Modulation of α-synuclein AGE-based cytotoxic aggregation by zinc oxide nanoparticles: a potential therapeutic approach

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Figure S1: 10 % SDS-PAGE gel and HPLC chromatogram of purified αS; **Figure S2:** Characterisation of $ZnONP_P$, $ZnONP_Y$, and $ZnONP_W$ using (a) UV-Visible spectrum, (b) X-Ray diffraction spectrum, (c) Raman spectrum, (d-f) representative TEM micrographs in 200 nm range with SAED pattern insets; **Table ST1:** Determination of zeta potential for synthesised ZnONPs by DLS Zeta; **Figure S3:** Representative bar graph for calculated free lysine percentage estimated by fluorescamine assay; **Table ST2:** Characterisation of fluorescence intensity at 490 nm and absorbance at 370 nm for mentioned samples; **Figure S4:** CD spectra of αS or αS-ZnONP complexes with MGO at 0 h. **Figure S5A:** Thermograms depicting isothermal titration curves of αS titrated in the presence of $ZnONP_P$ and MGO; **Figure S5B:** Thermodynamic parameters for interaction profiling of αS in the presence of $ZnONP_P$ and MGO by ITC; **Figure S6:** EDS analysis of αS and αS-ZnONP complexes using TEM; **Table ST3:** EDS analysis of mentioned samples.

1. Methodology for expression and purification of αS

The recombinant human αS was expressed in *E.coli* BL21(DE3) cells carrying pET-28a plasmid that encodes for SNCA gene, following a previously established protocol with slight modifications [1]. For expression of αS, 2 % inoculum of transformed BL21(DE3) was inoculated in 1000 mL of LB broth containing 100 µg/mL ampicillin and grown till OD_{600} reaches 0.6. The expression of desired protein was induced with 0.5 mM IPTG at 37 °C for 5 h. After incubation, the cells were harvested by centrifugation at 7500 rpm for 20 min. The cell pellet was re-suspended in buffer containing 10 mM Tris-HCl, 1mM EDTA, and 1 mM PMSF (pH 7.5). The solution was ultra-sonicated on ice for 40 min at 30 s interval with 80 % amplitude. The other bacterial proteins were removed by acid precipitation at pH 3.5, followed by centrifugation at 4 °C with 14000 rpm for 30 min. The pellet was then discarded and the supernatant was adjusted back to pH 7.5. The obtained supernatant was allowed to bind with Q-Sepharose at 4 °C for 3-4 h, followed by elution with 100-500 mM NaCl gradient in 10mM Tris-HCl, pH 7.5. The eluents were checked for desired protein in 10 % SDS-PAGE and dialyzed (12-14 kDa MW cut-off) against milli-Q water for 16 h at 4 °C. The obtained protein fractions were then lyophilised and stored at -20 °C for further use.

1.1. αS purification and characterisation

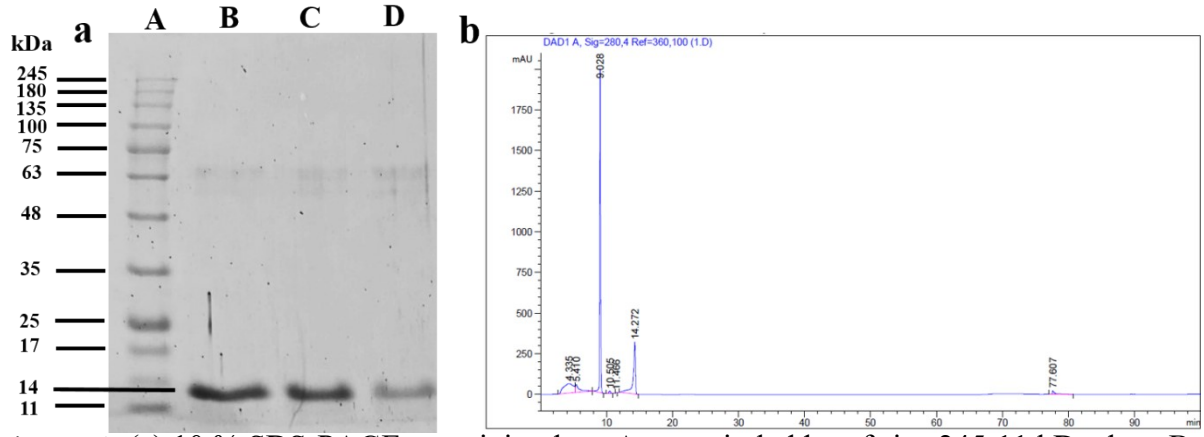


Figure S1: (a) 10 % SDS-PAGE containing lane A- protein ladder of size 245-11 kDa, lane B- 0.2 M, lane C- 0.25 M, and lane D- 0.3 M chromatographic elutes of purified αS. (b) HPLC chromatogram of purified αS done using C18 reverse phase column.

The chromatogram in Fig. S1b, showed that during the initial 60 min run with deionised water, most of the protein was eluted. This was followed by 30-70 % acetonitrile gradient run for 30 min to wash off about 40-50 % of weakly bound protein. In the last 10 min, 100 % acetonitrile was run down to completely remove any residual protein and for column

washing. The appearance of a single large peak in HPLC chromatogram during the initial 60 min run confirmed the presence of large monomeric population of αS, indicating purity of the synthesised protein.

2. Methodology for synthesis of bare and surface functionalised ZnONPs

The synthesised $ZnONP_P$ with positive surface potential was prepared using chemical precipitation method, following standardised protocol from our group [2]. Zinc acetate dihydrate and urea (0.1 M each) with volumetric ratio of 1:4 respectively was mixed and heated at 110 °C for 2 h. The solution was centrifuged at 5000 rpm for 30 min to obtain white precipitate. It was then sonicated, vortexed, and centrifuged with deionised water for several times until it reaches pH 7, to remove traces of urea. The pellet obtained was then dried at 100 °C for 12-14 h and calcinated at 300 °C for 2 h to synthesise $ZnONP_P$ of desired size.

For surface functionalisation of prepared $ZnONP_P$ to $ZnONP_Y$ (tyrosine coated) and $ZnONP_W$ (tryptophan coated) with negative surface potential, 20 mg of bare $ZnONP_P$ was incubated with 1 mM tyrosine/tryptophan in a reaction volume of 20 mL of 10 mM phosphate buffer, pH 7.4. The prepared solutions were mixed well and sonicated for 30 min, followed by centrifugation at 6000 rpm for 15 min to obtain white precipitate. It was then washed several times with deionised water to remove excess of unbound amino acids. The obtained pellets were then dried at 60 °C for 12-14 h and stored for characterisation [3].

2.1. Characterisation of synthesised ZnONPs

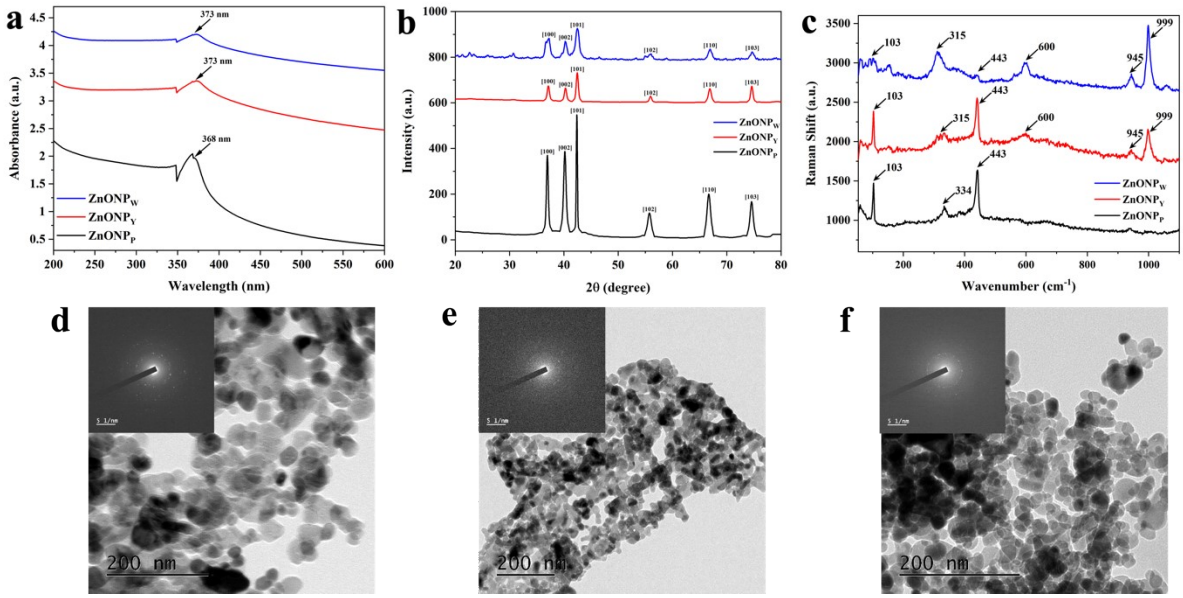


Figure S2: Characterisation of $ZnONP_P$, $ZnONP_Y$, and $ZnONP_W$ using (a) UV-Visible spectra, (b) X-Ray diffraction spectra, (c) Raman spectra, (d-f) representative TEM micrographs in 200 nm range along with SAED pattern insets for (d) $ZnONP_P$, (e) $ZnONP_Y$, and (f) $ZnONP_W$ respectively.

Table ST1: Determination of zeta potential for synthesised ZnONPs by DLS Zeta Analyser.

Sl. No.	**Nanoparticles**	**Zeta (mV) ± S.D.**
1	$ZnONP_P$	21.9 ± 6.4
2	$ZnONP_Y$	-18.7 ± 3.9
3	$ZnONP_W$	-15.1 ± 5.7

The photocatalytic properties of synthesised ZnONPs were determined using UV-Visible spectrophotometer by analysing ZnONP-specific localised Surface Plasmon Resonance (SPR) peaks. The X-ray Diffraction spectroscopy was used for determining crystalline nature, and Raman Spectrometer was used for determining the vibrational nodes of synthesised ZnONPs. The Transmission Electron Microscope was used for particle morphology and SAED pattern and DLS Zeta analyser was used for characterising the surface potential of different ZnONPs. The synthesised $ZnONP_P$ showed an SPR peak at 368 nm, and $ZnONP_Y$ or $ZnONP_W$ showed peak shifting to ~373 nm which can be attributed to respective amino acid coating (Fig. S2a). The XRD spectra showed hexagonal cubic crystalline structure with diffraction peaks at 37^o (100), 40^o (002), 43^o (101), 56^o (102), 57^o (110), and 75^o (103), which are characteristics of ZnO crystals (Fig. S2b). The characteristic ZnO peaks at ~103 cm^{-1} and ~330-340 cm^{-1} were observed in all three Raman spectra of ZnONPs. The peak at ~103 cm^{-1} has been associated with E2 (low) of nonpolar vibration for heavier Zn atoms and the peak at ~334 cm^{-1} has been assigned to the second order structure of ZnO. This peak at ~334 cm^{-1} was shifted in surface functionalised ZnONPs to ~315 cm^{-1} because of amino acid coating. The additional peak at ~443 cm^{-1} in ZnONPs was attributed to the E2 (high) mode of oxygen displacement. This peak had a lower intensity in $ZnONP_Y$ and was almost absent in $ZnONP_W$ due to surface functionalisation. The peak at ~600 nm was assigned to oxygen deficiency as

observed in ZnO of $ZnONP_Y$ and $ZnONP_W$. Additional peaks at ~940-1000 cm^{-1} corresponds to respective amino acids present in $ZnONP_Y$ and $ZnONP_W$ (Fig. S2c). The average size of $ZnONP_P$ was ~30-40 nm, and ~50-60 nm for $ZnONP_Y$ or $ZnONP_W$ as determined from TEM micrographs (Fig. S2d-f). The SAED pattern (Selected Area Electron Diffraction) from TEM (Fig. S2d-f insets) along with XRD spectra confirmed the crystalline nature of synthesised ZnONPs. The zeta potential was +21.9 mV for $ZnONP_P$, -18.7 mV for $ZnONP_Y$, and -15.1 mV for $ZnONP_W$ respectively (Table ST1). Further detailed descriptions on characterisation of ZnONPs has been reported from our group in previous studies [3, 4].

3. Estimation of free amines and carbonyl content in αS-ZnONP complexes

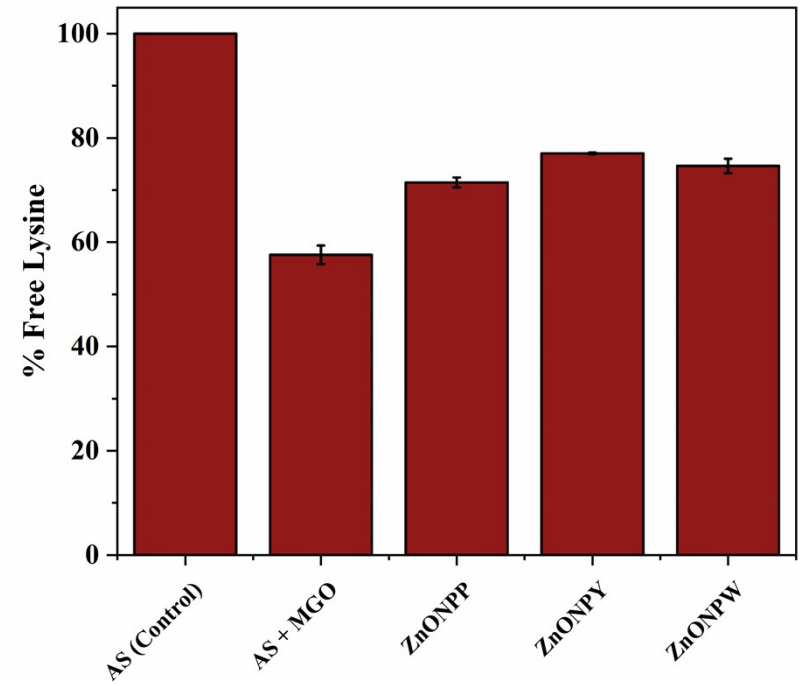


Figure S3: Representative bar graph for calculated free lysine percentage that reacted with fluorescamine of 50 µM αS control, αS or αS complexed ZnONP (30 µg/mL $ZnONP_P$, $ZnONP_Y$, $ZnONP_W$) in the presence of 5 mM MGO. αS is represented as AS. AS control refers to non-glycated αS, incubated for 144 h under similar conditions.

Table ST2: Characterisation of fluorescence intensity at 490 nm (glycated lysine) and absorbance at 370 nm (carbonyl content) for mentioned samples.

Sl. No.	Samples	Fluorescence Intensity at 490 nm	Absorbance at 370 nm
1	αS (Control)	23565 ± 35	0.043 ± 0.012

2	αS + MGO	13567 ± 420	0.381 ± 0.003
3	αS + MGO + $ZnONP_P$	16845 ± 224	0.280 ± 0.008
4	αS + MGO + $ZnONP_Y$	18158 ± 34	0.228 ± 0.007
5	αS + MGO + $ZnONP_W$	17589 ± 326	0.242 ± 0.020
6	αS Monomer (0 h)	23745 ± 220	0.039 ± 0.009
7	MGO	341 ± 6	0.048 ± 0.019
8	MGO + $ZnONP_P$	348 ± 12	0.059 ± 0.015
9	MGO + $ZnONP_Y$	361.5 ± 12	0.046 ± 0.010
10	MGO + $ZnONP_W$	393 ± 59	0.045 ± 0.017

4. Circular Dichroism spectra at 0 h

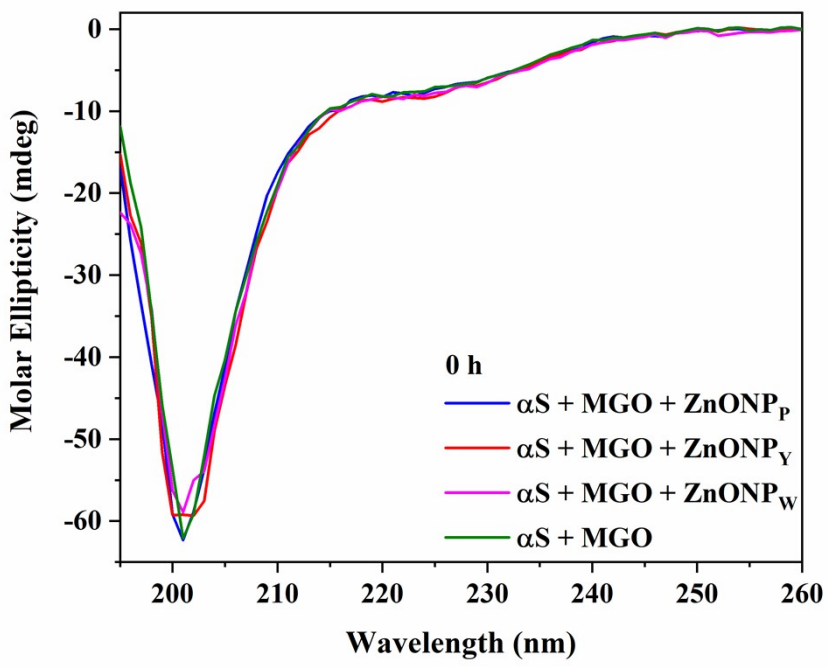


Figure S4: Circular Dichroism spectra of 50 µM αS or αS-ZnONP complexes in the presence of 5 mM MGO at 0 h. The ZnONPs used were 30 µg/mL of $ZnONP_P$, $ZnONP_Y$, and $ZnONP_W$ respectively.

5. Methodology for Isothermal titration calorimetry (ITC)

Isothermal titration calorimetry experiments were performed on Microcal PEAQ ITC (Malvern Panalytical, UK). 50 μM αS was titrated into the cell containing 30 μg/mL (~16.3 pM) $ZnONP_P$. In another experiment, 500 μM MGO was titrated into the cell containing 50 μM αS at 25 °C. A total of 20 injections were carried out, in which the first injection contained 0.4 μL of ligand, whereas the following 19 injections included 2 μL each of ligand solution. The time spacing between two consecutive injections was 180 s and the mixing speed was kept at 800 rpm. For each experiment, control titrations of αS, $ZnONP_P$, and MGO in 1X PBS buffer were performed. The obtained thermograms indicating the dilution-cum-solvation heat, were subtracted from the αS titration into $ZnONP_P$ and MGO titration into αS thermograms. The thermodynamic parameters were calculated using the stoichiometric binding model with one binding site, provided in instrument-integrated Malvern software.

5.1. Equations for one site binding model of multiple injection method in ITC

In ITC experiments involving one site binding model (1:1 stoichiometric binding) for calculation of thermodynamic parameters, the following equations were used by the software provided with the instrument:

$$K = \frac{\Theta}{(1-\Theta)[X]} \quad [Eq\ 1]$$

K = binding constant, Θ = fraction of sites occupied by titrant (ligand in syringe), [X] = free concentration of ligand in active volume

$$\Delta G = -nRTlnK \quad [Eq\ 2]$$

ΔG = Gibbs energy of binding, n= number of sites, T = temperature, R = gas constant.

$$\Delta H = \frac{Q}{n\Theta M_t V_0} \quad [Eq\ 3]$$

ΔH = molar heat of titrant binding, n= number of sites, [M] = free concentration of titrate in active volume, Q = total heat content of the solution present in V_0 (determined relative to zero for the unliganded population, i.e., heat change for each injection of the titrant) at fractional saturation Θ

$$\Delta S = \frac{(\Delta H - \Delta G)}{T} \quad [Eq\ 4]$$

In one-site binding model, ΔH is directly fitted as heat of 100 % binding and steepness of the rise to the saturation is related to binding affinity K_D. The steepness of the region is directly proportional to sample concentration. The stoichiometry (n) of experimental data is calculated as midpoint of titration which is in-between 100 % and 0 % binding.

5.2. Interaction profiling of αS and $ZnONP_P$ or MGO in 1X PBS buffer by ITC

Isothermal titration calorimetry (ITC) was used to investigate the interaction profiling of αS with $ZnONP_P$ and MGO at 25 °C for determining various thermodynamic parameters. For this study, 50 μM αS was titrated into the cell containing 30 μg/mL $ZnONP_P$. In another experiment, 500 μM MGO was titrated into the cell containing 50 μM αS. The Fig. S5A (top panels) represented the heat evolved/absorbed for each 2 μL injection of titrant (50 μM αS or 500 μM MGO) into the cell with respect to time. Additionally, the bottom panels of Fig. S5A represented the heat flow per mole of titrant against apparent molar ratio of titrant: titrate in the cell. The dissociation constant was in the range of micromolar, indicating higher affinity between the interfaces, i.e., αS and $ZnONP_P$ or αS and MGO interacting surfaces (Fig. S5B). The calculated amount of enthalpy change (ΔH), entropy change (TΔS), free energy change (ΔG), and apparent binding constant (K_D) are given in Fig. S5B. When αS was titrated against $ZnONP_P$ (Fig. S5Aa), the reaction was exothermic in nature while MGO titration against αS (Fig. S5Ab) was endothermic in nature. However, heat of dilution of MGO was also endothermic in nature. Therefore, after subtraction with MGO's heat of dilution, the resultant heat evolved was exothermic. The ΔH was favourable and TΔS was unfavourable for both the reaction titrations (Fig. S5B). Therefore, αS interaction with $ZnONP_P$ or MGO were enthalpically driven ($\Delta H < 0$) with favourable enthalpy contribution. Furthermore, entropy change substantiates with degree of randomness in the system, therefore more negative TΔS indicates conformational constraints in protein [5]. The data altogether indicated that αS interaction with $ZnONP_P$ or MGO showed electrostatic and Van der Waals interactions, along with release of caged water into the surrounding. The enthalpic and entropic factors compensated each other, making Gibbs free energy (ΔG) favourable. The ΔG was ~ -6.4 kcal/mol when αS adsorbed onto $ZnONP_P$ interface and ΔG was ~ -5.8 kcal/mol upon MGO interaction (Fig. S5B). The negative ΔG indicated spontaneous binding, suggesting multiple non-covalent interactions in the binding process [6]. Therefore, αS adsorption onto $ZnONP_P$ or αS interaction with MGO were non-specific in nature, with similar binding affinity. Therefore, in solution, $ZnONP_P$ and MGO would compete for αS

binding. Hence, glycating αS when complexed with ZnONP interfaces, will lead to flocs formation along with reduction in AGE formation. In summary, it can be concluded that αS adsorption onto ZnONP interface, inhibits glycation along with reduced self-assembly of protein monomers.

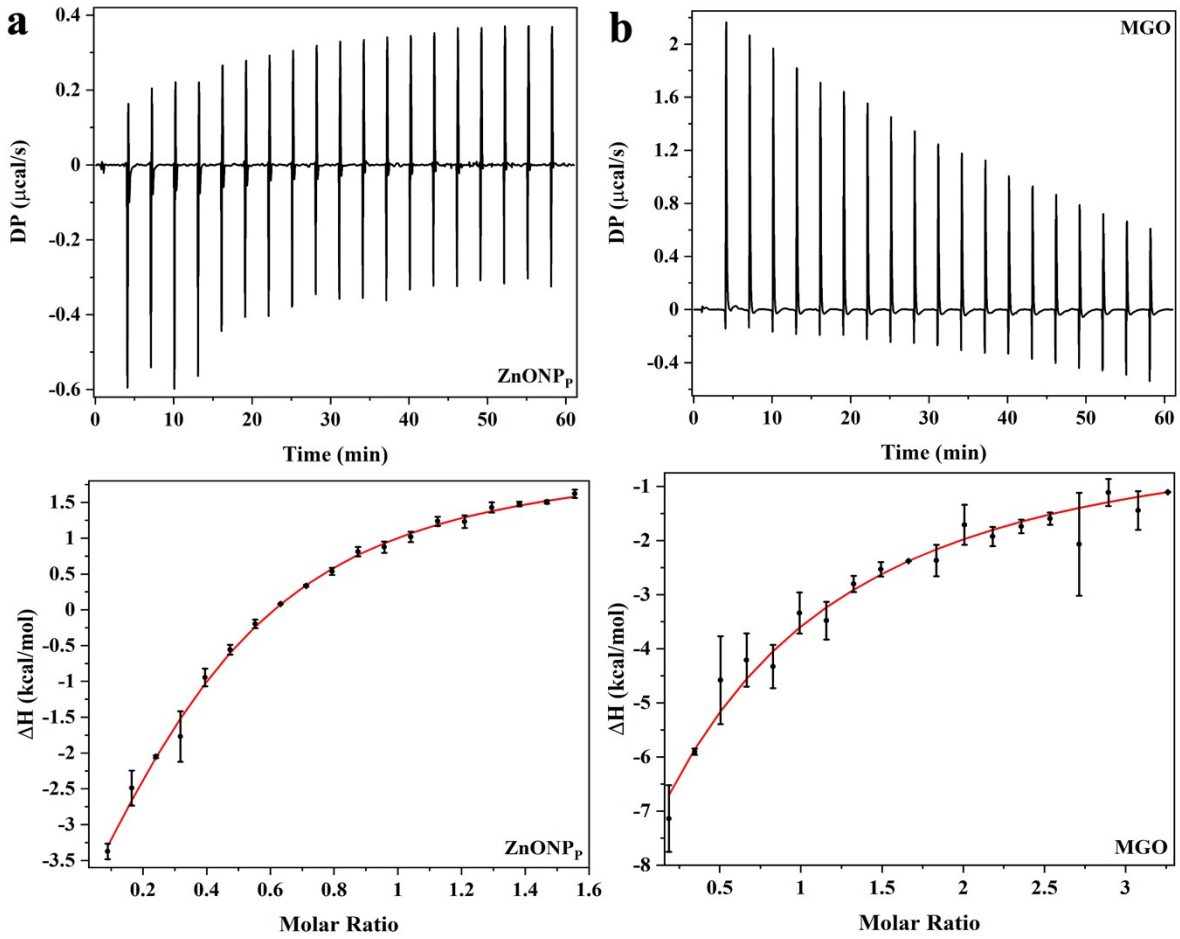
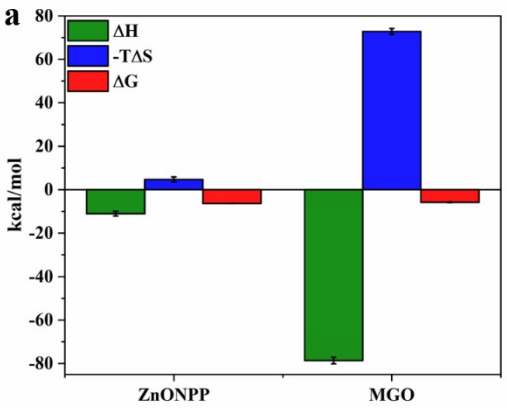


Figure S5A: Thermograms (top panels) and binding isotherms (bottom panels) depicting isothermal titration curves of 50 µM αS titrated in the presence of (a) 30 µg/mL ZnONP$_P$, and (b) 500 µM MGO at 25 ^oC. Red lines (bottom panels) represents the best fitting using one site binding models for data in panels (a-b).



b

Sl. No.		ΔH, kcal/mol	-TΔS, kcal/mol	-ΔS kcal/mol/K	ΔG, kcal/mol	Dissociation constant (K_D), μM
1	$ZnONP_P$	-11.05 ± 1.06	4.68 ± 1.17	0.02	-6.37 ± 0.08	21.95 ± 2.9
2	MGO	-78.65 ± 1.48	72.85 ± 1.34	0.24	-5.81 ± 0.13	65.05 ± 0.64

Figure S5B: (a) Signature plots calculated by applying 1:1 binding model to the experimental ITC measurements for interaction profiling of αS complexed with $ZnONP_P$ and MGO. (b) Representative mean and standard deviations of calculated thermodynamic parameters obtained from three independent experiments.

6. Energy Dispersive Spectroscopy (EDS) analysis from TEM micrographs

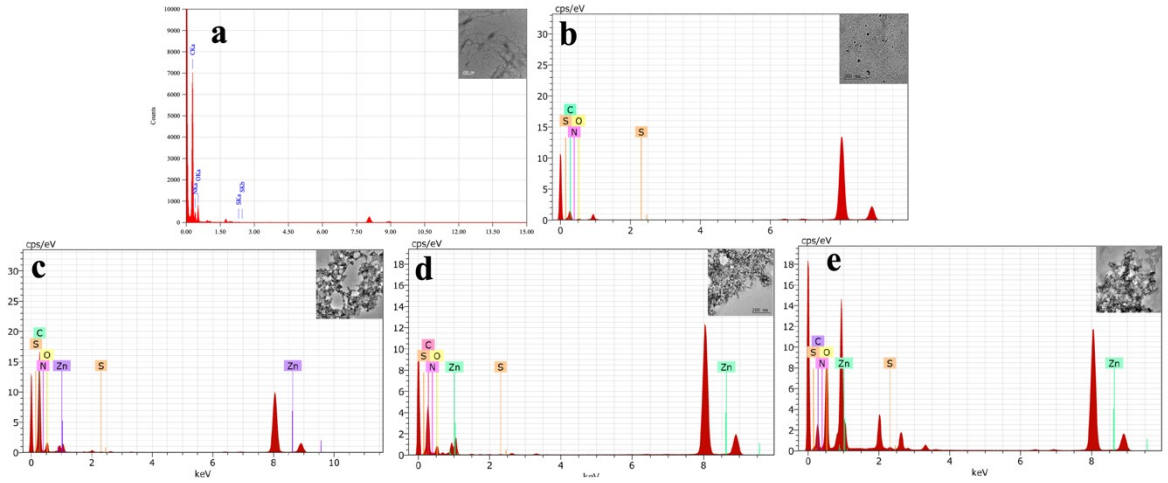


Figure S6: EDS analysis of 50 μM (a) αS only (fibrils), (b) glycated αS (oligomers), (c) αS-$ZnONP_P$, (d) αS-$ZnONP_Y$, and (e) αS-$ZnONP_W$. The ZnONPs and MGO concentrations were kept at 30 μg/mL and 5 mM respectively.

Table ST3: EDS analysis of αS and αS-ZnONP complexes using TEM

Sl.No.	Sample Name		Mass %	Atom %
1	αS Control (144 h)	C K	91.99	93.60
		N K	2.91	2.54
		O K	5.00	3.82
		S K	0.10	0.04

2	αS + MGO	C K	96.89	97.65
		N K	0.00	0.00
		O K	3.10	2.34
		S K	0.02	0.01
3	αS-ZnONP$_P$ + MGO	C K	97.73	98.42
		N K	0.22	0.19
		O K	1.74	1.32
		S K	0.06	0.02
		Zn K	0.25	0.05
4	αS-ZnONP$_Y$ + MGO	C K	96.32	97.81
		N K	0.00	0.00
		O K	2.56	1.95
		S K	0.12	0.05
		Zn K	1.00	0.19
5	αS-ZnONP$_W$ + MGO	C K	52.40	59.88
		N K	1.94	1.90
		O K	43.92	37.68
		S K	0.78	0.33
		Zn K	0.96	0.20

224

225 **7.1. Statistical analysis for fluorescence intensity data at 274 nm**

226 The Shapiro-Wilk test was performed to check for the normality of the groups.

	AS+MO +P	**AS+MO +Y**	**AS+MO +W**	**AS+MO**	**AS control**	**AS Monomer**	**MO**	**MO+P**	**MO+Y**	**MO+W**
Test for normal distribution										
Shapiro-Wilk test										
W	0.9177	0.9113	0.88	0.9078	0.9158	0.7798	0.9177	0.9714	0.9759	0.8898
P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0001	0.0006	<0.0001
Passed normality test	No	No	No	No	No	No	No	No	No	No

(alpha=0.05)?										
P value summary	****	****	****	****	****	****	****	***	***	****
Number of values	230	230	230	230	230	230	230	230	230	230

None of the groups followed normal distribution, thus a non-parametric ANOVA (Kruskal-Wallis) test was performed.

Kruskal-Wallis test	
P value	<0.0001
Exact or approximate P value?	Approximate
P value summary	****
Do the medians vary signif. (P < 0.05)?	Yes
Number of groups	10
Kruskal-Wallis statistic	1762
Data summary	
Number of treatments (columns)	10
Number of values (total)	2300

There was significant difference found between the groups, and thus group-wise comparisons were also performed and reported.

Number of families	1				
Number of comparisons per family	45				
Alpha	0.05				
Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary	Adjusted P Value	
AS+MO+P vs. AS+MO+Y	-48.91	No	ns	>0.9999	A-B
AS+MO+P vs. AS+MO+W	215.5	Yes	*	0.0226	A-C
AS+MO+P vs. AS+MO	-201.2	No	ns	0.052	A-D
AS+MO+P vs. AS incubated	-359.3	Yes	****	<0.0001	A-E
AS+MO+P vs. AS Fresh	-19.61	No	ns	>0.9999	A-F
AS+MO+P vs. MO	731.4	Yes	****	<0.0001	A-G
AS+MO+P vs. MO+P	1082	Yes	****	<0.0001	A-H
AS+MO+P vs. MO+Y	1085	Yes	****	<0.0001	A-I
AS+MO+P vs.	1311	Yes	****	<0.0001	A-J

MO+W					
AS+MO+Y vs. AS+MO+W	264.4	Yes	***	0.0009	B-C
AS+MO+Y vs. AS+MO	-152.3	No	ns	0.6258	B-D
AS+MO+Y vs. AS incubated	-310.4	Yes	****	<0.0001	B-E
AS+MO+Y vs. AS Fresh	29.3	No	ns	>0.9999	B-F
AS+MO+Y vs. MO	780.3	Yes	****	<0.0001	B-G
AS+MO+Y vs. MO+P	1131	Yes	****	<0.0001	B-H
AS+MO+Y vs. MO+Y	1134	Yes	****	<0.0001	B-I
AS+MO+Y vs. MO+W	1360	Yes	****	<0.0001	B-J
AS+MO+W vs. AS+MO	-416.7	Yes	****	<0.0001	C-D
AS+MO+W vs. AS incubated	-574.8	Yes	****	<0.0001	C-E
AS+MO+W vs. AS Fresh	-235.1	Yes	**	0.0066	C-F
AS+MO+W vs. MO	515.9	Yes	****	<0.0001	C-G
AS+MO+W vs. MO+P	866.2	Yes	****	<0.0001	C-H
AS+MO+W vs. MO+Y	869.1	Yes	****	<0.0001	C-I
AS+MO+W vs. MO+W	1096	Yes	****	<0.0001	C-J
AS+MO vs. AS incubated	-158.1	No	ns	0.481	D-E
AS+MO vs. AS Fresh	181.6	No	ns	0.1512	D-F
AS+MO vs. MO	932.6	Yes	****	<0.0001	D-G
AS+MO vs. MO+P	1283	Yes	****	<0.0001	D-H
AS+MO vs. MO+Y	1286	Yes	****	<0.0001	D-I
AS+MO vs. MO+W	1512	Yes	****	<0.0001	D-J
AS incubated vs. AS Fresh	339.7	Yes	****	<0.0001	E-F
AS incubated vs. MO	1091	Yes	****	<0.0001	E-G
AS incubated vs. MO+P	1441	Yes	****	<0.0001	E-H
AS incubated vs. MO+Y	1444	Yes	****	<0.0001	E-I

AS incubated vs. MO+W	1671	Yes	****	<0.0001	E-J
AS Fresh vs. MO	751	Yes	****	<0.0001	F-G
AS Fresh vs. MO+P	1101	Yes	****	<0.0001	F-H
AS Fresh vs. MO+Y	1104	Yes	****	<0.0001	F-I
AS Fresh vs. MO+W	1331	Yes	****	<0.0001	F-J
MO vs. MO+P	350.3	Yes	****	<0.0001	G-H
MO vs. MO+Y	353.2	Yes	****	<0.0001	G-I
MO vs. MO+W	579.8	Yes	****	<0.0001	G-J
MO+P vs. MO+Y	2.917	No	ns	>0.9999	H-I
MO+P vs. MO+W	229.5	Yes	**	0.0095	H-J
MO+Y vs. MO+W	226.6	Yes	*	0.0114	I-J

Test details	Mean rank 1	Mean rank 2	Mean rank diff.	n1	n2	Z
AS+MO+P vs. AS+MO+Y	1530	1579	-48.91	230	230	0.7898
AS+MO+P vs. AS+MO+W	1530	1315	215.5	230	230	3.48
AS+MO+P vs. AS+MO	1530	1731	-201.2	230	230	3.249
AS+MO+P vs. AS incubated	1530	1889	-359.3	230	230	5.802
AS+MO+P vs. AS Fresh	1530	1550	-19.61	230	230	0.3167
AS+MO+P vs. MO	1530	798.6	731.4	230	230	11.81
AS+MO+P vs. MO+P	1530	448.3	1082	230	230	17.47
AS+MO+P vs. MO+Y	1530	445.4	1085	230	230	17.51
AS+MO+P vs. MO+W	1530	218.8	1311	230	230	21.17
AS+MO+Y vs. AS+MO+W	1579	1315	264.4	230	230	4.27
AS+MO+Y vs. AS+MO	1579	1731	-152.3	230	230	2.46
AS+MO+Y vs. AS incubated	1579	1889	-310.4	230	230	5.012
AS+MO+Y vs. AS Fresh	1579	1550	29.3	230	230	0.4731
AS+MO+Y vs. MO	1579	798.6	780.3	230	230	12.6
AS+MO+Y vs. MO+P	1579	448.3	1131	230	230	18.26
AS+MO+Y vs. MO+Y	1579	445.4	1134	230	230	18.3

AS+MO+Y vs. MO+W	1579	218.8	1360	230	230	21.96
AS+MO+W vs. AS+MO	1315	1731	-416.7	230	230	6.729
AS+MO+W vs. AS incubated	1315	1889	-574.8	230	230	9.282
AS+MO+W vs. AS Fresh	1315	1550	-235.1	230	230	3.796
AS+MO+W vs. MO	1315	798.6	515.9	230	230	8.331
AS+MO+W vs. MO+P	1315	448.3	866.2	230	230	13.99
AS+MO+W vs. MO+Y	1315	445.4	869.1	230	230	14.03
AS+MO+W vs. MO+W	1315	218.8	1096	230	230	17.69
AS+MO vs. AS incubated	1731	1889	-158.1	230	230	2.553
AS+MO vs. AS Fresh	1731	1550	181.6	230	230	2.933
AS+MO vs. MO	1731	798.6	932.6	230	230	15.06
AS+MO vs. MO+P	1731	448.3	1283	230	230	20.72
AS+MO vs. MO+Y	1731	445.4	1286	230	230	20.76
AS+MO vs. MO+W	1731	218.8	1512	230	230	24.42
AS incubated vs. AS Fresh	1889	1550	339.7	230	230	5.485
AS incubated vs. MO	1889	798.6	1091	230	230	17.61
AS incubated vs. MO+P	1889	448.3	1441	230	230	23.27
AS incubated vs. MO+Y	1889	445.4	1444	230	230	23.32
AS incubated vs. MO+W	1889	218.8	1671	230	230	26.98
AS Fresh vs. MO	1550	798.6	751	230	230	12.13
AS Fresh vs. MO+P	1550	448.3	1101	230	230	17.78
AS Fresh vs. MO+Y	1550	445.4	1104	230	230	17.83
AS Fresh vs. MO+W	1550	218.8	1331	230	230	21.49
MO vs. MO+P	798.6	448.3	350.3	230	230	5.656
MO vs. MO+Y	798.6	445.4	353.2	230	230	5.704
MO vs. MO+W	798.6	218.8	579.8	230	230	9.363
MO+P vs. MO+Y	448.3	445.4	2.917	230	230	0.04711
MO+P vs. MO+W	448.3	218.8	229.5	230	230	3.707
MO+Y vs. MO+W	445.4	218.8	226.6	230	230	3.659

231

232 **7.2. Statistical analysis for fluorescence intensity data at 370 nm**

233 There was significant difference found between the groups, and thus group-wise comparisons
234 were also performed and reported.

	AS+M	AS+MO	AS+MO	AS+M	AS	AS	MO	MO+3	MO+3	MO+30

	O+P	+Y	+W	O	incubated	fresh		0P	0Y	W
Test for normal distribution										
Shapiro-Wilk test										
W	0.9161	0.9152	0.9153	0.9185	0.793	0.9192	0.8764	0.8801	0.8749	0.8753
P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Passed normality test (alpha=0.05)?	No	No	No	No	No	No	No	No	No	No
P value summary	****	****	****	****	****	****	****	****	****	****
Number of values	415	415	415	415	415	415	415	415	415	415

None of the groups followed normal distribution, thus a non-parametric ANOVA (Kruskal-Wallis) test was performed.

Kruskal-Wallis test	
P value	<0.0001
Exact or approximate P value?	Approximate
P value summary	****
Do the medians vary signif. (P < 0.05)?	Yes
Number of groups	10
Kruskal-Wallis statistic	2323
Data summary	
Number of treatments (columns)	10
Number of values (total)	4150

There was significant difference found between the groups, and thus group-wise comparisons were also performed and reported.

Number of families	1
Number of comparisons per family	45
Alpha	0.05

Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary	Adjusted P Value	
AS+MO+P vs. AS+MO+Y	40.57	No	ns	>0.9999	A-B
AS+MO+P vs. AS+MO+W	9.06	No	ns	>0.9999	A-C
AS+MO+P vs. AS+MO	-312.9	Yes	**	0.0076	A-D
AS+MO+P vs. AS incubated	1866	Yes	****	<0.0001	A-E

AS+MO+P vs. AS fresh	2588	Yes	****	<0.0001	A-F
AS+MO+P vs. MO	1215	Yes	****	<0.0001	A-G
AS+MO+P vs. MO+30P	1244	Yes	****	<0.0001	A-H
AS+MO+P vs. MO+30Y	1186	Yes	****	<0.0001	A-I
AS+MO+P vs. MO+30W	1277	Yes	****	<0.0001	A-J
AS+MO+Y vs. AS+MO+W	-31.51	No	ns	>0.9999	B-C
AS+MO+Y vs. AS+MO	-353.4	Yes	***	0.001	B-D
AS+MO+Y vs. AS incubated	1826	Yes	****	<0.0001	B-E
AS+MO+Y vs. AS fresh	2547	Yes	****	<0.0001	B-F
AS+MO+Y vs. MO	1175	Yes	****	<0.0001	B-G
AS+MO+Y vs. MO+30P	1204	Yes	****	<0.0001	B-H
AS+MO+Y vs. MO+30Y	1146	Yes	****	<0.0001	B-I
AS+MO+Y vs. MO+30W	1236	Yes	****	<0.0001	B-J
AS+MO+W vs. AS+MO	-321.9	Yes	**	0.0049	C-D
AS+MO+W vs. AS incubated	1857	Yes	****	<0.0001	C-E
AS+MO+W vs. AS fresh	2579	Yes	****	<0.0001	C-F
AS+MO+W vs. MO	1206	Yes	****	<0.0001	C-G
AS+MO+W vs. MO+30P	1235	Yes	****	<0.0001	C-H
AS+MO+W vs. MO+30Y	1177	Yes	****	<0.0001	C-I
AS+MO+W vs. MO+30W	1268	Yes	****	<0.0001	C-J
AS+MO vs. AS incubated	2179	Yes	****	<0.0001	D-E
AS+MO vs. AS fresh	2901	Yes	****	<0.0001	D-F
AS+MO vs. MO	1528	Yes	****	<0.0001	D-G
AS+MO vs. MO+30P	1557	Yes	****	<0.0001	D-H
AS+MO vs. MO+30Y	1499	Yes	****	<0.0001	D-I
AS+MO vs. MO+30W	1590	Yes	****	<0.0001	D-J
AS incubated vs. AS fresh	721.6	Yes	****	<0.0001	E-F
AS incubated vs. MO	-651.1	Yes	****	<0.0001	E-G
AS incubated vs. MO+30P	-621.8	Yes	****	<0.0001	E-H
AS incubated vs. MO+30Y	-680	Yes	****	<0.0001	E-I
AS incubated vs. MO+30W	-589.3	Yes	****	<0.0001	E-J
AS fresh vs. MO	-1373	Yes	****	<0.0001	F-G
AS fresh vs. MO+30P	-1343	Yes	****	<0.0001	F-H
AS fresh vs. MO+30Y	-1402	Yes	****	<0.0001	F-I
AS fresh vs. MO+30W	-1311	Yes	****	<0.0001	F-J
MO vs. MO+30P	29.23	No	ns	>0.9999	G-H
MO vs. MO+30Y	-28.94	No	ns	>0.9999	G-I
MO vs. MO+30W	61.77	No	ns	>0.9999	G-J
MO+30P vs. MO+30Y	-58.17	No	ns	>0.9999	H-I
MO+30P vs. MO+30W	32.54	No	ns	>0.9999	H-J
MO+30Y vs. MO+30W	90.71	No	ns	>0.9999	I-J

240

Test details	Mean rank 1	Mean rank 2	Mean rank diff.	n1	n2	Z
AS+MO+P vs. AS+MO+Y	2987	2946	40.57	415	415	0.4878

AS+MO+P vs. AS+MO+W	2987	2978	9.06	415	415	0.1089
AS+MO+P vs. AS+MO	2987	3300	-312.9	415	415	3.761
AS+MO+P vs. AS incubated	2987	1121	1866	415	415	22.44
AS+MO+P vs. AS fresh	2987	399	2588	415	415	31.11
AS+MO+P vs. MO	2987	1772	1215	415	415	14.61
AS+MO+P vs. MO+30P	2987	1742	1244	415	415	14.96
AS+MO+P vs. MO+30Y	2987	1801	1186	415	415	14.26
AS+MO+P vs. MO+30W	2987	1710	1277	415	415	15.35
AS+MO+Y vs. AS+MO+W	2946	2978	-31.51	415	415	0.3789
AS+MO+Y vs. AS+MO	2946	3300	-353.4	415	415	4.249
AS+MO+Y vs. AS incubated	2946	1121	1826	415	415	21.95
AS+MO+Y vs. AS fresh	2946	399	2547	415	415	30.62
AS+MO+Y vs. MO	2946	1772	1175	415	415	14.12
AS+MO+Y vs. MO+30P	2946	1742	1204	415	415	14.47
AS+MO+Y vs. MO+30Y	2946	1801	1146	415	415	13.77
AS+MO+Y vs. MO+30W	2946	1710	1236	415	415	14.86
AS+MO+W vs. AS+MO	2978	3300	-321.9	415	415	3.87
AS+MO+W vs. AS incubated	2978	1121	1857	415	415	22.33
AS+MO+W vs. AS fresh	2978	399	2579	415	415	31
AS+MO+W vs. MO	2978	1772	1206	415	415	14.5
AS+MO+W vs. MO+30P	2978	1742	1235	415	415	14.85
AS+MO+W vs. MO+30Y	2978	1801	1177	415	415	14.15
AS+MO+W vs. MO+30W	2978	1710	1268	415	415	15.24
AS+MO vs. AS incubated	3300	1121	2179	415	415	26.2
AS+MO vs. AS fresh	3300	399	2901	415	415	34.87
AS+MO vs. MO	3300	1772	1528	415	415	18.37
AS+MO vs. MO+30P	3300	1742	1557	415	415	18.72
AS+MO vs. MO+30Y	3300	1801	1499	415	415	18.02
AS+MO vs. MO+30W	3300	1710	1590	415	415	19.11
AS incubated vs. AS fresh	1121	399	721.6	415	415	8.676
AS incubated vs. MO	1121	1772	-651.1	415	415	7.828
AS incubated vs. MO+30P	1121	1742	-621.8	415	415	7.476
AS incubated vs. MO+30Y	1121	1801	-680	415	415	8.176
AS incubated vs. MO+30W	1121	1710	-589.3	415	415	7.085
AS fresh vs. MO	399	1772	-1373	415	415	16.5
AS fresh vs. MO+30P	399	1742	-1343	415	415	16.15
AS fresh vs. MO+30Y	399	1801	-1402	415	415	16.85
AS fresh vs. MO+30W	399	1710	-1311	415	415	15.76
MO vs. MO+30P	1772	1742	29.23	415	415	0.3515
MO vs. MO+30Y	1772	1801	-28.94	415	415	0.3479
MO vs. MO+30W	1772	1710	61.77	415	415	0.7427
MO+30P vs. MO+30Y	1742	1801	-58.17	415	415	0.6994
MO+30P vs. MO+30W	1742	1710	32.54	415	415	0.3912
MO+30Y vs. MO+30W	1801	1710	90.71	415	415	1.091

241

242 **7.3. Statistical analysis for fluorescence intensity spectral data of Thioflavin T assay**

243 The Shapiro-Wilk test was performed to check for the normality of the groups.

	AS + MO + 30P Mean	**AS + MO + 30Y Mean**	**AS + MO + 30W Mean**	**AS + MO Mean**	**AS Incubated Mean**	**AS Fresh Mean**	**MO Mean**	**MO + 30P Mean**	**MO + 30Y Mean**	**MO + 30W Mean**	**ThT Mean**
Test for normal distribution											
Shapiro-Wilk test											
W	0.9119	0.9141	0.9176	0.9086	0.8775	0.9083	0.9356	0.9308	0.9326	0.9264	0.9371
P value	0.0002	0.0002	0.0003	0.0001	<0.0001	0.0001	0.002	0.0012	0.0014	0.0008	0.0023
Passed normality test (alpha=0.05)?	No	No	No	No	No	No	No	No	No	No	No
P value summary	***	***	***	***	****	***	**	**	**	***	**
Number of values	66	66	66	66	66	66	66	66	66	66	66

244 None of the groups followed normal distribution, thus a non-parametric ANOVA (Kruskal-
245 Wallis) test was performed.

Kruskal-Wallis test	
P value	<0.0001
Exact or approximate P value?	Approximate
P value summary	****
Do the medians vary signif. (P < 0.05)?	Yes
Number of groups	11
Kruskal-Wallis statistic	586.6
Data summary	
Number of treatments (columns)	11
Number of values (total)	726

246 There was significant difference found between the groups, and thus group-wise comparisons
247 were also performed and reported.

Number of families	1
Number of comparisons per family	55
Alpha	0.05

248

Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary	Adjusted P Value	
AS + MO + 30PMean vs. AS + MO + 30YMean	32.66	No	ns	>0.9999	A-B
AS + MO + 30PMean vs. AS + MO + 30WMean	31.17	No	ns	>0.9999	A-C
AS + MO + 30PMean vs. AS + MOMean	-78.51	No	ns	>0.9999	A-D
AS + MO + 30PMean vs. AS IncubatedMean	-135	Yes	*	0.012	A-E
AS + MO + 30PMean vs. AS FreshMean	360.3	Yes	****	<0.0001	A-F
AS + MO + 30PMean vs. MOMean	344.9	Yes	****	<0.0001	A-G
AS + MO + 30PMean vs. MO + 30PMean	357.3	Yes	****	<0.0001	A-H
AS + MO + 30PMean vs. MO + 30YMean	198.2	Yes	****	<0.0001	A-I
AS + MO + 30PMean vs. MO + 30WMean	273.5	Yes	****	<0.0001	A-J
AS + MO + 30PMean vs. ThTMean	407.2	Yes	****	<0.0001	A-K
AS + MO + 30YMean vs. AS + MO + 30WMean	-1.492	No	ns	>0.9999	B-C
AS + MO + 30YMean vs. AS + MOMean	-111.2	No	ns	0.128	B-D
AS + MO + 30YMean vs. AS IncubatedMean	-167.6	Yes	***	0.0002	B-E
AS + MO + 30YMean vs. AS FreshMean	327.6	Yes	****	<0.0001	B-F
AS + MO + 30YMean vs. MOMean	312.2	Yes	****	<0.0001	B-G
AS + MO + 30YMean vs. MO + 30PMean	324.7	Yes	****	<0.0001	B-H
AS + MO + 30YMean vs. MO + 30YMean	165.6	Yes	***	0.0003	B-I
AS + MO + 30YMean vs. MO + 30WMean	240.9	Yes	****	<0.0001	B-J
AS + MO + 30YMean vs. ThTMean	374.5	Yes	****	<0.0001	B-K
AS + MO + 30WMean vs. AS + MOMean	-109.7	No	ns	0.1465	C-D
AS + MO + 30WMean vs. AS IncubatedMean	-166.1	Yes	***	0.0003	C-E

AS + MO + 30WMean vs. AS FreshMean	329.1	Yes	****	<0.0001	C-F
AS + MO + 30WMean vs. MOMean	313.7	Yes	****	<0.0001	C-G
AS + MO + 30WMean vs. MO + 30PMean	326.2	Yes	****	<0.0001	C-H
AS + MO + 30WMean vs. MO + 30YMean	167.1	Yes	***	0.0003	C-I
AS + MO + 30WMean vs. MO + 30WMean	242.4	Yes	****	<0.0001	C-J
AS + MO + 30WMean vs. ThTMean	376	Yes	****	<0.0001	C-K
AS + MOMean vs. AS IncubatedMean	-56.47	No	ns	>0.9999	D-E
AS + MOMean vs. AS FreshMean	438.8	Yes	****	<0.0001	D-F
AS + MOMean vs. MOMean	423.4	Yes	****	<0.0001	D-G
AS + MOMean vs. MO + 30PMean	435.8	Yes	****	<0.0001	D-H
AS + MOMean vs. MO + 30YMean	276.7	Yes	****	<0.0001	D-I
AS + MOMean vs. MO + 30WMean	352	Yes	****	<0.0001	D-J
AS + MOMean vs. ThTMean	485.7	Yes	****	<0.0001	D-K
AS IncubatedMean vs. AS FreshMean	495.2	Yes	****	<0.0001	E-F
AS IncubatedMean vs. MOMean	479.8	Yes	****	<0.0001	E-G
AS IncubatedMean vs. MO + 30PMean	492.3	Yes	****	<0.0001	E-H
AS IncubatedMean vs. MO + 30YMean	333.2	Yes	****	<0.0001	E-I
AS IncubatedMean vs. MO + 30WMean	408.5	Yes	****	<0.0001	E-J
AS IncubatedMean vs. ThTMean	542.2	Yes	****	<0.0001	E-K
AS FreshMean vs. MOMean	-15.4	No	ns	>0.9999	F-G
AS FreshMean vs. MO + 30PMean	-2.917	No	ns	>0.9999	F-H
AS FreshMean vs. MO + 30YMean	-162	Yes	***	0.0005	F-I
AS FreshMean vs. MO + 30WMean	-86.72	No	ns	0.9642	F-J
AS FreshMean vs. ThTMean	46.94	No	ns	>0.9999	F-K
MOMean vs. MO + 30PMean	12.48	No	ns	>0.9999	G-H
MOMean vs. MO + 30YMean	-146.6	Yes	**	0.0032	G-I
MOMean vs. MO + 30WMean	-71.32	No	ns	>0.9999	G-J
MOMean vs. ThTMean	62.34	No	ns	>0.9999	G-K
MO + 30PMean vs. MO + 30YMean	-159.1	Yes	***	0.0007	H-I

MO + 30PMean vs. MO + 30WMean	-83.8	No	ns	>0.9999	H-J
MO + 30PMean vs. ThTMean	49.86	No	ns	>0.9999	H-K
MO + 30YMean vs. MO + 30WMean	75.32	No	ns	>0.9999	I-J
MO + 30YMean vs. ThTMean	209	Yes	****	<0.0001	I-K
MO + 30WMean vs. ThTMean	133.7	Yes	*	0.0138	J-K

249

Test details	Mean rank 1	Mean rank 2	Mean rank diff.	n1	n2	Z
AS + MO + 30PMean vs. AS + MO + 30YMean	526.4	493.7	32.66	66	66	0.8946
AS + MO + 30PMean vs. AS + MO + 30WMean	526.4	495.2	31.17	66	66	0.8537
AS + MO + 30PMean vs. AS + MOMean	526.4	604.9	-78.51	66	66	2.15
AS + MO + 30PMean vs. AS IncubatedMean	526.4	661.4	-135	66	66	3.697
AS + MO + 30PMean vs. AS FreshMean	526.4	166.1	360.3	66	66	9.868
AS + MO + 30PMean vs. MOMean	526.4	181.5	344.9	66	66	9.446
AS + MO + 30PMean vs. MO + 30PMean	526.4	169	357.3	66	66	9.788
AS + MO + 30PMean vs. MO + 30YMean	526.4	328.2	198.2	66	66	5.429
AS + MO + 30PMean vs. MO + 30WMean	526.4	252.8	273.5	66	66	7.493
AS + MO + 30PMean vs. ThTMean	526.4	119.2	407.2	66	66	11.15
AS + MO + 30YMean vs. AS + MO + 30WMean	493.7	495.2	-1.492	66	66	0.04088
AS + MO + 30YMean vs. AS + MOMean	493.7	604.9	-111.2	66	66	3.045
AS + MO + 30YMean vs. AS IncubatedMean	493.7	661.4	-167.6	66	66	4.592
AS + MO + 30YMean vs. AS FreshMean	493.7	166.1	327.6	66	66	8.973
AS + MO + 30YMean vs. MOMean	493.7	181.5	312.2	66	66	8.551
AS + MO + 30YMean vs. MO + 30PMean	493.7	169	324.7	66	66	8.893
AS + MO + 30YMean vs. MO + 30YMean	493.7	328.2	165.6	66	66	4.535
AS + MO + 30YMean vs. MO + 30WMean	493.7	252.8	240.9	66	66	6.598
AS + MO + 30YMean vs.	493.7	119.2	374.5	66	66	10.26

ThTMean						
AS + MO + 30WMean vs. AS + MOMean	495.2	604.9	-109.7	66	66	3.004
AS + MO + 30WMean vs. AS IncubatedMean	495.2	661.4	-166.1	66	66	4.551
AS + MO + 30WMean vs. AS FreshMean	495.2	166.1	329.1	66	66	9.014
AS + MO + 30WMean vs. MOMean	495.2	181.5	313.7	66	66	8.592
AS + MO + 30WMean vs. MO + 30PMean	495.2	169	326.2	66	66	8.934
AS + MO + 30WMean vs. MO + 30YMean	495.2	328.2	167.1	66	66	4.576
AS + MO + 30WMean vs. MO + 30WMean	495.2	252.8	242.4	66	66	6.639
AS + MO + 30WMean vs. ThTMean	495.2	119.2	376	66	66	10.3
AS + MOMean vs. AS IncubatedMean	604.9	661.4	-56.47	66	66	1.547
AS + MOMean vs. AS FreshMean	604.9	166.1	438.8	66	66	12.02
AS + MOMean vs. MOMean	604.9	181.5	423.4	66	66	11.6
AS + MOMean vs. MO + 30PMean	604.9	169	435.8	66	66	11.94
AS + MOMean vs. MO + 30YMean	604.9	328.2	276.7	66	66	7.58
AS + MOMean vs. MO + 30WMean	604.9	252.8	352	66	66	9.643
AS + MOMean vs. ThTMean	604.9	119.2	485.7	66	66	13.3
AS IncubatedMean vs. AS FreshMean	661.4	166.1	495.2	66	66	13.57
AS IncubatedMean vs. MOMean	661.4	181.5	479.8	66	66	13.14
AS IncubatedMean vs. MO + 30PMean	661.4	169	492.3	66	66	13.49
AS IncubatedMean vs. MO + 30YMean	661.4	328.2	333.2	66	66	9.127
AS IncubatedMean vs. MO + 30WMean	661.4	252.8	408.5	66	66	11.19
AS IncubatedMean vs. ThTMean	661.4	119.2	542.2	66	66	14.85
AS FreshMean vs. MOMean	166.1	181.5	-15.4	66	66	0.4219
AS FreshMean vs. MO + 30PMean	166.1	169	-2.917	66	66	0.07989
AS FreshMean vs. MO + 30YMean	166.1	328.2	-162	66	66	4.438
AS FreshMean vs. MO + 30WMean	166.1	252.8	-86.72	66	66	2.375
AS FreshMean vs. ThTMean	166.1	119.2	46.94	66	66	1.286
MOMean vs. MO + 30PMean	181.5	169	12.48	66	66	0.342
MOMean vs. MO + 30YMean	181.5	328.2	-146.6	66	66	4.017
MOMean vs. MO + 30WMean	181.5	252.8	-71.32	66	66	1.953

MOMean vs. ThTMean	181.5	119.2	62.34	66	66	1.708
MO + 30PMean vs. MO + 30YMean	169	328.2	-159.1	66	66	4.359
MO + 30PMean vs. MO + 30WMean	169	252.8	-83.8	66	66	2.295
MO + 30PMean vs. ThTMean	169	119.2	49.86	66	66	1.366
MO + 30YMean vs. MO + 30WMean	328.2	252.8	75.32	66	66	2.063
MO + 30YMean vs. ThTMean	328.2	119.2	209	66	66	5.724
MO + 30WMean vs. ThTMean	252.8	119.2	133.7	66	66	3.661

7. References

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