Molecular modeling and spectroscopic study of quinone-protein adducts: insight to toxicity, selectivity, and reversibility

Mohamed Saleh Elgawish[a,b*], Naoya Kishikawa[a], Mohamed A. Helal[b], Kaname Ohyama[a], and Naotaka Kuroda[a,]

[a] Graduate School of Biomedical Sciences, Course of Pharmaceutical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan

[b] Medicinal Chemistry Department, Faculty of Pharmacy, Suez Canal University, Ismailia 41522, Egypt

*** Corresponding Author**

E-mail: Mohamed_elgawish@pharm.suez.edu.eg

Tel: +818033744881

Fax: +81958192445

Abstract

Supplementary material of the toxicological study on quinon-protein adducts includes Experimental section and some supporting figures.

EXPERIMENTAL SECTION

Chemical and reagents

All chemicals and solvents were of extra pure grade. MQ, acetonitrile (ACN), and methanol (HPLC grade) were supplied by Kanto Chemical Company (Tokyo, Japan). DTT, acetone, hydrochloric acid (HCl), trifloroacetic acid (TFA) and human serum albumin (HSA) were from Nacalai Tesque (Kyoto, Japan). Luminol, lyzozyme, cytochrome C, Ellman's reagent; 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), and phosphate buffer saline (PBS) powder (0.01 mol/L) were from Wako Pure Chemical Industry (Osaka, Japan). Sinapinic acid was from TCI (Tokyo, Japan). *β*-lactoglobulin, and fluorescamine were from Sigma Aldrich (St. Louis, MO, USA). Tris (hydroxymethyl) aminomethane (Tris) was obtained from INC biomedical Co (Eschwege, Germany). GSH was from Kohjin Co. Ltd (Tokyo, Japan). 8-Anilino-1-naphthalenesulfonic acid magnesium salt (ANS) was purchased from Nakarai Chemical; LTD (Kyoto, Japan). Alcohol dehydrogenase was from Oriental Yeast Co; Ltd (Tokyo, Japan). Quick Start™ Bradford Dye Reagent (Bio-Rad, Hercules, CA). Dialysis tubing 12-14 kDa molecular weight cutoff (Fisher brand, Pittsburgh, USA). Milli-Q water was purified by a Simpli Lab-UV (Millipore, Bedford, MA, USA) an ultrapure water system.

Modification of albumin with MQ

A solution of albumin (20 mg/ml, 0.3 mM) in PBS (pH 7.4) was incubated with different concentration of MQ at molar ratio ranged from 1:1 to 1:50 for 24 hr at 37^{o}C. For control incubation, quinone was omitted. The excess quinone was removed by dialysis vs 2 L PBS for 24 hr at 4 ^{o}C with three buffer changes. A large excess of MQ and long exposure time were chosen in order to force protein modification to completion, resulting in adduction of all reactive amino acids nucleophiles and thereby preventing any unwanted side reaction. The amount of protein was determined after dialysis by Bradford assay according to the standard microtiter plate protocol from the manufacturer (Bio-Rad, Hercules, CA) using commercial BSA for the standard curve[1]. The standard protocol was performed in a 250 µl microplate assay format. The linear range of these assays for BSA is 125–1,000 µg/ml. 2 mg/ml BSA standard tube was used and diluted using PBS to give different proteins' concentrations. 5 µl of each concentration was added to microplate with 250 µl of

Coomassie Brilliant Blue G-250 dye. The samples were mixed using microplate mixer and stand for 5 minute at room temperature before UV measurement at 595 nm.

Modification of proteins mixture with MQ

A solution of HSA, lyzozyme, cytochrome *C*, and alcohol dehydrogenase (0.25 mM/each protein) in PBS (pH 7.4) was incubated with 2 mM MQ for 24 hr at 37^{o}C. For control incubation, MQ was omitted. The excess MQ was removed by dialysis vs 2 L PBS for 24 hr at 4^{o}C with three buffer changes. The protein concentration after dialysis was determined in triplicate as illustrated above[1].

Quinoproteins determination by redox-cycling based CL

To small disposable test tube, 50 µl of different concentration (50-200 µg/ml) of modified proteins, 50 µl of 100 µM luminol (in 0.1 M carbonate buffer pH 9.4), and 100 µl of 100 µM DTT were added. The mixture was mixed and CL was measured by Berthold luminometer[1]. Each sample was measured in triplicate.

UV-Vis analysis of modified albumin

UV-Vis spectra of modified albumin were obtained using a shimadzu UV-1800 spectrophotometer. Fifteen µM of modified and unmodified albumin was scanned from 250 to 500 nm in a 1 cm x 2cm quartz cuvette. Each sample was measured in triplicate.

Fluorescence spectroscopy of modified albumin

Fluorescence spectra of 1 µM modified albumin were obtained using shimadzu RF-1500 spectrophotometer. All the fluorescence samples were excited at a wavelength of 290 nm for selective excitation of tryptophan residues. The emission spectra were recorded from 300 to 450 nm.

MALDI-TOF mass spectrometric analysis

Briefly, 1µL of sample (10 pmol) was mixed with 29 µL of a solution of (ACN/0.3% aqueous TFA saturated with sinapinic acid = 3:7, v/v). One microliter was applied on a MALDI-TOF-MS plate, and allowed to dry and crystallize. Each sample was applied in triplicate. Mass analysis was performed using Ultra Flex^{TM} (BRUKER DALTONICS) and Mascot search (Matrix Science Inc.). MALDI-TOF

mass spectrometer equipped with a nitrogen laser (337 nm, 3 ns pulse width). Spectra were generated by averaging 50-100 laser shots for each sample.

Thiol determinations by Ellman's reagents

To each well of microtiter plate, 10 µl of protein or protein adduct and 200 µl of 0.5 mM DTNB in phosphate buffer pH 8 were added. The absorbance was measured at 412 nm after incubation for 30 min in the dark at room temperature. Thiol concentration was calculated using GSH for standard curve[1]. Each concentration was measured in triplicate.

Estimation of protein lysine residue using fluorescamine fluorescence

To each well of microtiter plate, 10 µL of protein adduct, 70 µl of 0.2 M borate buffer (pH 9), and 25 µL of 1 µM fluorescamine solution dissolved in acetone were added. The emission was measured at 475 nm after excitation at 390 nm[2]. Sample was incubated at room temperature for 10 min before measurement. Each concentration was measured in triplicate.

ANS binding

A stock solution of 1 mM ANS was prepared. A suitable amount of ANS was added to each cuvette to achieve a final concentration of 10 µM. 1 µM of albumin or albumin adducted quinone complex was added and ANS binding was studied using 370 nm as the excitation wavelength and 480 nm as emission wavelength[3]. Each concentration was measured in triplicate.

All statistical analysis was performed using Microsoft Excel 2007.

Automated Docking Simulations

Albumin crystal structure was retrieved for the online Protein data bank (PDB code: 2XVV). All hydrogen atoms were added and the whole protein was subjected to a stepwise energy refinement in Macromodel using the OPLS-2005 force field and PRCG method with distance-dependent dielectric constant and extended cutoff [4,5]. Minimization consists of 1000 steps of steepest descent algorithm to a gradient of 0.01 kcal/mol/Å followed by 5000 steps of conjugate gradient algorithm to a gradient

of 0.001 kcal/mol/Å. The entire protein was kept free during these simulations, except for a mild positional restraint of 5 kcal/mol/Å2 on Cα atoms of the backbone in order to preserve the original 3D fold of the crystal structure.

Menadione (MQ) was built and energy-minimized using MMFF charges and the MMFF force field as implemented in Sybyl-X 1.1 with 2000 steps of conjugate gradient method to a gradient of 0.001 kcal/mol/Å. GOLD 3.2 software was used for docking of the minimized compounds into the albumin binding site defined as a 5 Å cavity around the sulphur atom of Cys34. Docking was carried out using standard mode setting and the GoldScore function[6]. A distance constraint of 1.5-5.0 Å between Cys^{34} sulfur and the Michael acceptor carbon of MQ was used to guide the docking algorithm.

Molecular dynamics simulation

The complex of albumin with the highest scoring pose of MQ was used for further study. This complex was inserted into a pre-equilibrated TIP3P water orthorhombic box using the system builder tool within Desmond software as implemented in Maestro interface (Schrodinger Inc., Portland, OR, USA). The size of the box was set to have a distance of 15 Å between the boundary and any atom of the protein with a total volume of 988,774 $Å^2$. To attain a net neutral charge, 12 Na^+ ions were added to the system. MD simulation was carried out using Desmond application in Maestro, employing OPLS-AA force field parameters. Prior to the simulation, five rounds of minimization were carried out, each consisted of 1000 steepest descents steps, and L-BFGS steps with gradually reducing constraints. The final minimized structure was then used for the MD simulation. The Particle-mesh Ewald (PME) method was used to handle long-range electrostatic interactions. Time step of simulation was set to 2.0 fs and a 10 Å cut off was used for non-bonded interactions. Shake algorithm was employed to keep all bonds involving hydrogen atoms rigid. Constant-Volume (NVT) MD simulation was performed for the first 100 ps, during which temperature of the system was raised from 0 to 310 K. The system temperature was maintained at 310 K for the remainder of the simulation using Langevin dynamics[7]. Then, the system was equilibrated in an NPT ensemble using the same gradually reducing harmonic constraints as in the minimization steps over a time of 1 ns. The production run was

then carried out for 20 ns using NPT ensemble with target pressure of 1.01325 bar. The evaluation of the trajectory was carried out using Maestro trajectory player.

References

1. M. S. Elgawish, N. Kishikawa, K. Ohyama, and N. Kuroda, *J. Chromatogr. A*, submitted.
2. P. Bohlen, S. Stein, W. Dairman and S. Udenfriend, *Arch. Biochem. Biophys*., 1973, **155**, 213-220
3. G. Weber and E. Daniel, *Biochemistry*, 1966, **5**, 1901-1907.
4. N. Souihi, A. Lindegren, L. Eriksson and J. Trygg, *Anal Chim Acta*, 2015, **857**, 28-38.
5. T. Harte, G. D. Bruce, J. Keeling and D. Cassettari, *Opt Express*, 2014 22, 26548-26558.
6. M. K. Annamala, K. K. Inampudi and L. Guruprasad, *Bioinformation*. 2007, 1, 339-350.
7. P. Anjukandi, G. G. Pereira and M. A. Williams, *J Theor Biol*., 2010, **265**, 245-249.

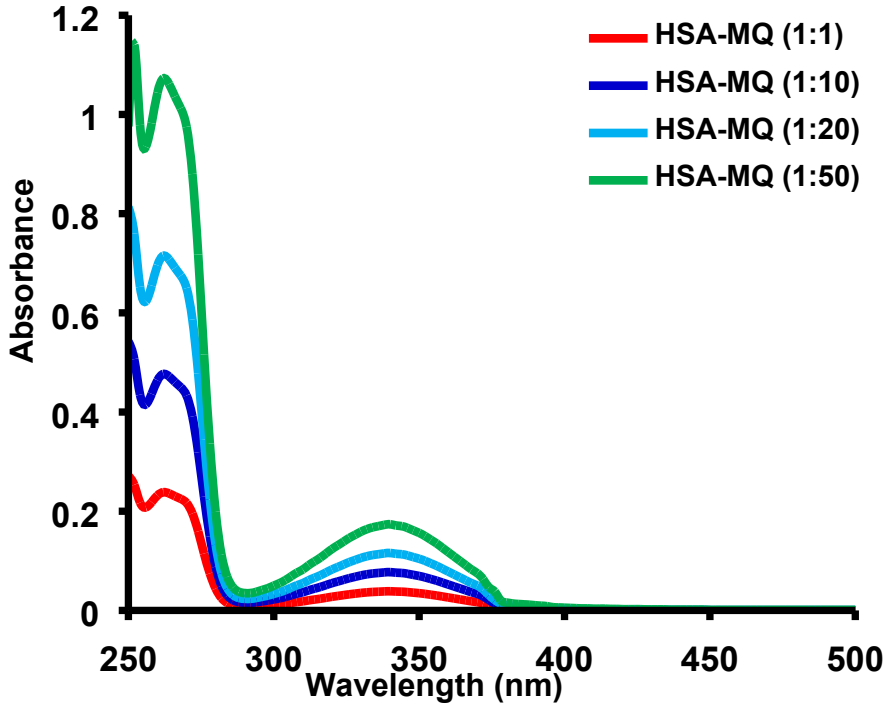

Figure S1. Absorption spectra of HSA-MQ at different molar ratio of MQ to HSA

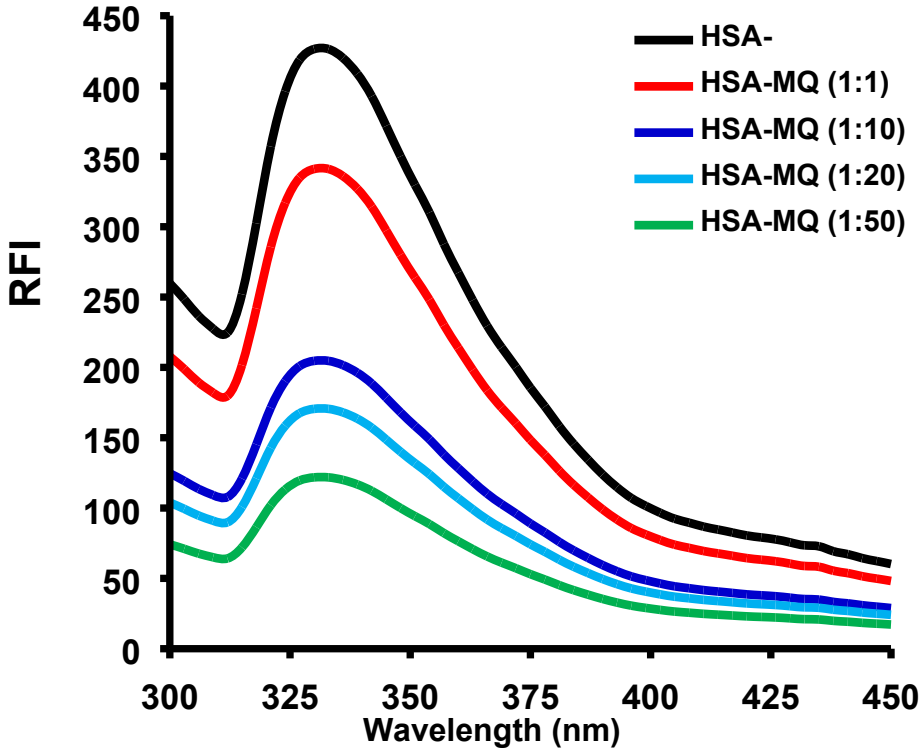

Figure S2. Emission spectra of HSA-MQ at different molar ratio of MQ to HSA

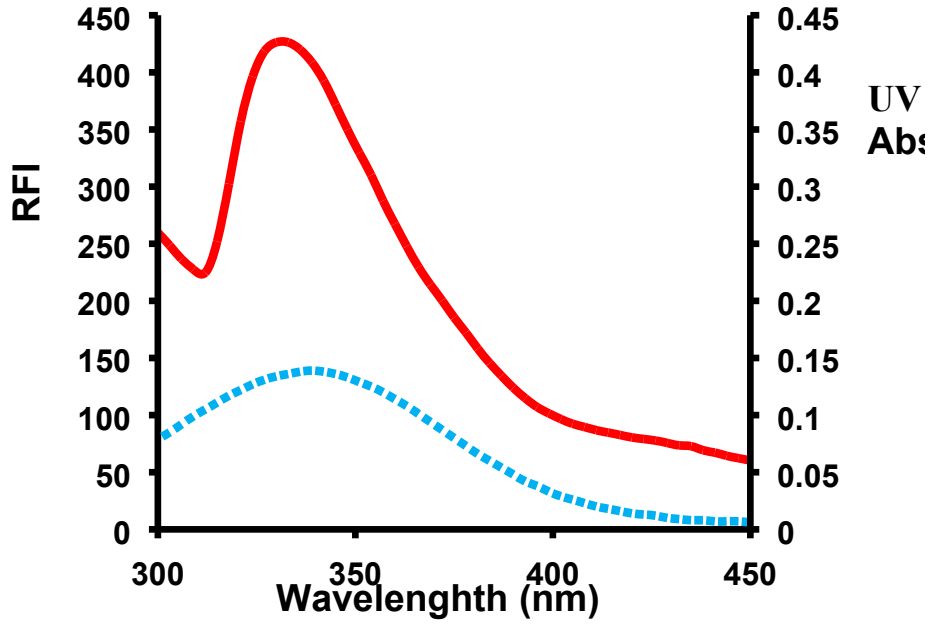

Figure S3. The overlap of UV absorption spectrum of MQ with the fluorescence emission spectrum of HSA

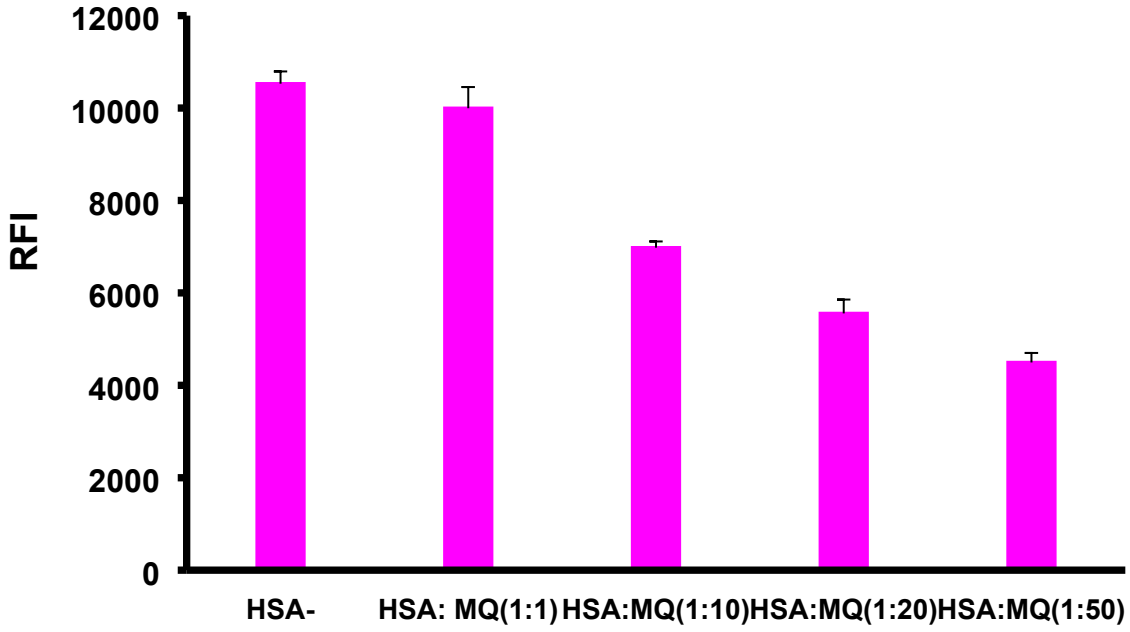

Figure S4. RFI of HSA and HSA-MQ at different molar ratio after reaction with fluorescamine (Ex 390 nm and Em 475 nm) (n = 3)

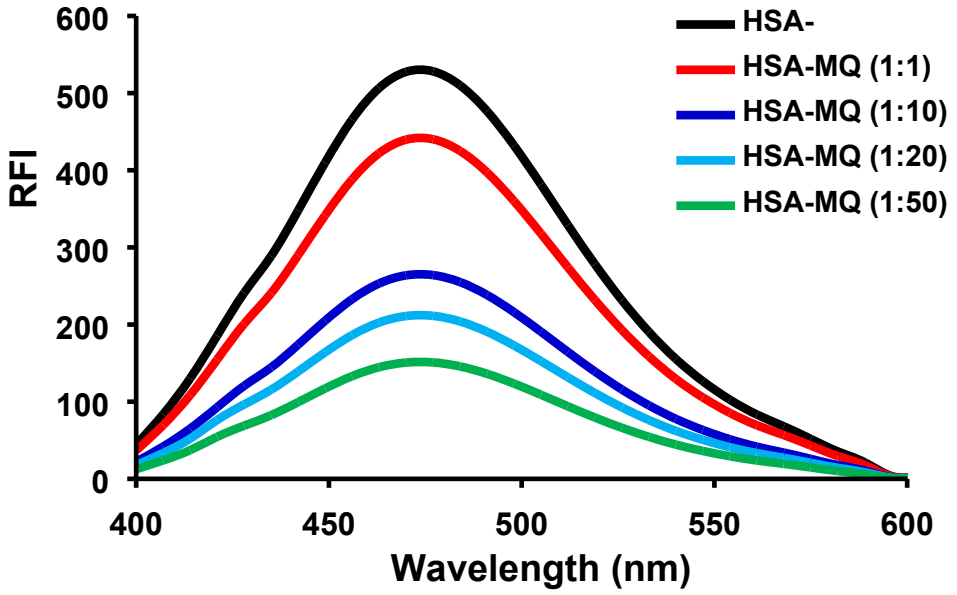

Figure S5. Emission spectra of HSA and HSA-MQ at different molar ration after reaction with 8-anilino-naphthalene sulfonic acid (Ex 370 Em 480 nm)

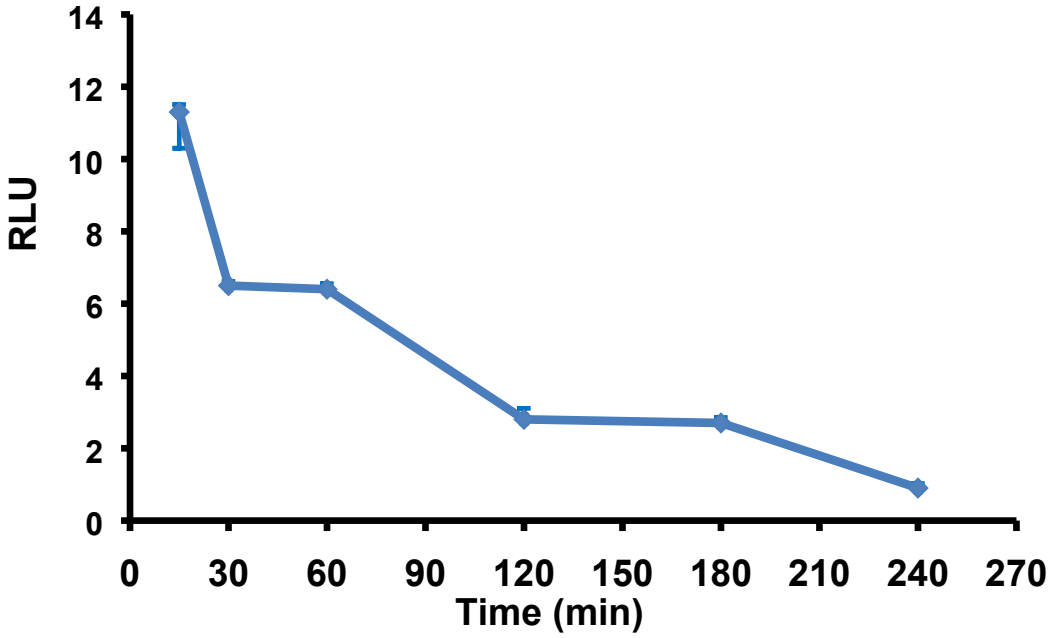

Figure S6. Time profile of rat plasma (2.5 µg/ml) after different time intervals of MQ administration (50 mg/kg) (n =3)