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

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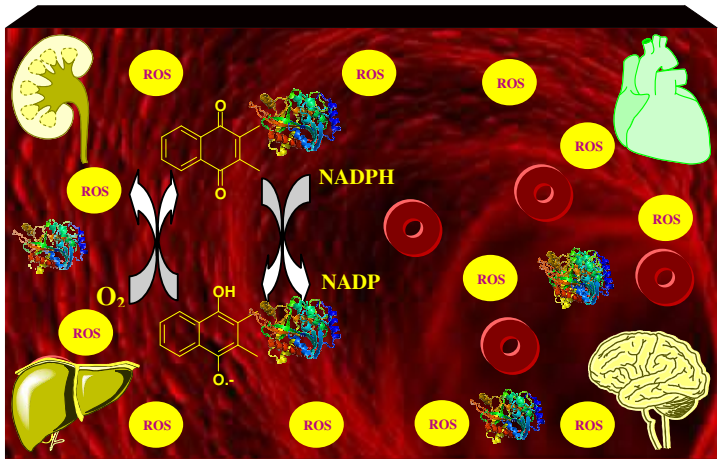
Toxicity, reversibility, selectivity of quinone-protein adducts were studied by molecular modeling and molecular spectroscopy. Adduction of quinones by proteins could affect their redox potential, bioavailability, and intracellular distribution.



Abstract

The important biological and toxicological roles of quinones could be attributed to their versatile electrophilic and oxidative properties. Quinones are able to Michael addition with cellular thiols such as glutathione and proteins, and promote electron transfer in living system via the redox-cycling. Although the proteins adduct of quinones are assumed as a part of their metabolic fate, the adducts retain the redox-cycling capability of parent quinones, and thus considering adduct as type of active metabolites. Herein, the toxicity, reversibility, and selectivity of protein adducts were studied by molecular spectroscopy, and molecular modeling.

Introduction

Quinones, a well-known class of electrophilic species, are of interest from biological, medical, and toxicological perspective due to their unique reactivity and high prevalence in nature. They are widely distributed in biology where they participate in diverse physiological processes, including electron transporting in cell membranes, posttranslational modifications (PTM) of proteins, activating biological signaling pathways in response to stress stimulation, and curing cancer and other fatal diseases[1, 2]. Unfortunately, the potential usefulness of quinones is limited by their toxicity. Quinones can disrupt cellular functions *via* two distinct chemical pathways: as a redox cycling quinone that promotes the generation of reactive oxygen species (ROS) or as Michael acceptors, in which quinones covalently modifies cellular nucleophiles, most prominently sulfur nucleophiles, thereby creating potentially damaging arylation adducts[3].

In general, thiol conjugates are considered products of a detoxification reaction. More recently, however, interest in the structure of thiol conjugates has been heightened by the finding that some classes of conjugates are themselves toxic as a consequence of certain mechanisms. Initial nucleophilic addition of thiol to quinones makes adduct a better redox reagent than quinone itself [4-6]. This is because the addition of an electron donating group to quinones can decrease their redox potential; consequently the rate constant for the reaction of a semiquinone with dioxygen to form a superoxide anion and the hydroquinone auto-oxidation are increased. Therefore, the conjugation of quinones with GSH and proteins thiols cannot be considered as a true detoxification reaction, although conjugation can affect both the intracellular distribution and bioavailability of quinones[4].

Acute cytotoxicity can occur via protein covalent binding by reactive metabolites of drugs, chemicals and natural products. It has been known that some compounds that give rise to covalently bound residues do not cause toxicity while some of the adducts do trigger events leading to toxicity. Many of quinones compound can form covalent adducts with proteins, and can produce toxicological effects via such adduction[1]. They may subsequently alter the structure and function of target proteins. Such functional alterations may disrupt protein-protein binding,

protein organelle localization patterns, and interfere with appropriate cell signalling pathways leading finally to cellular death[3].

Quinon-proteins adducts show long lived span compared with parent and quinone conjugating non-protein thiols as shown in this study[7]. The long lifespan of proteins adduct ensures the sustained release of ROS which then transfer damage to other biomolecules such as DNA, lipids, and other crucial proteins. Moreover, some electrophiles permanently and irreversibly modify a target protein and others induce more short-lived and reversible adduction of the target protein[8]. The reversible electrophilic adduction of proteins-cysteine can include inter-molecular or intra-molecular electrophile exchange between different thiols, and this is expected with some quinon-protein adducts. Besides, we confirmed that quinones-nonprotein thiols adduct show addition-elimination reaction toward thiol groups of proteins and non-proteis, which suggests that these quinones may be transferred between proteins[5]. These results could contribute to the understanding of biological activities, toxicity, and the anticancer mechanism of quinones and thioether quinone adducts. It is vital therefore to look at the toxicological effects of these compounds.

Identification of target proteins might reveal protein factors that distinguish target vs. non-target proteins and enlighten mechanisms connecting cytotoxicity to covalent binding. Although the established dogma has announced the protein adduction seems a random event[8], this brief study has shown that quinones adduction is surprisingly selective. Quinones as a strong electrophile could selectively modify specific proteins both *in vitro* and *in vivo*. Moreover, the selectivity is supposed to be within the protein itself; some nucleophilic sites could be conjugated and the other couldn't. To grasp the quinones cytoxic mechanism, human serum albumin (HSA) and menadione (MQ) are selected as examples for proteins and quinones, respectively. Molecular spectroscopy, molecular modeling, and mass analysis are done to study the toxicity, selectivity and reversibility of quinone-protein adducts.

Result and Discussion

Incubation of HSA with MQ at different molar ratio showed that, a direct relationship between the adduct formation and concentration of MQ in the reaction media was achieved. Quinon-proteins adduct formation was confirmed by many techniques such as chemiluminescence (CL), UV absorption, fluorescence spectrometry, and MALDI-TOF mass analysis. CL was released by mixing the adduct with dithiotheritol (DTT), reducing agent, and luminol. The mechanism of CL relied mainly on how the adduct can react as oxidizing agent with DTT to generate ROS. Furthermore, CL intensity HSA-MQ adducts was increased with increasing the number of MQ adducted to HSA[9]. UV absorption at 340 nm, peak maxima of MQ, was increased (Figure S1, Supporting information), whereas fluorescence intensity from 310-450 nm, attributed to tryptophane, was decreased (Figure S2, Supporting information). The tryptophan emission maxima of HSA in the HSA–MQ complex remained unaltered showing that the decrease in fluorescence intensity was mainly

due to fluorescence resonance energy transfer (FRET)[10] which explained by the overlaps of the fluorescence spectrum of HSA and the absorption spectrum of MQ (Figure S3, Supporting information). Molecular weight (MW) of HSA determined by MALDI-TOF-MS was also increased after incubation with MQ. For each MQ adduct, an increase in MW of 172 (m/z) Da should be obtained. The number of MQ complexed with HSA increased with increased molar ratio of HSA to MQ. For a 1:1 molar ratio of HSA to MQ, the number of MQ adducted was 1; for 1:10 ratio, the number was 3; for 1:20, it was 4 and that for 1:50 it was 5. All these results indicate that more than one MQ molecule was conjugated with HSA via Michael addition.

Modification sites

Reaction between electrophiles and nucleophiles of equal softness is favored because the high energy transition state of the molecular orbital sharing of reactants in transition to formation of the new molecular orbital of the products requires less energy input. Although the modification by quinones is selective to the free thiol form of cysteine (HSA-Cys34)[11], this study demonstrated other sites, in addition to the thiol that could be potentially adducted. The thiol modification was confirmed by Ellman's reagent; UV absorbance was decreased at 412 nm when HSA incubating with MQ[11]. Naturally, as the amount of MQ was increased in incubation media, it was possible to identify more modification sites besides the thiol such as the histidine, lysine, and N-terminus of amino acids. From our results, we can deduce that, ε-amino group of lysine under our reaction condition can form covalent bond with MQ. This confirmed by quenching of fluorescamine fluorescence at 475 nm with increasing the MQ concentration in incubation media (Figure S4, Supporting information)[12]. HSA has 59 ε-amino groups of lysine[13]. However, a small number of lysine could be modified by MQ because the presence of a predetermined nucleophilic amino acid does not necessarily resolve the incidence of an adduct formation. The terminal amino group in lysine has a p*Ka* of approximately 10. So at physiological pH, the group is almost completely charged and can't attack the electrophiles unless a neighboring function can lower its p*Ka*. For instance, the high nucleophilic reactivity of Lys199 of HSA has been already studied, and this is due to its unusual low p*Ka* (of ≈8) [14]. Besides, the influence of neighbor amino acids on the reactivity, the accessibility of proteins' nucleophilic groups (amino and sulfhydryl groups) play also a vital role in the reactivity. Accessibility elucidates which proteins are selectively adducted as shown in next section.

Selectivity of adduct formation

Four proteins including HSA, cytochrome C, Lyzozyme, and alcohol dehydrogenase were incubated with MQ at molar ratio 1:2 of protein to quinones. The proteins mixture was dialyzed and injected to gel filtration chromatography coupling to CL detector[9]. HSA was found the only protein forming adduct with MQ. Other proteins in the mixture lacked the reactivity with MQ at low concentration. The high reactivity of HSA could be attributed to the acidic nature of free cysteine of albumin

(i.e., p*K*a equal to 6.55 instead of 8.4 of cysteine). This acidity was enhanced by the closeness of one tyrosine and one histidine residue able to accept thiol proton and stabilize the thiolate anion. The accessible surface of Cys34 is significantly greater in the thiolate than in neutral form[15] and this accessibility confirm the high and fast reactivity of albumin with MQ comparing to other protein in the mixture. Cytochrome C, like albumin, could be reacted preferentially with free thiol when lacking the attachment with heme[16]. It could also prefer the formation of cyclised diquinone adduct in lysine rich regions which oxidized to form the final stable ring structure lacking quinone moiety[17]. Lyzozyme reacted only with quinone after disulfide bond reduction[11]. Although alcohol dehydrogenase might contain free thiols, it showed no reactivity with MQ. The microenvironment of a thiol on a cysteine can also influence the adduction of available cysteines. Glyceraldehydes phosphate dehydrogenase, which contain three cysteine residues, is selectively alkylated at one cysteine, Cys-52, by quinone [18]. This phenomenon would be discussed sooner by computational study. In addition we confirmed the high reactivity of albumin with quinone to form adduct *in vivo* after MQ administration via i.p injection and *in vitro* after incubation with human serum[9]. Albumin might be the main nucleophilic target in rat and human plasma that is able to form adduct with quinone. This result agrees with the previous information which confirms the predominant reactivity of albumin with quinone and other electrophiles[19,20].

Molecular modelling

MQ was found to fit nicely into the proposed adduct site with a GOLD docking score of 32. The interactions with the protein are mainly hydrophobic with one H bond with Thr79 side chain oxygen. This interaction was also noticed in a previous modeling study of albumin and may play a role in its selectivity[15]. Intriguingly, we noticed that the Michael acceptor carbon of MQ was placed close to Cys34 sulfur with a distance of 3.1 Å. The remainder of the compound makes hydrophobic contacts with Val77 and Leu42 which act as a hydrophobic clamp stabilizing the aromatic scaffold of MQ within the binding site of albumin. MD simulations showed that the adduct distance between MQ and Cys34 remained stable fluctuating between 2.7 Å and 3.3 Å. Also, as expected, the H bond with Thr79 was stable around 1.9 Å.

Docking of MQ into the nucleophilic site of albumin followed by MD simulation was carried out to investigate the mechanism and the selectivity of adduct formation at the molecular level. As mentioned above, after incubation of MQ with HSA, lysozyme, cytochrome C, and alcohol dehydrogenase, adduct formation was noticed only with HSA. The published crystal structures of these proteins show that both HSA and alcohol dehydrogenase contain free cysteine residues. The latter possesses one solvent-accessible cysteine residue, namely, Cys241. However, this residue was not able to form adduct with MQ. This observation highlights the effect of the neighboring residues on the nucleophilicity of Cys34 in albumin (Figure 1). A

catalytic triad, similar to that of cysteine proteases, is obvious within the adduct site of albumin. This triad, consisting of Asp38, His39, and Cys34, could enhance the acidity of the thiol function and the nucleophilicity of the sulphur atom of Cys34. In addition, it was reported that Tyr84, whose phenate anion is stabilized by its closeness to Lys41, plays the key role in the deprotonation of Cys34. After deprotonation, the thiolate anion of Cys34 easily attacks the Michael acceptor carbon of MQ yielding the adduct.

Our docking study shows the proximity of Cys34 sulfur to the less hindered Michael acceptor carbon of MQ, in addition to several favourable interactions, including H bonding and hydrophobic contacts (Figure 1). This arrangement sets the stage for adduct formation in the next step. MD simulation was performed to test the stability of the HSA-MQ complex. The distance between Cys34 sulfur and the accessible electrophilic carbon of MQ was monitored (green dashed line), as well as the H bond between one of the MQ carbonyl and Thr79 (red dashed line). Throughout the simulation, the monitored H-bond remained stable around 1.9 Å as well as the distance between the Michael acceptor carbon and Cys34 (Figure 2). This MD simulation study allowed us to investigate the role of all the binding site residues including the hydrophobic side chains and the H bonding Thr79 which could help in fine-tuning the affinity in certain cases. Generally, the simulation study has proved the stability of the proposed binding mode and rationalized the selective adduct formation with HSA.

Toxicity of quinon-proteins adduct

Although free quinones are short lived *in vivo* owing to its high reactivity, the binding of quinone to protein may dramatically extend the half life of these active species[7]. Modification of quinone nucleus by nucleophilic sites of HSA can affect the redox potential of parent quinone and this was clearly appeared in time profile curve of MQ and HSA-MQ (Figure 3). Quinon-proteins showed slow release of ROS within very long period of time compared to parent quinone which showed rapid release of ROS in short time. The lifespan of adduct based mainly on the half life of proteins. For instance, half life of HSA is 28 days compared to few hours for glutathione adduct. Additionally, redox-cycling is likely to be more pronounced with quinon-proteins than with free quinones in solution[21]. HSA-MQ adduct would undergo continuous redox-cycling with sustained release of ROS that could contribute to depletion of cellular energy stores, endogenous reductant, and inhibit mitochondrial respiration. Certainly, redox-cycling proteins have been implicated in several forms of neurotoxicity [22]. Additionally, quinon-proteins could increase the yield of 8-oxo-2-deoxyguanosine. Thus they are able to promote further radical-generating events, which then transferred damage to other biomolecules such as DNA [23].

Furthermore, the quenching of the tryptophan-214 (Figure S2, Supporting information) and 8-Anilino-1-naphthalenesulfonic acid magnesium salt (ANS) fluorescence means the alteration in albumin conformation and in hydrophobic environment upon binding with quinone as observed in this study. The ANS is a well

known and much utilized "hydrophobic probe" for proteins. ANS is sensitive to micro-environmental changes and can serve as a suitable reporter of interaction in the neighborhood of protein tryptophan residue[24]. HSA contains two hydrophobic patches located at sub-domain IIA (site 1) and sub-domain IIIA (site 2). These patches are the principal site for ligand binding[13]. Changes seen in the fluorescence intensity of protein bound ANS may be attributed to alterations of accessibility of hydrophobic regions and ligand binding capacity of HSA–MQ complex (Figure S5, Supporting information). This alteration of albumin structure and conformation may impair the binding, transportation, and distribution of many drugs and/ or endogenous molecules.

The reversibility of quinone thiol adducts was carefully studied under our investigation[5]. The new finding herein was the capability of HSA to rapidly attack MQ-GSH forming HSA-MQ adduct and free GSH. Low pK_a and accessibility of HSA Cys34 gave it the high and fast reactivity with electrophile compared to GSH. In contrast, the interconversion of HSA-MQ and NAC to release MQ-NAC and free HSA had occurred very slowly. We also examined the fate of quinone adduct in rat plasma after MQ administration and found a time-dependent loss in protein adduct[9] (Figure S6, Supporting information). This phenomenon could be attributed to the reversibility or the cellular degradability of adducted proteins. Taken together, the modification of albumin by MQ exemplified here could be generalized to other cellular proteins in various organs. Generation of redox-cycling quinon-proteins adduct could induce oxidative and quinone mediated toxicity. The ability of qinones species to form protein adducts has been implicated in a number of disease processes, including diabetes, atherosclerosis, and neurodegenerative diseases[3]. Therefore, the identification of quinone-derived protein adducts is of great importance to understand their biological and/or toxicological role in human beings.

Conclusion

The inevitable exposure to quinones and the high concentration of cellular nucleophiles virtually guarantees human exposure to the potential adverse effects of quinone adducts. The tendency of quinones to bind to nucleophilic functional groups represents the most popular mechanistic theory underlying their toxicity. Therefore, more detailed information on the characters, occurrence, and fate of these adducts is important for understanding their mechanism of action. Our study demonstrates that, with molecular modeling and spectroscopy, adduction appears to be very selective because adduct formation was only detected on some of the nucleophilic sites. It was also noticed that there is no clear relationship between the number of nucleophilic sites and the number of adduct identified. Hence, more experiments using LC-MS/MS is required to identify the site specific modification on amino acid level. Moreover, to the best of our knowledge, the stability and reversibility of quinone- conjugates in a biological fluid is also announced herein.

References

1. Y. Kumagai, Y. Shinkai, T. Miura and A. K. Cho, *Annu. Rev. Pharmacol. Toxicol.*, 2012, **52**, 221-247.
2. J. P. Klinman and D. Mu, *Annu. Rev. Biochem.*, 1994, **63**, 299-344.
3. J. Bolton, J. Trush, T. Penning and T. J. Monaks, *Chem. Res. Toxicol.*, 2000**,** **13**, 135-160.
4. P. C. Jocelyn and T. J. Monks, *Curr. Drug. Metab.*, 2002, **3**, 425-443.
5. M. S. Elgawish, C. Shimomai, N. Kishikawa, K. Ohyama, M. Wada and N. Kuroda, *Chem. Res. Toxicol.*, 2013, **26**, 1409-1416
6. T. J. Monks and S. S. Lau, *Crit. Rev. Toxicol.*, 1992, **22**, 243-264.
7. D. G. Graham, *Mol. Pharmacol.*, 1978, **14**, 633-643.
8. T. K. Rudolph and B. A. Freeman, *Sci. Signal*, 2009, **2**. re7
9. M. S. Elgawish, N. Kishikawa, K. Ohyama, and N. Kuroda, *J. Chromatogr. A*, submitted.
10. A. Banerjee, K. Basu and P. K. Sengupta, *J. Photochem. Photobiol. B*, 2008, **90**, 33-40.
11. J. K. Diedrich and R. R. Julian. *Anal. Chem.*, 2010, **82**, 4006-4014.
12. C. L. Hawkins, P. E. Morgan and M. J. Davies, *Free. Radic. Biol. Med.*, 2009, **46**, 965-988.
13. T. Jr. Peters, All about Albumin. Biochemistry, Genetics, and Medical Applications, Academic Press: San Diego, 1996.
14. N. Diaz, D. Suarez and T. L. Sordo, *J. Am. Chem. Soc.*, 2001, **123**, 7574-7583.
15. G. Aldini, G. Vistoli, L. Regazzoni, L. Gamberoni, R. M. Facino, S. Yamaguchi, K. Uchida and M. Carini, *Chem. Res. Toxicol.*, 2008, **21**, 824-835.
16. W. Li, J. Heinze and W. Haehnel, *J. Am. Chem. Soc.*, 2005, **127**, 6140-6143.
17. M. D. Person, T. J. Monks, S. S. Lau, *Chem. Res. Toxicol.*, 2003, **16**, 598-608.
18. T. Miura, H. Kakehashi, Y. Shinkai, Y. Egara, R. Hirose, A. K. Cho, Y. Kumagai, *Chem. Res. Toxicol.*, 2011, **24**, 1836-1844.
19. C. Lin, D. R. Chen, S. L. Wang, W. C. Hsieh, W. F. Yu, T. W. Wang, C. H. Tsai, H. H. Wei, B. J. Tsuang and P. L. Lin, *J. Environ. Sci. Health. A*, 2014, **49**, 634-640.

20. S. Waidyanatha, Y. Zheng, B. Serdar and S. M. Rappaport, *Cancer. Epidemiol. Biomarker. Prev*., 2004, **13**, 117-124.

21. D.M. Kuhn, R. E. Arthur, D. M. Thomas and L. Elferink, *J. Neurochem*., 1999, **73**, 1309-1317

22. A. Brunmark, E. Cadenas, *Free. Radic. Biol. Med*., 1989, **7**,435-447.

23. B. Morin, M. J. Davies, R. T. Dean, *Biochem. J*., 1998, **330**, 1059-1067.

24. G. Weber and E. Daniel, *Biochemistry*, 1966, **5**, 1901-1907.

Figure Captions

- **Figure 1 a)** Proposed binding interactions of MQ with albumin. Cartoon representation was used for the backbone atoms in grey. Only residues interacting with the ligands were shown as green sticks. Ligands are shown as capped sticks and colored by atom types with carbon atoms in cyan.
  b) The accessible cysteine residue of alcohol dehydrogenase (Cys241). The surrounding amino acids (green sticks) do not include any activating residues as in the case of albumin.

- **Figure 2**. **Blue**: H bond distance between one of the MQ carbonyl oxygen and Thr79 side chain hydroxyl group; **red**: distance between the accessible Michael acceptor atom of MQ and the sulfur atom of Cys34.

- **Figure 3.** Time profile of MQ, HSA-MQ adduct, and MQ in presence of native HSA.
  50 µl of (200 µg/ml HSA-MQ (1:20), or MQ-GSH (2 µM) or MQ (2 µM), or MQ (2 µM) + native HSA (200 µg/ml), or HSA (200 µg/ml)), **50 µl** of 100 µM luminol (in 0.1M carbonate buffer pH 9.4), and **100 µl** of 100 µM DTT were added. The mixture was mixed and luminescence was measured by Berthold luminometer. (n=3)

Figures

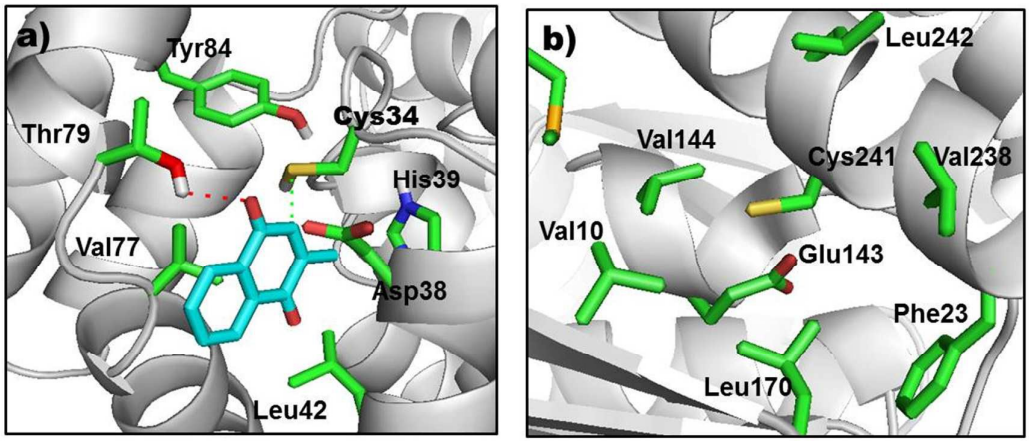


Figure 1

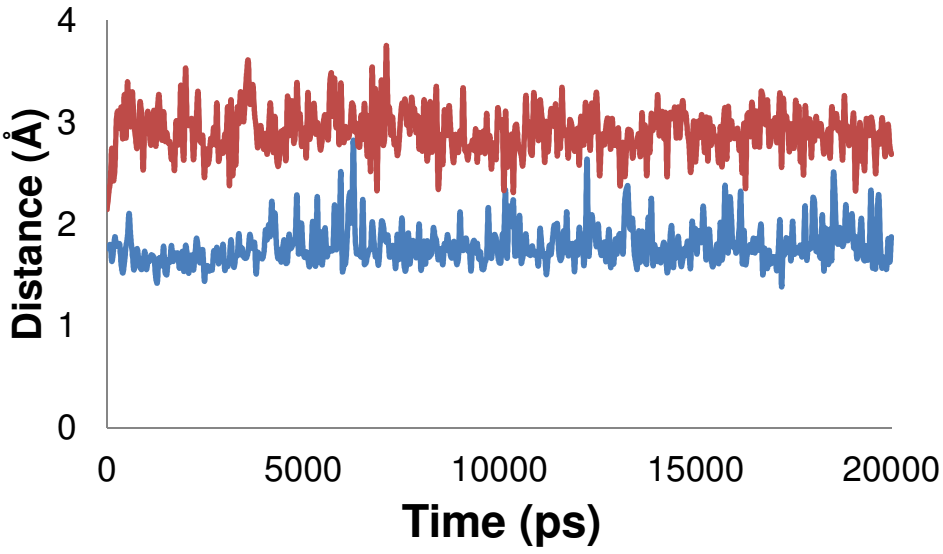


Figure 2

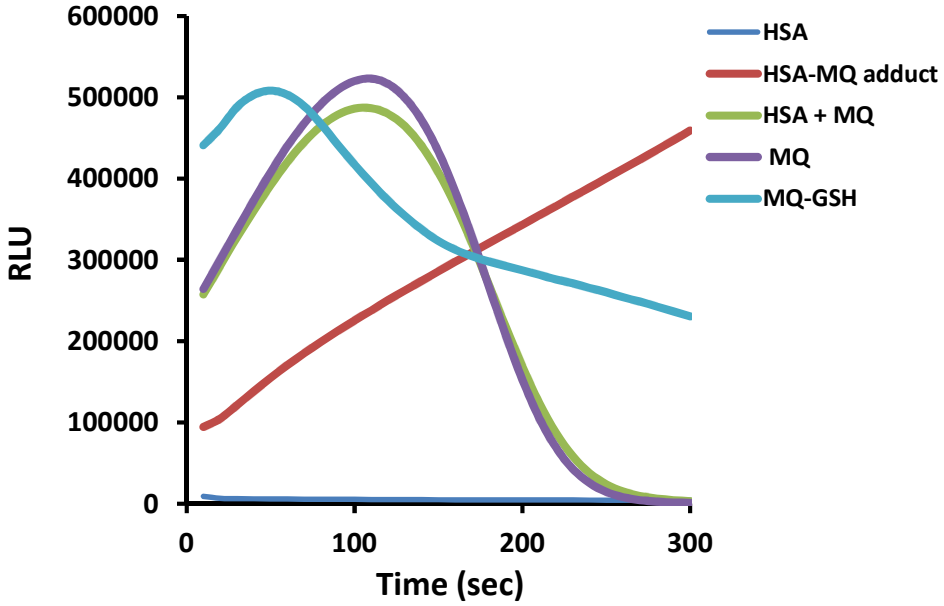


Figure 3