A comparison study on ribonuclease A modifications induced by substituted p-benzoquinones

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In this paper, we present our investigation on ribonuclease A (RNase) modifications induced by 1,4-benzoquinone (PBQ), 2-methyl-1,4-benzoquinone (MBQ), and 2-chloro-1,4-benzoquinone (CBQ). The goal of the study was to evaluate quinone-induced protein modifications as well as substituent effects, utilizing several techniques such as SDS–PAGE, fluorescence spectroscopy, microscopy, and LC-ESI−QTOF-MS. SDS–PAGE experiments revealed that all quinones modify RNase through oligomerization as well as polymeric aggregation; with CBQ functioning as the most efficient quinone while MBQ was least efficient. The confocal imaging analysis showed that the presence of CBQ resulted in massive RNase aggregation, while PBQ-treated RNase formed much smaller aggregates. MBQ-treated RNase exhibited micrographic features that closely resembled those of the unmodified RNase. LC-ESI−QTOF-MS studies indicated the nature of PBQ- and CBQ-induced RNase modifications are complex mainly due to simultaneously occurrence of both adduct formation and oligomerization. Kinetic studies on quinone reactivity toward lysine revealed the rank order of CBQ > PBQ > MBQ, based on the second-order rate constants. We also utilized scanning electron microscopy in order to investigate the effect of modified RNase on the biomineralization of salts.

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1. Introduction

Quinones are commonly found in nature [1–3] and the general environment [4]. Some quinones exist as an integral part of biological systems such as quinoprotein-bound cofactors [1] or electron carriers in the mitochondrial electron transport chain [3], while others occur naturally as free (i.e., unbound) quinones in many organisms [2] or as the metabolites of polycyclic aromatic hydrocarbons (PAHs) from industrial processes [4]. Due to their redox ability and structure containing conjugated carbonyl groups in a cyclic ring, quinones exhibit interesting biological activities and cytotoxicity [4]. For instance, protein-bound quinones are known to oxidize protein substrates and amines; producing the corresponding aldehydes, ammonia, and hydrogen peroxide [1]. Unlike protein-bound quinones which have limited structural variations, free quinones appear in many different structures with distinctive chemophysical properties, whether generated endogenously [1–3] or introduced exogenously in living cells [4]. However, the free quinones' biological effects remain to be studied due to the overwhelmingly diverse structural variations. Accordingly, many studies have been focused mostly on the biological outcome of a few PAH quinones. PAH quinones are responsible for the observed toxicity of PAHs by reacting with cellular proteins and nucleic acids through redox cycling [5,6] and adduct formation [7–23]. Among these lines, our recent study showed that PBQ, one of the benzene metabolites [8], was able to modify a model protein, ribonuclease A (RNase), in a complex manner including adduct formation and protein aggregation intervened by redox-cycling [24]. In addition to PBQ, halogenated benzoquinones have been the subject of recent studies. For example, chlorinated benzoquinones were...
found to be produced during drinking water disinfection processes [25–28] and to modify DNA as well as the building blocks of DNA [4,18,19], and bromobenzoquinone was identified as a metabolite of bromobenzene and found to be involved in adduct formation with cellular proteins [13,20].

The above-mentioned results on quinones, each containing different structures and different biological outcomes, motivated us to devise a comparison study to establish a reactivity profile of quinones with structural differences affecting their biological activities. In this regards, we selected PBQ, MBQ, and CBQ in order to evaluate substituent effects playing a role toward quinone induced-protein modifications in a model system. RNase was chosen for the study since it was used for the previous report showing RNase modifications induced by PBQ [24]. We examined the modification nature of RNase, upon exposure to PBQ, MBQ, and CBQ, using SDS–PAGE, fluorescence spectroscopy, UV–Vis spectroscopy, confocal microscopy, LC–MS, and scanning electron microscopy. Fig. 1 shows the proposed action of quinones leading to oxidative damage, adduct formation, lysine oxidation, and protein crosslinking, which are intermediated by redox-cycling. Lysine oxidation can consequently lead to either intra- or intermolecular protein crosslinking and redox cycling leads to the generation of hydroquinone (HQ) which can be detected using a fluorescence approach. Herein, the current study mainly focuses on adduct formation and protein crosslinking induced by quinones and their substituents. SDS–PAGE was utilized for investigating the change in the molecular mass of RNase upon modifications. Monitoring the structural and morphological changes of RNase was carried out utilizing fluorescence spectroscopy coupled to anisotropic measurements and confocal microscopy. UV–Vis spectroscopy was utilized to assess adduct formation. Together with the techniques mentioned above, a LC-ESI–QTOF-MS approach was utilized to determine an accurate mass of the modified RNase adducts in the monomer level as well as in the dimer level. Kinetic analyses were carried out based on the reactions of each quinone and lysine. Lastly, we examined biomineralization of salts affected by the presence of RNase whether unmodified or modified by each quinone.

2. Materials and methods

All chemicals were purchased from Fisher and were of reagent grade unless specified otherwise. The water used in the study was deionized water (d-water) purified by a Millipore system (Milli-Q water). Ribonuclease A (from bovine pancreas) was purchased from Sigma. Electrophoresis units for minigels were purchased from Fisher. Dialysis was carried out using a 3 ml Float-A-Lyzer with a molecular weight (MW) cutoff of 3.5 kDa, which was purchased from Spectrum Laboratories. Spin column was purchased from Millipore (Millipore Microcon-YM3) with a MW cutoff of 3 kDa.

2.1. RNase modifications detected by SDS–PAGE

Aliquots of the RNase stock solution were diluted to have a final concentration of 0.145 mM RNase and treated with each quinone (1.0 mM) in phosphate buffer (pH 7.0, 50 mM). Then, each solution was incubated at 37 °C for various time periods, followed by immediate cooling at 0 °C. These samples underwent trichloroacetic acid (TCA) precipitation according to protocol [24]. Then, the TCA-treated samples were submitted to SDS–PAGE analysis. For the SDS–PAGE analysis, all proteins were separated on a 10% SDS–PAGE gel according to the method of Laemmli [29]. Electrophoresis was performed for 2.5 h at 100 V and 60 mA for two gels (30 mA per gel). Pierce Blue Prestained Protein Molecular Weight Marker Mix (Cat. # 26681) was used as a reference to determine molecular weight (MW) of protein bands. Protein bands were visualized by staining the gels with 0.1% coomassie brilliant blue G-250 blue. The molecular mass (MM) of each protein band was determined by plotting the mobility (i.e. represented in the distance each band traveled) against the logarithm of the proteins’ MM using the Protein Molecular Weight Marker [30].

2.2. RNase modification detected by fluorescence spectroscopy

Fluorescence spectra and anisotropy values were obtained at 37 °C using a Horiba Jobin Yvon Fluorolog-3 spectrophotometer with a fluorescence polarization accessory and a full-spectrum, xenon-source lamp. The samples were recorded in a 1 cm × 1 cm quartz cuvette using an excitation wavelength of 280 nm. All emission spectra were recorded over the 290–550 nm range in increments of 1 nm, with a band pass of 2 nm for both excitation and emission, and were intensity corrected. The spectrum of unmodified RNase was recorded with an integration time of 0.5 s, while the spectra of modified (i.e., post-dialysis) RNase and RNase-quinone reaction mixtures were recorded with an integration time of 0.1 s. The spectra of the unmodified and the post-dialysis RNase were normalized in the 430–460 nm range, where the tyrosine emission is negligible. The anisotropy values were determined with a band pass of 5 nm for both excitation and emission and with an integration time of 5 s.

All fluorescence studies were carried out in a phosphate buffer (pH 7.0, 50 mM) solution. The concentration of RNase in the reaction mixture was always 0.050 mM and was obtained by transferring aliquots of the RNase stock solution. The quinone concentrations were 0.50 mM (for a quinone:RNase molar ratio of 10:1), and were obtained from fresh quinone solutions that were prepared each time and used immediately after the 10 min sonication. The reaction was initiated by adding the appropriate volume (always less than 100 μL) of quinone solution to a solution containing RNase that was equilibrated at 37 °C in the temperature-controlled cell holder of the fluorimeter for 15 min. The RNase modifications were monitored at one-hour increments for 24 h.

Following the 24-h fluorescence monitoring, the reaction mixture was dialyzed against phosphate buffer (pH 7.0, 50 mM, 3 × 1000 mL per 3 mL sample) at 4 °C for 24 h. The dialyzed samples, which will be called further as modified RNase or post-dialysis RNase, were submitted to additional analysis as presented below in Sections 2.3–2.6. Control RNase was subjected to the same procedure except the addition of quinone.

Fig. 1. Proposed action of quinones (X = H for PBQ, = CH3 for MBQ, = Cl for CBQ).
2.3. RNase modification detected by UV–Vis spectroscopy

UV–Vis spectra were obtained in a 1 cm × 1 cm quartz cuvette using BioSpec-1601 spectrophotometer by Shimadzu and UV Probe Soft. With a jacketed (temperature controlled) cell compartment to maintain the physiological temperature.

2.4. LC-ESI®-QTOF-MS studies on RNase modifications

Following the procedures described in sections 2.2 and 2.4, the samples for LC-MS studies were further purified with spin columns to remove residual salts, lyophilized, and resuspended in an eluting buffer prior to submission to a chromatographic separation using C18 column. To note, the RNase control sample was solubilized well with the eluting buffer while both RNase + PBQ and RNase + CBQ samples were only partially solubilized.

LC-MS analyses were carried out with an Agilent 6520 QTOF coupled to 1290 UHPLC operated in ESI® mode. Chromatographic separation was achieved using an Agilent Poroshell-C18 column (2.1 mm × 100 mm, 2.7 μm) eluted at a flow rate of 0.5 mL/min. The mobile phase was composed of solvents A and B. Solvent A was 0.1% formic acid in water and solvent B was 0.1% formic acid in acetonitrile. The gradient condition for analyses was as follows: 0% to 2% solvent B from 0 to 2 min, 2% to 10% solvent B from 2 to 2.1 min, 10% to 25% solvent B from 2.1 to 15 min, 25% to 95% solvent B from 15 to 20 min, and 95% to 2% solvent B from 20 to 20.1 min. The column temperature was maintained at 75 °C using a thermostated column compartment. Under these conditions, RNase control was eluted as a single peak with a retention time (RT) of 11.88 min, RNase + PBQ was eluted as multiple peaks ranging from 11.80 to 16.14 min, and RNase + CBQ was eluted as multiple peaks ranging from 11.76 to 16.40 min. The mass spectrometer was operated in the positive mode with nitrogen gas as a nebulizing and drying gas (55 psi, 12 L/min). The total eluent flow was directed to the ESI source. The Vcap voltage was set to 5000 V, while the fragmentor voltage was set to 300 V. The drying gas temperature was set to 350 °C. The acquisition rate was set at 1 scan/s. The data was analyzed using MassHunter Workstation Qualitative Analysis software 4.0 and MassHunter BioConfirm software 4.0 (Agilent) Spectra were generated over the full LC peak. The acquired mass spectra were deconvoluted using the maximum entropy algorithm utilizing the intense peaks with the charge states from +7 to +13. The parameters for the maximum entropy calculation were as follows: For RNase control, range 10,000 to 14,000 Da, mass step 1.0000 Da, and S/N threshold 30; For RNase + PBQ and RNase + CBQ, range 10,000 to 100,000 Da, mass step 1.0000 Da, and S/N threshold 30.

2.5. Confocal microscopy analysis of the modified RNase

Aliquots (20 μL) of the post-dialysis RNase solutions were placed on microscopic slides, covered with a cover slip (22 mm in diameter), and sealed. Images were captured with a SIM (Simultaneous) Scanner on an Olympus Fluoview 1000 laser scanning confocal microscope (Olympus B × 61 microscope with a 10 × dry objective lens) and analyzed using Olympus Fluoview acquisition software. Scale bar representations are specified under the figure caption. The reference control micrograph was obtained using a solution containing the unmodified RNase (0.050 mM).

2.6. Detection of biomineralization by scanning electron microscopy

Aliquots (5 μL) of the unmodified RNase and the modified RNase solutions, after dialysis, were deposited on a microscopic cover glass (22 mm in diameter) and fixed with an equal volume of the SEM matrix made of 4% formaldehyde in phosphate-buffered saline (PBS) containing [NaCl] = 137 mM, [KCl] = 2.7 mM, [Na2HPO4] = 10 mM, [KH2PO4] = 2 mM, followed by air-drying for 2 h at room temperature. Then, the samples were dehydrated in ethanol in ascending percentage concentrations (30%, 50%, 70%, 80%, 90%, 100%) for 20 min each. For a control sample, an aliquot of the SEM matrix was mixed with an equal volume of milliQ water, and the residue was submitted to the same dehydration procedure described above. Scanning electron micrographs (SEMs) were collected using a JEOL Neoscope microscope operating with an accelerating voltage of 10 kV.

2.7. Kinetic studies for the reactions of each quinone and lysine detected by UV–Vis spectroscopy

The reactions of each quinone (0.010 mM) and lysine (20 mM) were monitored by UV–Vis spectroscopy under pseudo first-order conditions with excess lysine in phosphate buffer (pH 7.0, 50 mM) in a 1.0 mL volume, 1 cm path-length quartz cuvette. The reaction was initiated by adding an aliquot of a freshly prepared quinone solution to the amino acid-containing solution which was equilibrated at 37 °C for 10 min and submitted to UV–Vis scanning. The pseudo-first-order rate constants (kobs) of the reactions of quinone and amino acids were calculated from the rate of a decrease in the intensity of the absorption due to quinone using the first-order rate equation:

\[ k_{obs} = -\ln\left(\frac{A_t - A_0}{A_u - A_0}\right), \]

where t is time (min), A_u is absorbance at infinity which was recorded at Shimadzu UV–Vis spectrophotometer. The pseudo-first-order rate constants (kobs) of the reactions of quinone and amino acids were calculated from the rate of a decrease in the intensity of the absorption due to quinone using the first-order rate equation:

\[ k_{obs} = -\ln\left(\frac{A_t - A_0}{A_u - A_0}\right), \]

where t is time (min), A_u is absorbance at infinity which was obtained at 8 × t_{1/2} (t_{1/2}, half-life) or longer, A_t is absorbance at time t, and A_0 is absorbance at the initial time point. The disappearance of quinone in the reaction was monitored by following the change in A_{355} recorded at Shimadzu UV–Vis spectrophotometer. The pseudo-first-order rate constants were converted to the second-order rate constants in units of M⁻¹ s⁻¹ by dividing the used amino acid concentration.

2.8. RNase structure modeling and solvent accessibility scoring

The structure of RNase was generated using the RNase A sequence obtained from the protein data bank (PDB ID: 3MZQ) and viewed with the DeepView/Swiss-PdbViewer 4.1.0 available at the Swiss-Prot server [31]. In order to predict the solvent accessibility of amino acid residues in RNase, we submitted the RNase sequence to the I-TASSER on-line platform [32]. Then, a RNase solvent accessibility scale plot was constructed with the solvent accessibility scale on y-axis and the amino acid residue number on x-axis, where the scale 9 is for a highly exposed residue and 0 is for a highly buried residue.

3. Results

3.1. RNase modification detected by SDS–PAGE

SDS–PAGE analysis was carried out after RNase was incubated with 1.0 mM PBQ, MBQ, or CBQ, respectively, up to 5 h in phosphate buffer (pH 7.0, 50 mM) at 37 °C. Fig. 2 represents the gel features of RNase treated with PBQ, MBQ, and CBQ, which are labelled as RNase + PBQ, RNase + MBQ, and RNase + CBQ, respectively. RNase + MBQ presented a simplest gel feature with two major bands around 14 and 30 ± 3 kDa (Fig. 2A). However, RNase + PBQ and RNase + CBQ exhibited multiple protein bands around 14, 30 ± 3, 53 ± 3, and 70 ± 3 kDa (Fig. 2B/C). In addition, for RNase + CBQ, a smearing band was observed at the high MW region in the gel (Fig. 2C). Major modifications appeared to occur within 1 h of incubation for all reactions.
3.2. Fluorescence spectroscopy and anisotropy determination

The RNase modifications induced by quinones were further analyzed by fluorescence experiments in which RNase (0.050 mM) was treated with each quinone (0.50 mM), respectively, in phosphate buffer (pH 7.0, 50 mM) for 24 h at 37 °C. The fluorescence spectrum of the unmodified RNase, as well as those of the modified RNase, shows a band centered around 300 nm. Because this band partially overlaps with bands from other reaction products, like the band around 330 nm of hydroquinone (HQ, the product of PBQ reduction), the monitoring of the changes in the RNase fluorescence intensity was performed at 295 nm and at 300 nm. In addition, fluorescence anisotropy values were also determined and monitored for 24 h at the same wavelengths.

Fig. 3 shows the changes in the fluorescence intensity at the observed wavelengths as the protein modification occurred over 24 h as well as the changes observed in the anisotropy. The fastest and the most significant decrease in the fluorescence intensity occurred for the reaction mixture with CBQ. Fig. 3 also shows that anisotropy values, at both investigated wavelengths, tend to decrease as the reaction progresses. It was found however that the anisotropy values for the modified RNase, obtained after removal of side products through dialysis, were larger than those of the reaction mixture at 24 h, and even slightly larger than these values of the unmodified RNase. The anisotropy values for the unmodified RNase and the modified RNase by MBQ, PBQ and CBQ are 0.169, 0.184, 0.185, and 0.214, respectively, at 295 nm and 0.166, 0.185, 0.188, and 0.209, respectively at 300 nm.

The fluorescence spectra of the unmodified and the modified (i.e., post-dialysis) RNase are shown in Fig. 4A. The fluorescence intensity of the modified RNase was lower than that of the unmodified RNase, and the intensity decreased in the order MBQ > PBQ > CBQ. The integrated fluorescence intensity in the 290–360 nm range, normalized to a value of 1 for unmodified RNase, are 0.336, 0.163, and 0.109 for MBQ, PBQ and CBQ, respectively.

3.3. UV–Vis spectroscopic analysis

Fig. 4B shows the representative UV–Vis spectra of the incubation reactions after dialysis. A control sample containing the unmodified RNase (0.050 mM) has the maximum absorption at 280 nm. As each quinone was added to RNase, there was distinguishable change in the spectral feature, accompanying an additional chromophore that developed at 346 nm for all three quinone-treated RNase. The absorbance values were 0.357, 0.837, 1.075, and 1.306 at 280 nm, and 0.023, 0.464, 1.632, and 1.926 at 346 nm for unmodified RNase, RNase + MBQ, RNase + PBQ, and RNase + CBQ, respectively.

3.4. Confocal microscopic analysis

Fig. 5 illustrates the confocal micrographs of post-dialyzed RNase whether RNase was unmodified (RNase control) or modified. RNase control appeared as small-sized-protein dots in a relatively uniform shape (Fig. 5A), while the micrographs of the quinone-treated RNase showed recognizable differences in terms of RNase size and shape. RNase + MBQ exhibited relatively similar micrographic feature to RNase control. However, noticeable changes were observed for both RNase + PBQ and RNase + CBQ. In particular, RNase + CBQ showed extensive RNase modifications based on the large sized-RNase aggregates (Fig. 5D).

3.5. LC-ESI−QTOF-MS results

LC-MS studies were carried out with the modified RNase by PBQ and CBQ (denoted as RNase + PBQ and RNase + CBQ, respectively), in addition to the unmodified RNase (denoted RNase control), in order to determine molecular mass of modified RNase via deconvolution.

Fig. 6 presents the total ion chromatograms (TICs) in relative intensity vs. retention time (RT) in min, for RNase control, RNase + PBQ and RNase + CBQ, respectively. The TIC of RNase control has a single peak at a retention of 11.88 min (Fig. 6A), which reflects that the sample consists exclusively of an intact unmodified RNase. TICs of RNase + PBQ (Fig. 6B) and RNase + CBQ (Fig. 6C) exhibited more complicated features with many peaks spreading over a range of RT from 11.80 to 16.14 min for RNase + PBQ and 11.76–16.40 min for RNase + CBQ. Those features were labelled Pp1–Pp5 for RNase + PBQ and Pc1–Pc4 for RNase + CBQ, respectively. The Pp1 in RNase + PBQ and Pc1 in RNase + CBQ have the same retention time as the single RNase control peak suggesting the presence of remaining unmodified RNase.

Figs. 7 and 8 represent the acquired MS (left-panel) and the corresponding deconvoluted spectra (color-highlighted right panel) for each assigned peak in Ppi series and Pci series, respectively. In order to obtain the experimental mass values for the unmodified
as well as the modified RNase, maximum entropy deconvolution algorithm was used by deconvoluting a multiply charged protein spectrum into a deconvoluted zero-charge mass spectrum. Table 1 shows the theoretical mass to charge ratio ($m/z$) values of the native RNase in multiple-charged states along with the experimentally observed $m/z$ values of RNase control. Supplementary content contains the acquired MS and the deconvoluted MS for RNase control. The major observed $m/z$ values at the acquired MS for RNase control are 1141.1766, 1244.8170, 1369.2091, 1521.2339, 1711.2504, and 1955.5683 corresponding to the charge states of RNase from +12 to +7, respectively. All $m/z$ values and deconvoluted masses are represented in Dalton (Da). The observed $m/z$ values of the multiply charged RNase control are in great agreement with the theoretically calculated $m/z$ values of intact RNase (Table 1). The deconvoluted mass of RNase control was found to be 13682.3277, and this is consistent with the theoretically calculated average mass value of RNase of 13682.2846. Left panels (A to E) of Fig. 7 show the acquired MS of RNase + PBQ at various RTs, labeled as Pp1 (11.89 min), Pp2 (12.58 min), Pp3 (13.40–14.04 min), Pp4 (14.34–15.54 min), and Pp5 (15.80 min), respectively. As RT in TIC increases, the acquired MS peak feature was altered with shifting $m/z$ values compared to the values of RNase control and this altering trend is more evident for the acquired MS for the region Pp5, containing many peaks overlapping together. For instance, the $m/z$ values of the multiply charged species at the acquired MS of Pp1 (Fig. 7A and Table 1) were 1141.1682, 1244.8148, 1369.2091, 1521.2247, 1711.2425, and 1955.5662, and these values are in good agreement with the values for the multiply charged RNase control (Table 1). At the later RT, such as Pp2 and Pp3, the $m/z$ values (for a charge state of +12) increased to 1149.8339 for Pp2 and 1158.4969 for Pp3, respectively. This increasing trend in $m/z$ values was consistently observed for the charge states of +11 to +7 as well. The deconvoluted spectra for each Ppi region provided a single deconvoluted mass per region: 13682.33 for Pp1 (Fig. 7A), 13786.41 for Pp2 (Fig. 7B), and 13890.40 for Pp3 (Fig. 7C), respectively. However, the deconvoluted spectrum for Pp4 (Fig. 7D) presents several deconvoluted mass values at 13890.49, 13980.54, 13994.62, 14010.75, and 14104.75, respectively, for the multiple peaks appearing around 13980.54. Pp5 exhibits a similar trend with Pp4, however with many peaks of low intensity both at the acquired MS and deconvoluted MS mode; with the deconvoluted masses of 13994.07, 14084.84, 27781.04, and 27886.09.

Similarly, left panels of Fig. 8 show the acquired MS of RNase + CBQ acquired at various RT, labeled as Pc1 (11.86 min), Pc2 (12.59 min), Pc3 (13.23–15.31 min), and Pc4 (15.84 min), respectively. Fig. 8A represents the acquired MS at the earlier RT,
Pc1 and the m/z values for the acquired MS were determined to be 1141.1712, 1244.8213, 1369.2077, 1521.2295, 1711.2482, and 1955.5610, which are consistent with the m/z values of RNase control (Table 1). Starting from Pc2, the m/z values of the multiple-charged species in the acquired MS deviate significantly from the values obtained for the RNase control. The m/z value differences between RNase control and Pc2 of RNase + PBQ at the charge states of +12, +11, +10, +9, +8, and +7 are 8.7, 9.5, 10.4, 2.6, 13.0, and 14.9, respectively. This increasing trend in m/z values continued at Pc3 and Pc4, as RT increased. In addition, the apparent presence of bulging peaks at the acquired MS was observed for the later RT in the region Pc4. The deconvoluted spectrum for Pc1 region (right panel of Fig. 8A) exhibited a single deconvoluted mass of 13682.33. However, the deconvoluted spectrum for Pc2 (Fig. 8B) resulted in two deconvoluted masses of 13786.40 and 13820.69, respectively. Deconvolution of Pc3 and Pc4 resulted in several deconvoluted mass values: 13786.40, 13820.32, 13890.36, 13925.06, 13942.35, 13958.84, and 13976.53 for Pc4, and 14048.86, 28094.78, and 28297.70 for Pc5, respectively.

3.6. Biomineralization affected by the presence of RNase

In order to see if the modified RNase affects the biomineralization of salts, we monitored scanning electron micrograph (SEM) features of crystalized salts in the absence and presence of RNase. Fig. 9A represents the micrograph of SEM matrix comprised of PBS and 4% formaldehyde, showing four crystals of salts in a pyramidal shape with slight variation and a length of each crystal at 1 mm. When RNase was introduced to the SEM matrix, there was some change in the micrographic feature showing the decrease in the size of salt crystals (Fig. 9B). However, the micrograph of the SEM matrix containing the modified RNase exhibited polymorphism in the formed salt crystals with respect to their sizes and shapes. This altered feature with much smaller crystals in various shapes was more evident when the SEM matrix was treated with CBQ-treated RNase.

3.7. Kinetic analysis for the reactions of quinone and lysine

The relative reactivity of the investigated quinones of interest toward lysine, as a model for biologically available nucleophiles,
was investigated by carrying out the reactions of each quinone (0.010 mM) and lysine (20 mM) in phosphate buffer (pH = 7.0, 50 mM) at 37 °C. The second-order rate constants for the reactions of each quinone and lysine were determined to be 0.019, 0.078, and 0.20 M⁻¹ s⁻¹ for MBQ, PBQ, and CBQ, respectively.

### Table 1

<table>
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<th>Charge on RNase</th>
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<th>Experimental m/z of RNase + PBQ at Pp1</th>
<th>Experimental m/z of RNase + CBQ at Pc1</th>
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a The value represents the mass of RNase in its neutral state whether theoretical or experimental.

In order to visualize protein modifications induced by quinones, SDS–PAGE analysis was utilized after incubating RNase with MBQ, PBQ, and CBQ, respectively. SDS–PAGE results show that all three quinones modify RNase significantly but with different magnitudes. For the incubation of RNase with MBQ, two protein bands appeared (Fig. 2): one at 14 kDa corresponding to RNase monomer and the other at 30 ± 3 kDa due to a protein dimer suggesting that only dimerization is occurring. For the incubation of RNase with PBQ or CBQ, more bands were observed at 14, 30 ± 3, 53 ± 3, and 70 ± 3 kDa, suggesting a more complex protein oligomerization. Furthermore, the gel feature of CBQ-treated RNase display a smearing band at the high MW region indicating that RNase further underwent the formation of polymeric aggregate which is not observed for MBQ- or PBQ-treated RNase under the same reaction conditions. Our previous study showed indeed that 34 equivalents of PBQ (at reaction conditions of RNase:PBQ = 0.145 mM:5.0 mM) was required to induce the formation of the smearing band due to RNase polymerization [24]. This finding implies that CBQ is more efficient than PBQ in inducing RNase polymerization while effort to further understand the nature of the quinone-induced protein modifications, we expanded our investigation to include substituted quinones, MBQ and CBQ, which are metabolites of commonly found PAH in the general environment. In the current study, we discuss our findings on the quinone-induced protein modifications, employing a number of techniques that provide comprehensive and complementary information on the nature and degree of the modification.

![Fig. 8. Acquired MS (left panel) and deconvoluted MS (right panel) of RNase + CBQ. RNase (0.050 mM) was dialyzed after treated with CBQ (0.50 mM) at pH = 7.0 and 37 °C prior to submission to LC-MS analysis. (A) Pc1, retention time at 11.86 min, (B) Pc2, retention time at 12.59 min, (C) Pc3, retention time range at 13.23–15.31 min, (D) Pc4, retention time at 15.84 min.](image-url)
RNase aggregates in the micrographic image (Fig. 5D). Again, RNase was treated with CBQ, showing the formation of much larger protein dots (Fig. 5C). This trend became even more evident when RNase was incubated with PBQ, there was increase in the size of the observed oligomerization or polymerization. However, when RNase was modified by PBQ, MBQ and CBQ, the MW estimates of the observed bands in SDS–PAGE are a bit higher than the estimated MW of RNase oligomers (namely 27.4 kDa for dimer, 41.04 kDa for trimer, and 54.72 kDa for tetramer, respectively). SDS–PAGE experiments suggest that adduct formation/alkylation of amino acids in RNase occurred simultaneously with RNase crosslinking.

The RNase oligomerization and polymerization were further evidenced by confocal microscopic analysis. The confocal image of RNase + MBQ sample (Fig. 5B) has similar features (i.e., small-sized dots) as the unmodified RNase (Fig. 5A) suggesting no oligomerization or polymerization. However, when RNase was incubated with PBQ, there was increase in the size of the observed protein dots (Fig. 5C). This trend became even more evident when RNase was treated with CBQ, showing the formation of much larger RNase aggregates in the micrographic image (Fig. 5D). Again, similar to SDS–PAGE, the confocal microscopy reveals that CBQ is the most efficient in RNase polymerization.

The relative reactivity of PBQ, MBQ, and CBQ toward the nucleophilic reaction with lysine was also investigated as model reactions similar to reactions of quinones with lysine residues in RNase, which is believe to be the first step in crosslinking and oligomerization. The second-order rate constants, at 37 °C in phosphate buffer, were determined to be 0.078, 0.019, and 0.20 M⁻¹ s⁻¹ for the reactions of lysine with PBQ, MBQ, and CBQ, respectively. These values are consistent with the relative reactivity of the investigated quinones toward RNase and show that CBQ is the most reactive quinone while MBQ is the least reactive in the reaction with nucleophilic lysine.

As RNase oligomerization and polymerization involve lysine oxidation in intermediary steps, we examined the locations of all lysine residues in RNase (namely residues 1, 7, 31, 37, 41, 61, 66, 91, 98, and 104, respectively) using DeepView-SwissPDB Viewer [31] (Fig. 10) and evaluated the solvent accessibility of lysine residues in RNase. The rendered RNase structure revealed that most of lysine residues are located at the surface of RNase. Fig. 11A shows a profile of the solvent accessibility of all residues of RNase, generated by I-TASSER [32] on the scale of 0–9, where a value of 0 represents a buried residue and a value of 9 represents a highly exposed residue. Lysine residues 1, 61, 66, 91, and 98 scored higher than 6 as expected since all these residues are completely exposed to the exterior of RNase. Lysine residues 7, 31, 37, and 41 scored in the range of 3–5. These scores suggest that these residues may be protected from solvent slightly better compared to the completely exposed residues. The I-TASSER profile suggests that quinones should be able to access lysine residues and convert them into oxo-lysine since most of lysine residues in RNase are solvent exposed, and these oxo-lysine residues would participate in intermolecular crosslinking leading to oligomerization as well as polymerization of RNase, as observed by SDS–PAGE. The main factor governing the degree of crosslinking appears to be the reactivity of quinone as determined by the substituent effect rather than the positions of lysine residues that can participate in crosslinking.

To obtain an overall degree of modification (at least for or around the RNase fluorophores), fluorescence spectroscopy was employed. In monitoring the fluorescence of reaction mixture up to 24 h, the fluorescence intensity decreased in time and the intensity decrease was much faster for CBQ than PBQ than MBQ (Fig. 3). Anisotropy determination also shows a slow decrease although the determination at 300 nm for reaction of RNase with PBQ might be affected by fluorescence of hydroquinone, the product PBQ reduction. The modified RNase samples obtained after dialysis have lower fluorescence intensity than the unmodified RNase (Fig. 4A). Again the loss of fluorescence is more significant for CBQ, suggesting greater modification than PBQ or MBQ. Based on the normalized integrated fluorescence intensity (NIFI) of the unmodified and the modified RNase, one can estimate a degree of protein modification (DPM) defined as DPM = 1 – NIFI. DPM is expected to be larger for the more reactive quinone in the reaction mixture and smaller for the less reactive quinone. Indeed, DPM based on integrated fluorescence intensity in the 290–360 nm range is estimated to be 0.66, 0.84, and 0.89 for MBQ, PBQ and CBQ, respectively. Considering that the RNase fluorescence is primarily due to six tyrosine residues and that the decrease in fluorescence is most

![Fig. 10. Model of RNase structure showing the locations of lysine residues, rendered at DeepView/Swiss-PdbViewer. LYS, lysine residue.](image)
likely a combination of quenching from all six tyrosine residues, the calculated DPM values are equivalent to the quenching of fluorescence from 4, 5 and 5.5 tyrosine residues for MBQ, PBQ and CBQ, respectively.

The anisotropy values determined at 295 and 300 nm for the modified, post-dialysis RNase were higher compared to those of the unmodified RNase as presented above. A higher anisotropy value is associated with a larger rotational correlation time and, assuming similar lifetimes, with a larger molecular volume for the modified RNase. This finding suggests an increase in the MW of the modified RNase through adduct formation and aggregation, which is consistent with the findings of SDS–PAGE and confocal microscopy.

UV–Vis spectral feature of the unmodified, post-dialysis RNase control (Fig. 4B, black line) shows a typical protein spectrum for an intact protein with $\lambda_{\text{max}}$ at 280 nm and $A_{280}$ of 0.357. This was found to be lowest value compared to modified, post-dialysis RNase obtained from reaction with each of the three quinones. However, the modified RNase exhibited also a different spectral profile that includes a new chromophore at 346 nm when compared to RNase control. As dialysis removed all unbound quinones or their redox-cycling products, this spectral feature is most likely due to quinones (or their derivatives) covalently linked to RNase supporting the idea of adduct formation. Among modified RNase, both $A_{280}$ and $A_{346}$ were highest for CBQ-treated RNase, while lowest for MBQ-treated RNase, consistent with results from other methods.

The nature of the adduct formation as well as RNase oligomerization, with dimerization in particular, was investigated even further by LC-MS of PBQ- and CBQ-modified RNase. In principle, the adduct formation of RNase can occur either through the nucleophilic attack of amino acid residues in RNase containing $-\text{NH}_2$, $-\text{OH}$, or $-\text{SH}$ group to one of the four available $\beta$-carbons of quinone or through the condensation of a lysine residue with a quinone carbonyl group.

One way to monitor the nature of adduct is to analyze the mass difference in Da (denoted, for simplicity, as $^{\text{PBQ}}\Delta m$) between the deconvoluted mass spectrum of RNase + PBQ and that of RNase control. For the Ppi series of RNase + PBQ (Fig. 7), at Pp2, $^{\text{PBQ}}\Delta m$ is 104.08 (the difference between 13786.41 for RNase + PBQ in Fig. 7B and 13682.33 for RNase in Fig. 7A). This value is only about 4 Da less than the mass of PBQ (i.e., 108.02 Da), suggesting that RNase + PBQ in Pp2 represents RNase monoalkylated by one PBQ molecule. The loss of about 4 Da suggests that the adduct is formed by losing 4 hydrogen atoms altogether from PBQ and two nucleophilic amino acids in RNase (i.e., by forming 2 new covalent bonds between PBQ and RNase). Furthermore, this suggests that adduct formation occurred through the nucleophilic attack at two of the four available $\beta$-carbons of PBQ. Similarly, RNase + PBQ at Pp3 (Fig. 7C), with the deconvoluted mass of 13890.40 and $^{\text{PBQ}}\Delta m$ of 208.07, is consistent with an adduct formed via dialkylation of RNase by two PBQ molecules. The deconvoluted MS spectrum in Fig. 7D, obtained for Pp4, is more complex, and it shows modified RNase with the deconvoluted masses of 13890.49, 13980.54, 13994.62, 14010.75, and 14104.73, respectively. These values correspond to $^{\text{PBQ}}\Delta m$ of 208.17 (equivalent to two times PBQ with 4 less H atoms, denoted $(2 \times [\text{PBQ} - 4\text{H}])$, 298.21 $(2 \times [\text{PBQ} - 4\text{H}] + 90.17)$, 312.29 $(3 \times [\text{PBQ} - 4\text{H}])$, 328.42 $(3 \times [\text{PBQ} - 4\text{H}] + [\text{H}_2\text{O} - 2\text{H}])$, and 424.41 $(4 \times [\text{PBQ} - 4\text{H}] + 6 \times [\text{H}])$, respectively. These values of $^{\text{PBQ}}\Delta m$ are consistent with RNase being di-, tri-, and tetra-alkylated by PBQ and a more fundamental change to the structure of RNase. For example, the $^{\text{PBQ}}\Delta m$ value of 328.42 suggests that the adduct is comprised of RNase and three PBQ molecules linked at two $\beta$-carbons each as well as an additional modification that adds a mass value of 16, equivalent to adding an O atom to the adduct. This addition of an O atom could be envisioned as occurring either on the PBQ part or on the RNase part of the adduct. The $^{\text{PBQ}}\Delta m$ value of 422.41 suggests that the adduct is comprised of RNase and four PBQ molecules which of these PBQ molecules are reduced into HQ form, thus adding two H atoms each. Finally, the deconvoluted MS for Pp5 (Fig. 7E) not only revealed complex adduct formation of RNase and PBQ but also confirmed the presence of RNase dimer linked through PBQ molecules. The observed deconvoluted masses for the PBQ-bound monomeric RNase are 13994.07 Da with $^{\text{PBQ}}\Delta m$ of 311.75 $(3 \times [\text{PBQ} - 4\text{H}])$ and 14084.84 Da with $^{\text{PBQ}}\Delta m$ of 402.51 $(3 \times [\text{PBQ} - 4\text{H}] + 90.45)$, respectively. The observed deconvoluted masses for the PBQ-bound dimeric RNase are 27781.04 Da, equivalent to $2 \times $[\text{RNase} (13682.33) + 416.37 (4 \times [\text{PBQ} - 4\text{H}]) + 27886.09 Da, equivalent to $2 \times [\text{RNase} + 521.45 (5 \times [\text{PBQ} - 4\text{H}])]$, respectively.

The protein modification induced by CBQ, as presented in the deconvoluted mass spectra (Fig. 8) of the Pci series, shows similar features to the modification by PBQ, however with a greater complexity in nature due to the presence of a Cl atom in the quinone structure and greater reactivity. Similar to Pp1 for RNase + PBQ, Pci1 for RNase + CBQ displays a peak with the deconvoluted mass

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig11.png}
\caption{(A) Solvent accessibility scale vs. residue number of RNase, determined by I-TASSER. Lysine residues are marked with \textbullet{}, where the scale 9 for a highly exposed residue and 0 for a highly buried residue. (B) RNase sequence.}
\end{figure}
of 13682.33 that is consistent with unmodified RNase. However, the deconvoluted mass spectra for other Pci series, Pci2-Pci4, present interesting findings (described again by the difference between the deconvoluted mass in the deconvoluted spectrum of RNase + CBQ and the unmodified RNase value of 13682.33 Da and denoted \( [CBQ] n \)), suggesting that RNase + CBQ adducts contain either dechlorinated CBQ or CBQ itself. The deconvoluted masses for RNase + CBQ at Pci2 (right panel of Fig. 8B) are 13786.37 and 13820.69 Da, resulting in \( [CBQ] n \) values of 104.04 and 138.36, respectively. The 104.04 value (which was also found for RNase + PBQ adduct) is consistent with the addition of one CBQ molecule that loses the Cl atom in the process, a possible result since Cl is a good leaving group. The \( [CBQ] n \) value of 138.36 at Pci2, however, is consistent with RNase being monoalkylated by one molecule of CBQ through two covalent bonds utilizing two nucleophilic amino acid residues of RNase. The deconvoluted MS of Pci3 in Fig. 8C is much more complex, and it further supports the evidence of adduct formation between RNase and CBQ with the deconvoluted masses of 13768.40, 13820.32, 13925.06, 13942.35, 13958.84, and 13976.53, respectively. The resulting \( [CBQ] n \) values are 104.07 (equivalent to a CBQ molecule with 1 Cl and 3 H atoms less, denoted \( [CBQ \text{--} \text{Cl} \text{--} 3\text{H}] \)), 137.99 (equivalent to a CBQ molecule with 4 less H atoms, \( [CBQ \text{--} 4\text{H}] \)), 208.04 \( (2 \times [CBQ \text{--} \text{Cl} \text{--} 3\text{H}]) \), 242.74 \( (1 \times [CBQ \text{--} \text{Cl} \text{--} 3\text{H}] + 1 \times [CBQ \text{--} 4\text{H}]) \), 260.02 \( (1 \times [CBQ \text{--} \text{Cl} \text{--} 3\text{H}] + 1 \times [CBQ \text{--} 4\text{H}] + 1 \times [H_2O]) \), 276.52 \( (2 \times [CBQ \text{--} 4\text{H}]) \), and 294.21 \( (2 \times [CBQ \text{--} 4\text{H}] + [H_2O]) \), respectively. The deconvoluted MS for Pci4 (Fig. 8D) confirmed also the adduct formation between RNase and CBQ as well as the presence of RNase dimer linked to CBQ or modified CBQ. The major identifiable deconvoluted mass for the CBQ-bound monomeric RNase at Pci4 is 14048.86, with \( [CBQ\text{--}\text{Cl}\text{--}3\text{H}] \) of 366.53 that is equivalent to \( 2 \times [CBQ \text{--} \text{Cl} \text{--} 3\text{H}] + 1 \times [CBQ \text{--} 4\text{H}] + [H_2O] + 2 \times [H] \). The observed deconvoluted masses for the CBQ-bound dimeric RNase at Pci4 are 28094.78 and 28237.70 Da, respectively. These peaks have lower relative intensity and are accompanied by many deconvoluted peaks around these values. The deconvoluted mass of 28094.78 corresponds to \( 2 \times [RNase + 730.12 \text{ equivalent to } 3 \times [CBQ \text{--} \text{Cl} \text{--} 3\text{H}] + 3 \times [CBQ \text{--} 4\text{H}] + 4 \times [H]) \). This value suggests that the formation of the RNase dimer can occur through CBQ bound to RNase in either dechlorinated form or not. The deconvoluted mass of 28237.70 corresponds to \( 2 \times [RNase + 873.04 \text{ equivalent to } 7 \times [CBQ \text{--} \text{Cl} \text{--} 3\text{H}] + 1 \times [CBQ \text{--} 4\text{H}] + 6] \).

Our MS studies presented here support indeed the complex nature of chemical modifications induced by quinones and are consistent with the various adduct structures of quinones and proteins characterized by several other groups. These known adducts include a reduced form of RNase covalently linked to PBQ [14], cytochrome C alkylated by \([\text{glutathione-S-yl}]-1,4\text{-benzoquinone [21], cytochrome C with a cyclized diquinone-lysine adduct [22], cytochrome C linked to PBQ at the solvent-exposed lysine-rich regions [23], and liver proteins linked to the bromobenzene-derived-quinones [20].}

Taken together, our findings suggest that the quinone substituent effect plays an important role on RNase modifications, with CBQ being the most reactive one while MBQ being the least reactive quinone. This is expected since Cl atom is known to withdraw electron density, making the carbonyl carbon more electrophilic, therefore more susceptible to a nucleophilic attack, while CH\(_3\) group is known to donate electron density, therefore providing more electron density to the carbonyl carbon and making it less electrophilic. The reduction potentials are also affected, with CBQ being more electrophilic, therefore more susceptible to a nucleophilic attack, while MBQ being more oxidizing (i.e., higher reduction potential) while MBQ being less oxidizing than PBQ.

Lastly, in addition to the investigation on the nature of RNase modifications, we studied the role of quinone’s substituent effect on biominalerization by utilizing SEM morphology analysis. The SEM study indicates that the presence of RNase in the SEM matrix, whether unmodified or modified by all three quinones, affected the way salts crystallized probably through non-covalent interactions or electrolyte shielding [33,34]. This is consistent with our previous findings which showed the presence of RNase affecting salts crystallization in the SEM matrix especially when a higher concentration of PBQ was used to modify RNase [24]. Furthermore, the type of the substituent group in quinone affecting RNase modifications appeared to play a significant role in inducing polymorphism in salt crystals. For example, CBQ-treated RNase, which was modified the most, appeared to result in extensive polymorphism in the SEM matrix and many small size crystals with the length less than 50 μm (Fig. 9E), while MBQ-treated RNase, which was modified the least, resulted in many bigger sized crystals (Fig. 5C) than the ones from either PBQ or CBQ-treated RNase. The SEM analysis supports the idea that the more modified RNase is, the more hydrophobic in nature it becomes, leading to more polymorphism in salt crystals and decrease in the crystal size.

5. Conclusions

The goal of this study was to investigate the modifications of RNase caused by a series of three quinones and to evaluate the quinone substituent effect in protein modifications. SDS–PAGE and confocal micrographic results show that CBQ is remarkably efficient in modifying RNase resulting in significant RNase polymerization, while MBQ is the least reactive one. This finding is consistent with fluorescence as well as anisotropy results showing the most dramatic change in CBQ-treated RNase. The second-order rate constants for the reactions of lysine and each quinone revealed that CBQ was the most reactive, while MBQ was the least reactive. UV–Vis data confirmed that all three quinones were able to induce adduct formation with RNase. LC-ESI+–QTOF-MS studies confirmed that RNase underwent adduct formation as well as oligomerization as evidenced by the high resolution acquired MS of the multiply charged RNase and the deconvoluted MS for RNase control, PBQ-modified RNase, and CBQ-modified RNase. SEM analysis indicated that CBQ-treated-RNase was the most efficient in altering biomineralization of SEM matrix. To summarize, we elucidated the complex nature of RNase modifications induced by the three quinones of interest and confirmed that the substituent effects play an important role in modifying RNase.

Conflict of Interest

The authors declare that there are no conflicts of interests.

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Appendix A. Supplementary material

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References