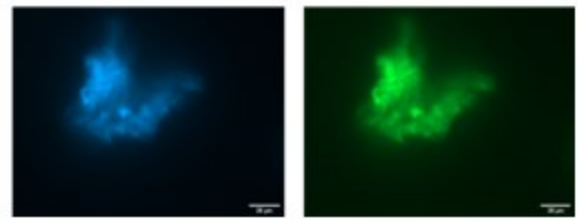


Supplementary Figure S2. Absorption spectra of the HEWL amyloid formation at day 0 and day 11. Protein samples were diluted 12-fold with glycine-HCl buffer, pH 2.2. The inset graph highlights the new

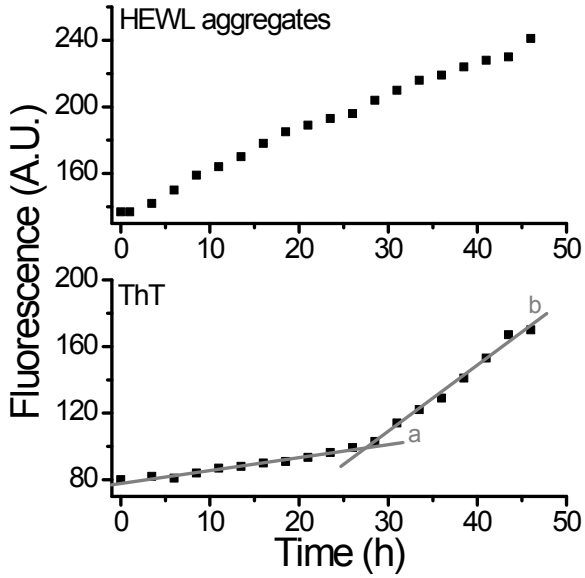
absorption band that is centered at approximately 350 nm and occurs during protein incubation at pH 2.2 and 50.0 ºC. This weak absorption band appears at edge of the typical protein absorption peak at 280 nm due to absorption of aromatic residues. *Asterisk marks instrumental artifact due to changing light sources (tungsten and deuterium lamps).



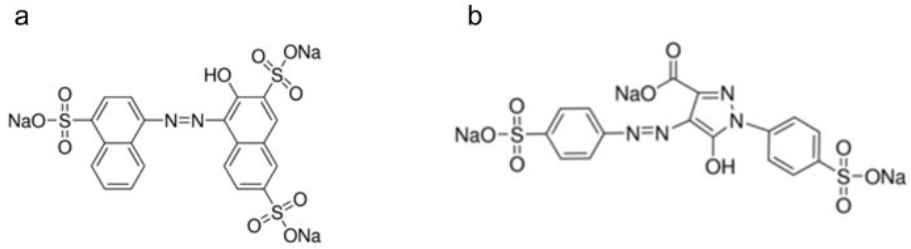
Supplementary Figure S3. Tryptophan fluorescence emission spectra of HEWL obtained at different incubation times. The excitation wavelength was 280 nm. Fluorescence spectrum of the monomeric protein (day 0) exhibits fluorescence maximum at approximately 340 nm. For HEWL aggregates, the wavelength of the maximum of its fluorescence spectrum undergoes a very slight red shift of approximately 3 nm whereas its fluorescence quantum yield is 1.1-fold lower than that observed for the monomeric protein. The changes in tryptophan photophysical properties suggests that tryptophan fluorescence quenching may occur during HEWL aggregation. These quenching interactions result probably from the intra- and/or inter-molecular interactions of the tryptophan microenvironment with neighbouring residues within the oligomeric forms of HEWL[1].



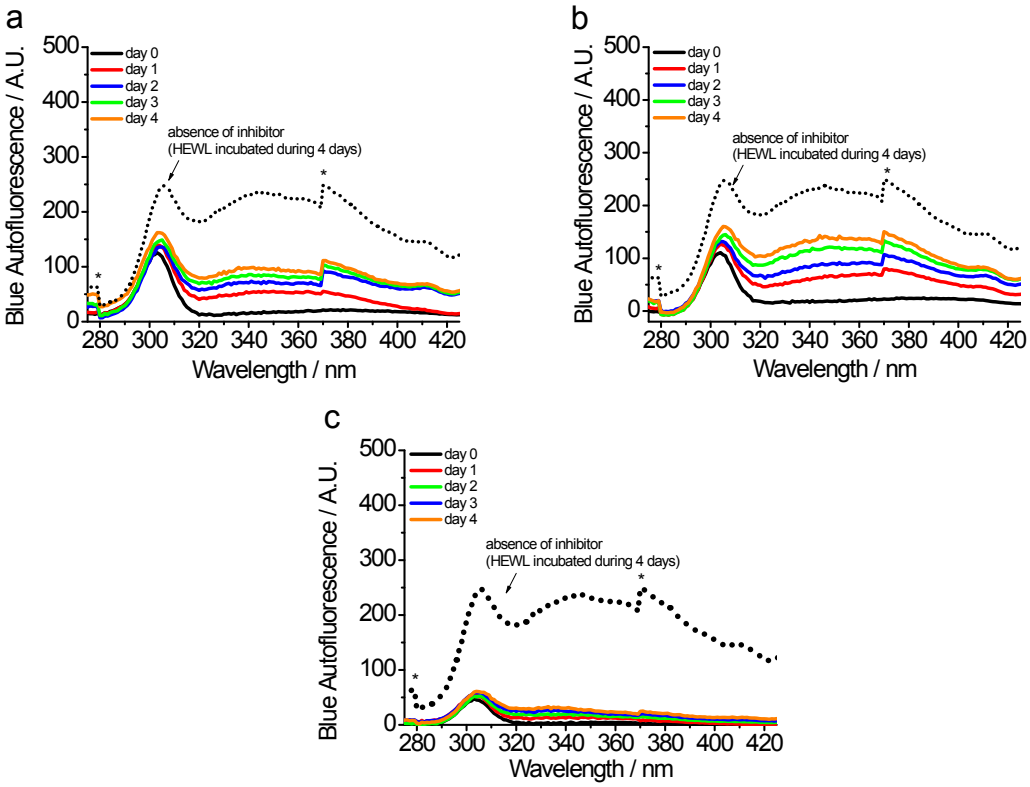
Supplementary Figure S4. Fluorescent microscopy images of HEWL aggregates using the intrinsic emitting light properties of amyloid (blue) and the ThT staining of amyloid (green); Scale bar, 25 μm.



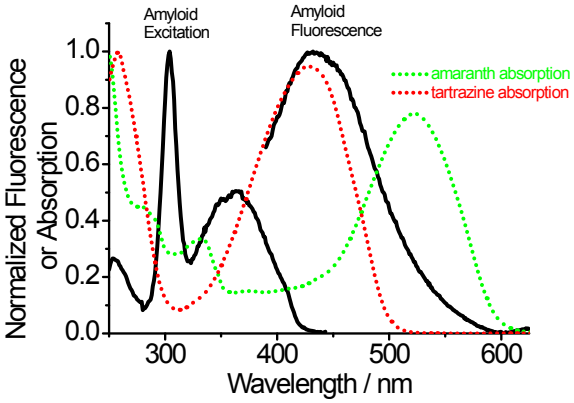
Supplementary Figure S5. Kinetics of the initial stages of HEWL aggregation through the detection of the blue autofluorescence and ThT fluorescence. Although the plateau region was not reached after 48 hours of incubation using the ThT assay, the lag time for the ThT fluorescence assay was estimated by fitting the straight line *a* to the baseline of the lag phase and *b* as a tangent to the steepest region of the growth phase curve. The time point of its intersection was taken as the lag time, which corresponds to approximately 26 hours.



Supplementary Figure S6. Chemical structures of amaranth (a) and tartrazine (b). These compounds are food colorant azo dyes with aromatic rings in conjunction with N=N linkages. While amaranth is a reddish or brownish dye used to color a variety of foodstuff, tartrazine is an orange-colored, mono azo pyrazolone dye present in soft drinks, cookies and cereals.

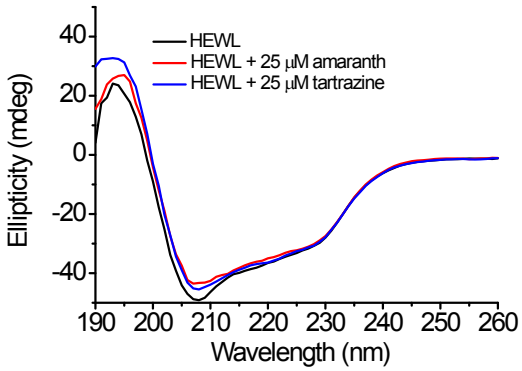


Supplementary Figure S7. Excitation spectra of HEWL incubated at 20 mM Glycine-HCl pH 2.2 and 50 ºC, in the presence of (a) 25 µM tartrazine, (b) 25 µM amaranth and (c) 100 µM tartrazine. The excitation spectra were measured with detection wavelength fixed at 470 nm, with the excitation and emission slits of 10 nm, PMT voltage 620 V. *Asterisk marks instrumental artifact.

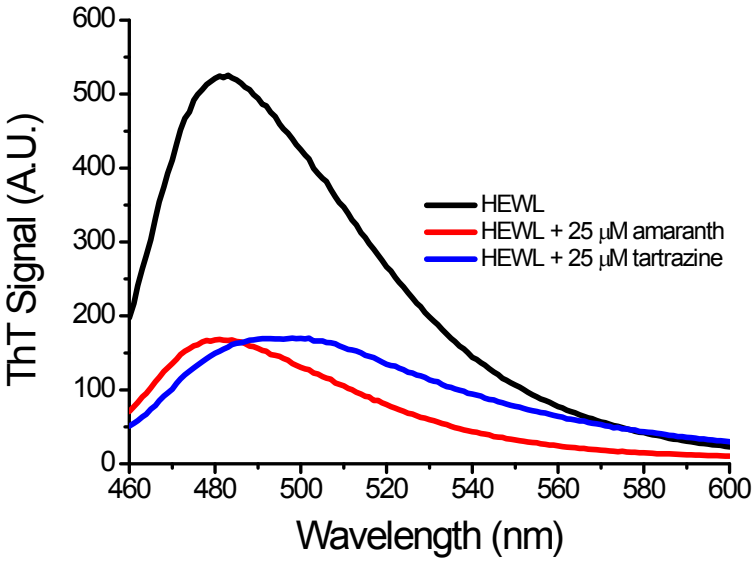


Supplementary Figure S8. Overlap of the UV-visible absorption spectrum of amaranth and tartrazine with both excitation and emission spectrum of HEWL amyloid aggregates. Amaranth (in red) presents residual absorption at excitation and emission wavelengths used to monitor HEWL amyloid formation. However, tartrazine (in green) has a broad absorption that significantly overlaps the fluorescence spectrum of amyloid from HEWL. The presence of high concentrations of inhibitors may perturb the observed fluorescence intensity due to the absorption of excitation or emission light through the so-called inner filter effects, that can be can be divided into the primary and secondary inner filter effects. While the former refers to the absorption of excitation radiation by various chromophores in solution or matrix, the latter is also termed reabsorption and relates to the absorption of emission radiation by these same chromophores[2–4]. The importance of the inner filter effect is dependent on solution concentration, and needs to be taken in to

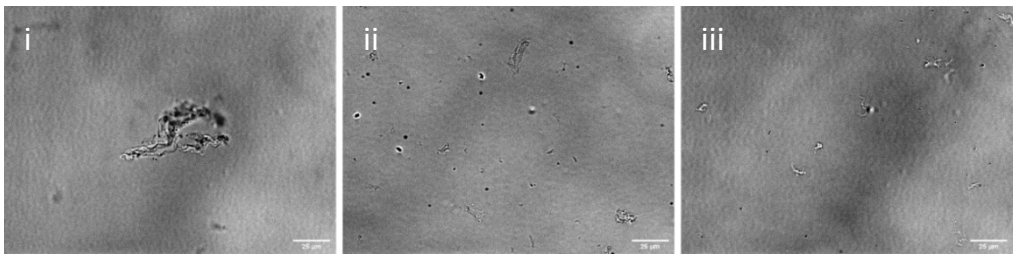
consideration for high solution concentrations[3]. The fluorescence inner filter effect appeared when the absorbance of sample is higher than 0.05. For these two compounds we therefore expect that the measured fluorescence of HEWL aggregation in the presence of high compound concentration is affected by the inner filter effects. Thus, fluorescence measurements were corrected for inner filter effect by applying the mathematical model developed by Albinsson et al.[5] (equation presented in Methods Section).



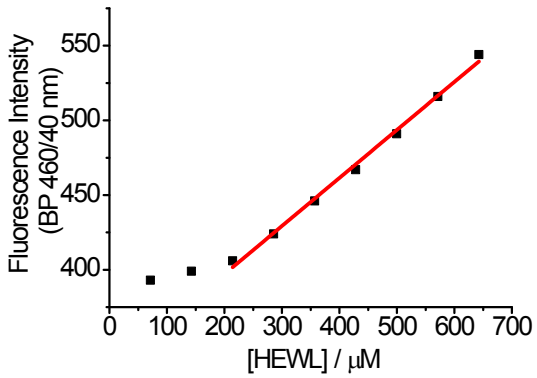
Supplementary Figure S9. Far-UV CD spectra of HEWL in the absence and in the presence of amaranth and tartrazine at pH 2.2.



Supplementary Figure S10. ThT fluorescence assays performed after four days of HEWL incubation at pH 2.2 and 50.0 ºC without stirring, in the absence and presence of amaranth and tartrazine. The excitation wavelength was 450 nm. The inhibition potential obtained for amaranth was 52.4 % and for tartrazine 51.4 %. These values were determined taking into consideration the inner filter effects.



Supplementary Figure S11. Brightfield microscopy images after 4 days of incubation of HEWL in the absence (left panel) and in the presence of 25 μM tartrazine (middle panel) and 25μM amaranth (right panel); scale bar, 25μm.



Supplementary Figure S12. Dependence of amyloid fluorescence as a function of HEWL concentration (as a monomer) after incubation for 24 hours at pH 2.2 and 50 ºC. The excitation filter was BP 360/40 nm and the emission filter a BP 460/40 nm. The measured fluorescence intensity has a linear dependence on initial HEWL concentration above 200 μM.

References

1. Chaari, A., Fahy, C., Chevillot-Biraud, A. & Rholam, M. Insights into kinetics of agitation-induced aggregation of hen lysozyme under heat and acidic conditions from various spectroscopic methods. *PLoS One* **10,** e0142095 (2015).

2. Lakowicz, J. R. *Principles of fluorescence spectroscopy*. (Springer US, 2006).

3. Wang, T., Zeng, L. H. & Li, D. L. A review on the methods for correcting the fluorescence inner-filter effect of fluorescence spectrum. *Applied Spectroscopy Reviews* **52,** 883–908 (2017).

4. Chen, S., Yu, Y. L. & Wang, J. H. Inner filter effect-based fluorescent sensing systems: A review. *Analytica Chimica Acta* **999,** 13–26 (2018).

5. Kubista, M., Sjöback, R., Eriksson, S. & Albinsson, B. Experimental correction for the inner-filter effect in fluorescence spectra. *Analyst* **119,** 417–419 (1994).