Supplementary Information

Non-enzymatic glycation of human serum albumin modulates its binding efficacy towards bioactive flavonoid chrysin: A detailed study using multi-spectroscopic and computational methods

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Running Head: Binding of chrysin with native and glycated HSA

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S1. Synthesis and characterization of glycated HSA

S1.1. Synthesis of glycated analogue of HSA using D-glucose

The gHSA was prepared by incubating 40 mM HSA with 0.2 M D-Glucose in 0.1 M phosphate buffer of pH 7.2 at 37 °C for 60 days. 1 mM sodium azide (NaN₃) was added to the solution to prevent bacterial growth. After 60 days the sample was dialyzed for two times to remove excess amount of glucose. Then the sample was lyophilized and stored at -20 °C for further use (Joseph, Anguizola & Hage 2011).

S1.2. Characterization of gHSA

S1.2.1. UV-vis studies

UV-vis spectra of 10 μM HSA and gHSA were recorded at room temperature. The absorbance of the glycated sample was found out to be more than its native form as shown in the Figure S1. Similar observation was reported by Roy et al. (Singha Roy, Ghosh & Dasgupta 2016). A visible browning of the glycated sample was also noticed.

![Figure S1](image)

**Figure S1.** UV-vis spectra of 10 μM HSA (black) and gHSA (red) in phosphate buffer.
S1.2.2. Fluorescence spectroscopic measurements

The Trp fluorescence intensity of the modified HSA was found lower than that of the native HSA. Similar observation was also reported earlier by Roy et al. and Coussons et al. (Coussons et al. 1997; Singha Roy, Ghosh & Dasgupta 2016). The formation of advanced glycation end products was confirmed by fluorescence spectroscopic studies. The fluorescence spectra of the protein samples incubated with glucose were monitored at $\lambda_{\text{ex}} = 295, 335, 350$ and $370$ nm respectively for Trp, pentosidine, other AGEs and malonaldehyde. The experimental observations are presented in the Figure S2 ($\lambda_{\text{ex}} = 295$ and $350$ nm). Figure S3 represents the bar diagram of fluorescence intensity of the glycated samples after 60 days.

**Figure S2.** Fluorescence emission intensity of 40 mM HSA upon incubation with 0.2 M D-glucose in 0.1 M phosphate buffer at (a) $\lambda_{\text{ex}} = 295$ nm and (b) $\lambda_{\text{ex}} = 350$ nm.
Figure S3. Fluorescence emission intensity comparison of gHSA sample after 60 days of incubation at $\lambda_{ex} =$ (a) 295, (b) 335, (c) 350 and (d) 370 nm in phosphate buffer.

S1.2.3. Excited state fluorescence lifetime measurements

The excited state lifetime of HSA and gHSA were determined on Pico Master time correlated single photon counting (TCSPC) lifetime instrument (PM-3) provided by Photon Technology International (PTI), USA. A magic angle of 54.7° was used in order to avoid any involvement of the anisotropic decay. The instrument response function (IRF) was measured using a dilute solution of non-dairy coffee whitener. The reliability of the graphical fits were analysed using the following parameters (i) Durbin-Watson (DW) parameter, (ii) $\chi^2$ values and (iii) a visual scrutiny of the fitted function to the data. The average lifetime of the fluorophore present in HSA and gHSA were estimated using the following equation (Eq. 1).
\[ \tau_{avg} = \frac{\sum_i \alpha_i \tau_i^2}{\sum_i \alpha_i \tau_i} \]  

(1)

where, \( \alpha_i \) is the pre-exponential factor associated with the \( i^{th} \) decay time constant \( \tau_i \). The decay profile of HSA and gHSA are shown in the Figure S4. The average lifetime of HSA was found to be 5.63 ns, which correlates well with the previously reported results (Pastukhov, Levchenko & Sadkov 2007; Wu et al. 2011). The glycated analogue of HSA possess an excited state lifetime of 4.05 ns (Table S1).

**Figure S4.** Excited state lifetime decay profiles of 3 µM HSA and gHSA in 20 mM phosphate buffer of pH 7.4. \( \lambda_{ex}=295\text{nm} \) and \( \lambda_{em}=347 \text{ nm} \).

**Table S1.** Excited state lifetime data of 3 µM HSA and gHSA in 20 mM phosphate buffer of pH 7.4.

| Proteins | \( \tau_1 \) (ns) | \( \alpha_1 \) | \( \tau_2 \) (ns) | \( \alpha_2 \) | \( \tau_3 \) (ns) | \( \alpha_3 \) | \( \tau_{avg} \) (ns) | \( \chi^2 \) |
|----------|-------------------|---------------|------------------|---------------|------------------|---------------|--------------------|---------|
| HSA      | 1.28              | 37.41         | 4.01             | 40.47         | 8.23             | 22.11         | 5.63               | 1.25    |
| gHSA     | 1.04              | 58.16         | 3.17             | 29.41         | 7.08             | 12.43         | 4.05               | 1.15    |

**S1.2.4.** Determination of carbonyl content in gHSA
To determine the carbonyl content of the glycated sample 2,4-dinitrophenyl hydrazine (DNPH) assay was performed.\textsuperscript{6} To a 0.1% DNPH solution in 2.5 M HCl, 0.2 mL of gHSA (10 mg/mL) was added. After 1.5 h of incubation at room temperature 1 mL of 20% trichloroacetic acid was added and further incubated for half an hour to form precipitate. The resultant mixture was centrifuged at 10,000 rpm to form pellets. The pellets were then washed with EtOH-H\textsubscript{2}O mixture (50:50 v/v) for two times. The pellets were resuspended with 2.5 mL of 6M guanidine hydrochloride. The absorbance of the resultant mixture was measured at 370 nm using molar extinction coefficient as 22,000 M\textsuperscript{-1} cm\textsuperscript{-1}. The carbonyl content was calculated and found out to be 14.226±0.193 nmol/mL.\textsuperscript{7}

\textbf{S1.2.5. Circular dichroism (CD) measurements}

The far-UV CD spectra were recorded on a JASCO-J1500 CD spectrophotometer using 0.1 cm quartz cuvette in the range of 190-240 nm. The scan rate of 100 nm/sec with 4s response time and 1 nm bandwidth was used for the data collection. The CD spectra of 3 μM HSA, gHSA were recorded in the range of 190-240 nm (Figure S5). The secondary structural components were analysed on a online server DICHROWEB.\textsuperscript{8} Blank subtractions and baseline corrections were performed for each sample. The % α-helix for HSA was found to be 55.7% which upon incubation with D-glucose for 60 days reduced to 40.4%. The % β-sheet increased from 8.3% to 13.6%. The decrease in the α-helical content can be attributed to the partial unfolding of HSA during glycation. Similar observations were reported by Roy \textit{et al} for the glycation of HSA (Singha Roy et al. 2016).
Figure S5. The CD spectra of 3 µM HSA and gHSA in phosphate buffer.

S1.2.6. MALDI-ToF analysis

The mass of HSA and its glycated analogue was measured using MALDI-ToF analyser (Autoflex speed, Bruker). The samples were mixed with the matrix in 50:50 v/v for the analysis. The matrix used for the analysis was prepared by dissolving sinapinic acid in 50:50 v/v solutions of water and acetonitrile containing 0.1% trifluoroacetic acid (TFA). HSA and gHSA were first mixed with the matrix and spotted on the MALDI plate and left for complete drying. Then the analyses were performed in the mass range of 30 to 90 kDa. The mass of HSA and gHSA sample were found to be 66408.200 Da and 68244.198 Da respectively. The increase in the mass corresponds to ten glucose moieties attached to the glycated form.

The tryptic digestion analysis was also carried out to determine the amino acid residues that were modified during glycation.

Preparation of buffer solutions for tryptic digestion

Preparation of the digestion buffer

The digestion buffer was prepared by dissolving 10 mg of ammonium bicarbonate in 2.5 mL of ultrapure water and stored at 4 °C. The concentration of the buffer was 50 mM.

Preparation of the reducing buffer
The reducing buffer was prepared by dissolving 8 mg of DTT (dithiothreitol) in 0.5 mL ultrapure water to obtain a 100 mM solution. The solution was kept at -20 °C for further use.

**Preparation of the alkylation buffer**

The alkylation buffer was prepared by dissolving 9 mg of iodoacetamide in 0.5 mL ultrapure water. The buffer was stored in dark.

The concentration of HSA and gHSA was taken as 1 mg/mL. The tryptic digestion of the samples were carried out by mixing 10 µL of the sample to a solution of 15 µL digestion buffer and 1.5 µL reducing buffer. The solutions were incubated at 90 °C for 5 minutes and allowed to cool down at room temperature. The resulting solution was further incubated in dark for 25 minutes after the addition of 3 µL alkylation buffer. Then 1 µL of trypsin solution (1 µg/mL) was added and incubated at 37 °C for 3 hours. After 3 hours, another 2 µL of trypsin solution was added and incubated further for overnight at 37 °C. The digested samples were mixed with the matrix (50:50 v/v) for MALDI-ToF analyses. The results from the tryptic digestion and MALDI-ToF analysis are given in the Table S1.

**Table S2.** Glycated peptides indentified from MALDI-ToF analyses of the tryptic digested product.

| Peptides | Theoretical Mass (Da) | Mass observed (Da) | Analysis          |
|----------|-----------------------|-------------------|-------------------|
| 11-20    | 1244.367              | 1445.267          | Lys 12 or Lys 20  |
| 146-159  | 1743.038              | 1913.238          | Lys 159           |
| 160-181  | 2455.888              | 2653.248          | Arg 160 or Lys 162|
| 196-209  | 1718.004              | 2047.564          | Lys 199, Lys 205  |
| 226-240  | 1668.905              | 1958.433          | Lys 233, Lys 240  |
| 473-485  | 1544.805              | 1707.875          | Arg 485           |
| 525-534  | 1146.392              | 1554.649          | Lys 525, Lys 534  |
Figure S6. MALDI-ToF spectrum of HSA.

Figure S7. MALDI-ToF spectrum of glycated HSA.
**Figure S8.** MALDI-ToF spectrum of the peptide fragments obtained from tryptic digested HSA sample.

**Figure S9.** MALDI-ToF spectrum of the peptide fragments obtained from tryptic digested glycated HSA sample.
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Table S3. The Stern-Volmer constants ($K_{SV}$) and bimolecular quenching constants ($k_q$) of HSA-chrys in and gHSA-chrysin systems at pH 7.4 and $\lambda_{ex} = 295$ nm

| System     | Temperature | $K_{SV} \times 10^5, M^{-1}$ | $k_q \times 10^{13}, M^{-1} s^{-1}$ |
|------------|-------------|------------------------------|--------------------------------------|
|            | 290 K       | 3.193±0.38                   | 5.671±0.690                          |
| HSA-chrysin| 300 K       | 2.573±0.20                   | 4.570±0.367                          |
|            | 310 K       | 1.723±0.29                   | 3.060±0.529                          |
|            | 290 K       | 1.795±0.06                   | 4.432±0.160                          |
| gHSA-chrysin| 300 K      | 1.470±0.10                   | 3.629±0.246                          |
|            | 310 K       | 1.300±0.01                   | 3.209±0.024                          |
Table S4. Energy transfer parameters for the interaction of HSA/gHSA and chrysin

| System       | $E$   | $J(\lambda)(10^{-14}, \text{M}^{-1} \text{cm}^{3})$ | $R_0$ (nm) | $r$ (nm) |
|--------------|-------|-------------------------------------------------|------------|---------|
| HSA-chrysin  | 0.478 | 1.701                                           | 2.674      | 2.714   |
| gHSA-chrysin | 0.399 | 1.598                                           | 2.647      | 2.833   |
| Rank | Binding energy (kcal/mol) | Calculated binding constant \( (K_b) \times 10^5 \text{ M}^{-1} \) | Rank | Binding energy (kcal/mol) | Calculated binding constant \( (K_b) \times 10^5 \text{ M}^{-1} \) |
|------|--------------------------|-------------------------------------------------|------|--------------------------|-------------------------------------------------|
| 1    | -8.60                    | 18.37                                           | 1    | -8.40                    | 13.86                                           |
| 2    | -8.00                    | 6.72                                            | 2    | -8.00                    | 7.07                                            |
| 3    | -8.00                    | 6.72                                            | 3    | -7.90                    | 5.97                                            |
| 4    | -7.90                    | 5.68                                            | 4    | -7.50                    | 3.05                                            |
| 5    | -7.60                    | 3.43                                            | 5    | -7.20                    | 1.84                                            |
| 6    | -7.50                    | 2.90                                            | 6    | -6.90                    | 1.11                                            |
| 7    | -7.20                    | 1.76                                            | 7    | -6.90                    | 1.11                                            |
| 8    | -7.20                    | 1.76                                            | 8    | -6.80                    | 0.94                                            |
| 9    | -7.10                    | 1.48                                            | 9    | -6.80                    | 0.94                                            |
| 10   | -6.80                    | 0.90                                            | 10   | -6.70                    | 0.79                                            |
| 11   | -6.80                    | 0.90                                            | 11   | -6.40                    | 0.48                                            |
| 12   | -6.60                    | 0.64                                            | 12   | -6.30                    | 0.40                                            |
| 13   | -6.50                    | 0.54                                            | 13   | -6.30                    | 0.40                                            |
| 14   | -6.40                    | 0.46                                            | 14   | -6.10                    | 0.29                                            |
| 15   | -6.40                    | 0.46                                            | 15   | -6.10                    | 0.29                                            |
| 16   | -6.40                    | 0.46                                            | 16   | -6.00                    | 0.24                                            |
| 17   | -6.20                    | 0.33                                            | 17   | -6.00                    | 0.24                                            |
| 18   | -6.10                    | 0.28                                            | 18   | -5.80                    | 0.17                                            |
| 19   | -5.90                    | 0.20                                            | 19   | -5.80                    | 0.17                                            |
| 20   | -5.90                    | 0.20                                            | 20   | -5.80                    | 0.17                                            |

**Table S5.** Docking parameters with rank for the lowest energy conformations at 300K.
Figure S10. The Stern-Volmer plots for the interactions of 3 μM (a) HSA and (b) gHSA with chrysin. \(\lambda_{ex} = 295\) nm.
Figure S11. Synchronous fluorescence spectra of 3 μM (a) HSA and (b) gHSA in the presence of 0-16.39 μM chrysin.
Figure S12. The fluorescence emission spectra (a, b) and the double logarithm plots (c, d) of 1:1 complex of HSA-warfarin and gHSA-warfarin in the presence of 0-16 μM chrysin at $\lambda_{\text{ex}} = 295$ nm and 300 K.
Figure S13. The fluorescence emission spectra (a, b) and the double logarithm plots (c, d) of 1:1 complex of HSA-FA and gHSA-FA in the presence of 0-16 μM chrysin at $\lambda_{ex} = 295$ nm and 300 K.
Figure S14. Time evolution of RMSDs of the HSA-chrysin and gHSA-chrysin complexes.