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Role of Membrane Charge and Semiquinone Structure on Naphthosemiquinone Derivatives and 1,4-Benzosemiquinone Disproportionation and Membrane–buffer Distribution Coefficients

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Semiquinone membrane/buffer partition coefficients have been determined for 1,2-naphthosemiquinone ($ONQ^{\cdot -}$), 1,4-naphthosemiquinone ($NQ^{\cdot -}$) and two of its hydroxylated derivatives, 5,8-dihydroxy-1,4-naphthosemiquinone ($NZQ^{\cdot -}$) and 5-hydroxy-1,4-naphthosemiquinone ($JQ^{\cdot -}$) as a function of membrane charge in multilamellar vesicles of phosphatidylcholine (PC) and equimolar mixtures of this lipid and phosphatidic acid (PC:PA) and cetyltrimethylammonium bromide (PC:CTAB) at physiological pH with the exception of values corresponding to PC:PA mixtures which were obtained at pH 9. These coefficients follow the order PC:PA<PC<PC:CTAB in agreement with the negative charge of the semiquinones. The disproportionation equilibria of the naphthosemiquinone derivatives are shifted to the semiquinone in the presence of neutral and positive membranes, being more pronounced in the latter. However, very low partition coefficients as well as small shifts in the semiquinone disproportionation equilibrium were observed for $ONQ^{\cdot -}$ as compared to the other semiquinones. No partition of 1,4-benzosemiquinone ($BQ^{\cdot -}$) into the lipid phase was detected for either charged or neutral lipid membranes. The presence of lipid membranes decreases the $BQ^{\cdot -}$ equilibrium concentration in the presence of all the types of membranes considered here.

Keywords: Quinone; Semiquinone; Electron paramagnetic resonance; Distribution constant; Disproportionation

INTRODUCTION

Semiquinones ($Q^{\cdot -}$), the one-electron reduction products of quinones (Q), are important intermediates in biological[1] and photochemical[2] processes. These are also postulated as cytotoxic

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intermediates in quinone-containing antitumor drug activity.[3,4] Semiquinones are readily reoxidized under aerobic conditions and, in biological systems, these can enter a redox cycle with molecular oxygen forming superoxide ions, hence, producing damaging hydroxyl radicals via the iron-catalyzed Haber–Weiss reaction.[5,6]

The efficiency of these electron-transfer processes should depend on the thermodynamic stability of the semiquinones which is governed by the combined relationship between semiquinone structure and environmental characteristics. Two major environments are present in biological systems. These are either hydrophylic or hydrophobic. A combination of these two environments is found in phosphatidylcholine (PC) membranes. Since oxygen is more soluble at the hydrophobic lipid moiety of PC membranes than in water,[7] the semiquinone–oxygen electron transfer process could be more effective in this site than in water. Thus, any study of the role of semiquinone membrane buffer partition on the semiquinone electron transfer efficiency should start with the knowledge of these partition coefficients and how semiquinone structure and membrane physical properties affect the partition process.

Semiquinone–membrane interaction could also affect the semiquinone disproportionation equilibrium (Eq. (1)). In a careful work by Roginsky *et al.*, it was demonstrated that ascorbate oxidation (or oxygen consumption) was accelerated by the presence of quinones.[8]

$$2Q^{\cdot -} + 2H^{+} \rightleftarrows Q + QH_2 \qquad (1)$$

However, at relatively large ascorbate/quinone mole ratios, ascorbate oxidation inhibition was observed after an initial short burst of oxygen consumption. This observation was more pronounced above a threshold quinone one-electron redox potential. Since both, more positive quinone redox potentials and larger ascorbate to quinone concentration ratios will favor the formation of the hydroquinone species, the observed ascorbate oxidation inhibition is attributed to hydroquinone formation.[8] This explanation is supported by the fact that direct oxidation of hydroquinones by oxygen is a very slow, spin-forbidden, reaction[9–12] and, thus, hydroquinone autoxidation is proposed to proceed via the semiquinone reduction of oxygen.[10,13,14] Therefore, semiquinone stabilization or destabilization should play a major role in controlling rates of oxygen consumption and production of oxygen radicals. A shift in equilibrium (Eq. (1)), or a change in the Gibbs free energy controlling this equilibrium, due to the semiquinone–membrane interaction, could modulate the rate of oxygen radicals production.

Evidence for the partition of anthracycline semiquinones into neutral PC membranes has been reported previously[15,16] and their membrane–buffer partition constants have been obtained.[16] However, no study on the effects that the type of substituents at the anthraquinone ring on these compounds could have on their semiquinone partition constants has been reported. In a previous work, we determined the membrane–buffer partition coefficients of 1,4-naphtho- ($NQ^{\cdot -}$), 1,4-benzo- ($BQ^{\cdot -}$) and 5,8-dihydroxy-1,4-naphthosemiquinones ($NZQ^{\cdot -}$) using neutral PC membranes.[17] Partition coefficients were found to be in the order $BQ^{\cdot -} < NZQ^{\cdot -} < NQ^{\cdot -}$. In this work, we have extended these measurements to include another hydroxy derivative of 1,4-naphthosemiquinone and 1,2-naphthosemiquinone (Fig. 1), in order to determine the role of hydroxy substitution and of the relative position of the semiquinone oxygen atoms on this partition process. Hydroxylated naphthosemiquinones are selected since these could serve as models for the antitumor anthracycline semiquinones of adriamycin and daunomycin which also contain hydroxy substituents at their quinone moiety.[18] In addition, we address the role of neutral, positive and negative charges at the membrane–buffer inter-

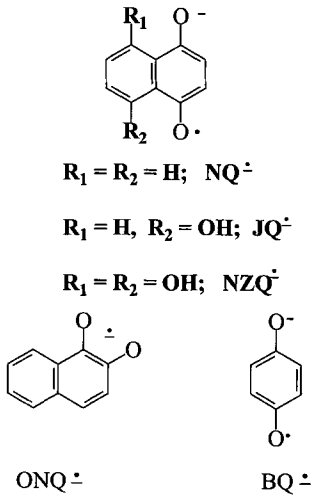


FIGURE 1 Semiquinones studied in this work. These are anions in the aqueous phase at the pH values used here (pK_a of $QH^{\bullet} \leq 5$).

face on the relative magnitudes of the corresponding partition coefficients as well as on the semiquinone disproportionation equilibrium. Although there are very few reports of positively charged domains in tissue or cell membranes,[19,20] in contrast to negatively charged or neutral membranes, positively charged membranes are included in this work as a means to aid in the interpretation of the observations.

MATERIALS AND METHODS

Materials

The compounds 1,4-benzoquinone (BQ), 1,4-napthtoquinone (NQ), 1,2-naphthoquinone (ONQ) and the hydroquinone of ONQ, $ONQH_2$, were purchased from Aldrich. Naphthazarin (NZQ), 5-hydroxynaphthoquinone (juglone, JQ), phosphatidic acid (PA) and cetyltrimethylammonium bromide (CTAB) were purchased from Sigma Chemicals. Egg-yolk phosphatidylcholine (PC) was purchased from both Sigma Chemical and Avanti Polar Lipids. Quinones and hydroquinones were purified by double sublimation. Fresh stock solutions of these compounds were prepared in water and used in the same day. Samples were prepared in 50 mM cacodylate buffer at pH 7.4 using distilled, deionized water.

EPR Measurements

First derivative EPR spectra were acquired and analyzed using an EMX X-band Bruker EPR spectrometer coupled to a computer. Well-resolved semiquinone first-derivative EPR spectra in the absence of lipids were simulated and optimized (best-fitted to the experimental spectra) using WINSIM,[21] starting with hyperfine coupling constants available in the literature corresponding to aqueous or water-containing samples.[22] First derivative EPR spectra corresponding to lipid-containing samples, in which both the membrane-bound and the free semiquinone spectra were observed superimposed (hereby known as the composite spectra), were simulated and optimized in the same fashion starting with two superimposed different spectra: the well-resolved spectrum corresponding to the semiquinone in the aqueous phase and a broad (membrane-bound) semiquinone spectrum. A single broad line with peak-to-peak width of 2 G with identical g-value to that of the aqueous phase species was used as the starting broad spectral component in the optimization process. Linewidths, relative g-values, lineshapes and relative intensities were optimized in the composite spectra. The optimization process produced the EPR spectral intensity ratios corresponding to membrane-bound semiquinones to those of semiquinones in the aqueous phase (I_m/I_{aq}) at appropriate aqueous to lipid volume proportions. Semiquinone membrane–buffer partition coefficients K_2 were determined using Eq. (2), where V_{aq} and V_m are the volumes of the aqueous and lipid phases, respectively. The lipid volume was determined from the lipid mass used and its density, assuming that the lipid phase density is equal to the aqueous

phase density.[23,24]

$$K_2 = \frac{I_m V_{aq}}{I_{aq} V_m} \quad (2)$$

Semiquinone Generation

Semiquinones were produced as previously described.[17,25] Since most of the quinones under study here are slightly soluble in the buffer phase, semiquinones were first generated in absolute methanol by reacting from 10 to 20 mg of quinone with $NaBH_4$ in a convenient molar ratio such that semiquinone spectral intensity is maximized when transferred to aqueous buffer. The solvent was then evaporated to dryness using a dry nitrogen flow. The sample was submitted to high vacuum for at least 30 min. A nitrogen-purged aqueous sodium cacodylate buffer solution was then added to the dry semiquinone sample and the resulting solution equilibrated to pH 7.4 by the addition of small aliquots of HCl or NaOH. This aqueous solution was diluted to 3.00 ml and centrifuged at 3500 rpm for 5 min. The supernatant was then submitted to EPR analysis or used in the multilamellar vesicles (MLVs) preparation.

The semiquinones $BQ^{-\cdot}$ and $ONQ^{-\cdot}$ were produced by the comproportionation reaction between the corresponding hydroquinones and quinones (reverse of reaction (Eq. (1))).

As observed previously, no spectral decrease in intensity was detected during the time period of the analysis of these samples.[17]

Preparation of Semiquinone-containing MLVs

These were prepared as previously described.[17] A thin film of PC (1.0–100.0 mg), with or without CTAB or PA in a 1:1 Mol ratio with PC, was prepared in a septum-stoppered test tube, by evaporating the solvent from its chloroform solution, using first a nitrogen gas flow followed by high-vacuum application for 30 min. A sample (400 μl) of an aqueous semiquinone solution, prepared as described above, was then added to this lecithin film and strongly vortexed under nitrogen until a stable suspension was obtained (*ca.* 3–4 min). This suspension was submitted for EPR analysis. Again, no spectral decrease in intensity was detected during the time period of the analysis of these samples.

Disproportionation Constants Determination in the Absence of Lipids

The semiquinone disproportionation constant, K_1, as defined by Eq. (3), was determined depending on the magnitude of the quinone solubility in buffer. Semiquinone concentrations

$$K_1 = \frac{[Q][QH_2]}{[Q^{-\cdot}]^2[H^+]^2} \quad (3)$$

were obtained by comparing the semiquinone over modulated EPR double-integrated spectral area with that corresponding to a 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrrolin-1-oxide spin standard.

The disproportionation constant of $ONQ^{-\cdot}$ was determined by mixing the corresponding quinone and hydroquinone as described previously for $BQ^{-\cdot}$ [26] or other semiquinones.[8] The equilibrium concentrations of the corresponding hydroquinones and quinones were obtained by subtracting half the corresponding semiquinone concentrations from the initial concentrations of these species.

In the case of $JQ^{-\cdot}$, which is produced from a slightly soluble quinone, the disproportionation constant was determined as described previously.[26] The semiquinone was generated as indicated above by reducing JQ with $NaBH_4$. In order to obtain the equilibrium hydroquinone concentration, the total quinone concentration, $[JQ]+[JQH_2]+[JQ^{-\cdot}]$, was determined. For this purpose, a 250 μl sample of the aqueous solutions containing the semiquinone was extracted with HPLC-grade methylene chloride until the aqueous phase was left colorless. After evaporating

the organic solvent, the remaining solid, composed of JQ, JQH_2 and $JQ^{\cdot -}$, was redissolved in 5 ml of dry benzene and all reduced species oxidized to JQ following the reported method of Ansell *et al.*[27] by addition of 0.3 g of AgO and 0.1 g of dry $MgSO_4$. The total quinone concentration, produced by this reaction was then determined by HPLC analysis. It was found that less than 30 min were needed until a maximum concentration of quinone was detected. The total quinone concentration, i.e. $[Q]_{eq} + [QH_2]_{eq} + [Q^{\cdot -}]_{eq}$, in the aqueous semiquinone solutions were interpolated from calibration curves of quinone concentration versus chromatographic peak area. The hydroquinone concentration was obtained from the total quinone, semiquinone equilibrium quinone concentrations.

The quinone concentration at equilibrium was assumed to be the solubility of each quinone in cacodylate buffer, pH 7.4, since undissolved solid quinone was always present in the precipitated solid after buffer addition to the solid mixture left in the test tube after methanol evaporation. Evidence for the presence of undissolved quinone was determined by washing several times this solid precipitate with buffer followed by dissolving this sample in the N_2-saturated $MeOH/H_2O$ solvent mixture used for HPLC analysis and detecting the quinone peak by HPLC. In this case, a helium-purged mobile phase was used to avoid air-oxidation of reduced species. No ESR signal was detected from the precipitated solids after submitting an N_2--saturated buffer dispersion of the water-washed solid in a quartz cell to EPR analysis. Thus, the quinone peak detected is not produced by semiquinone salt disproportionation or hydroquinone oxidation upon addition of the HPLC eluant solvent mixture to this solid.

The hydroquinone concentration at equilibrium is, therefore, obtained by subtracting the quinone solubility in buffer and the semiquinone concentration obtained by EPR analysis, from the total quinone concentration, obtained by oxidation.

Semiquinone Disproportionation as a function of Lipid Concentration

The effect of the presence of lipids on the semiquinone disproportionation was monitored by following the variation in semiquinone concentration upon addition of increasing amounts of phospholipid mixtures at pH 7.4.

HPLC Analyses

A Waters analytical HPLC system equipped with a Waters 600 solvent delivery pump, UV–Vis tunable detector and a μBondapack C_{18} (3.9 × 300 mm) column was used. A mobile phase of $MeOH/H_2O$ (85:15 v/v) was used at a flow rate of 1 ml/min.

RESULTS AND DISCUSSION

Semiquinone Membrane–buffer Partition

Free and membrane-bound semiquinones were generated using none and convenient amounts of lipids, i.e. at lipid concentrations where semiquinones in the two physical environments were detected (Fig. 2(c)). Both PA[28,29] and CTAB[30,31] form stable liposomes with PC, thus, providing the needed negative or positive charge, respectively, to the MLVs. Lipid concentrations varied with the charge of the membrane and the semiquinone under study such that both, membrane-bound and aqueous, species were observed simultaneously. The presence of PA or CTAB have no special effects in the shapes of neither the membrane-bound nor the free semiquinone spectra as compared to PC without these charged lipids. The composite spectra were simulated and optimized as described in the Experimental section (Fig. 2(c)), Table I. Thus, partition coefficients were extracted from the composite spectra and are shown in Table II.

The partition coefficients of $NQ^{\cdot -}$ and $NZQ^{\cdot -}$ in the presence of PC obtained in this work are larger than those reported

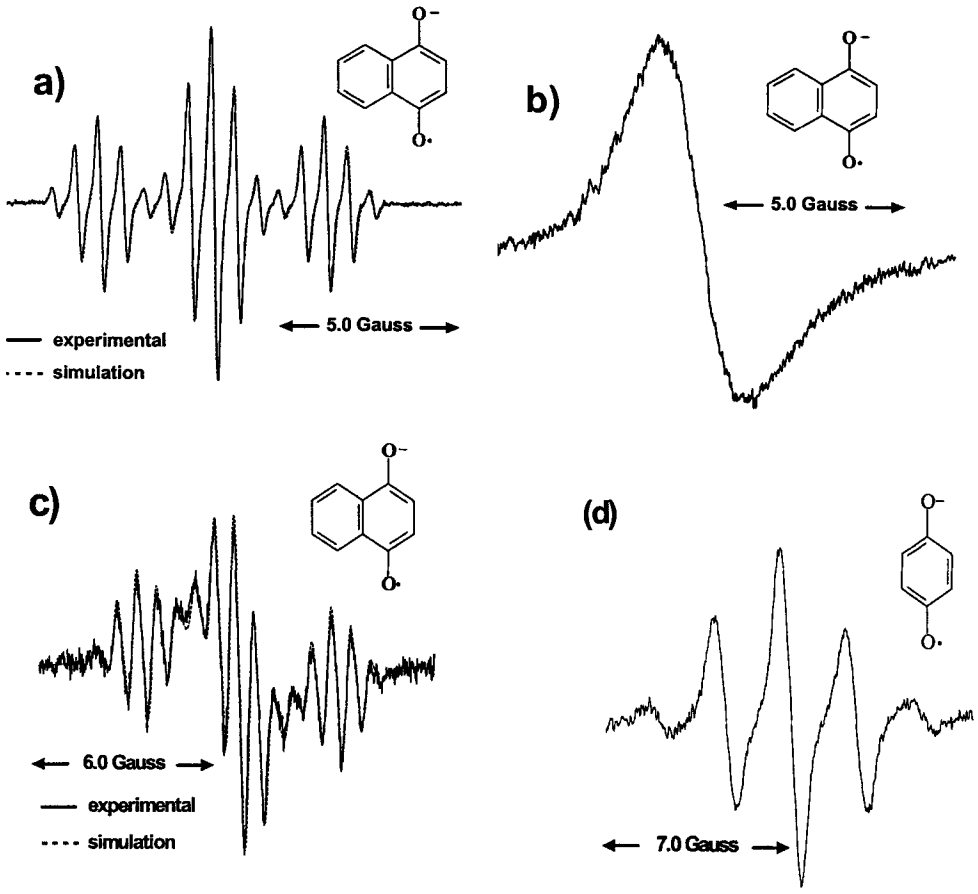


FIGURE 2 First derivative EPR spectra of $NQ^{\cdot -}$ (—) and simulations (· · ·) corresponding to $NQ^{\cdot -}$ in N_2-saturated 20 mM cacodylate buffer at pH 7.4 containing (a) no PC, (b) 100 mg PC/ml (only the membrane-bound semiquinone is observed here) and (c) 24 mg PC/ml. A value of 87/13 = 67 for the lipid/buffer semiquinone mol ratio was obtained from the best fit of spectrum (c). (d) EPR spectrum corresponding to an N_2-saturated 20 mM cacodylate solution of $BQ^{\cdot -}$ in the presence of 210 mM PC + CTAB with PC:CTAB 1:1 mol ratio.

previously ($K_2 = 130 \pm 30$ and 30 ± 20 for $NQ^{\cdot -}$ and $NZQ^{\cdot -}$, respectively), although the same relative order ($K_2(NQ^{\cdot -}) > K_2(NZQ^{\cdot -})$ is observed.[17] The reason for this discrepancy should be in the differences in which the I_m/I_{aq} ratios were determined in both works. In the previous work, K_2 was determined by subtracting the broad spectral component from that where the two species are observed and visually checking that the resulting spectrum is similar to the well-resolved one corresponding to the aqueous phase semiquinone. In order to obtain K_2, this was followed by subtracting the double integration of this spectrum from that of the composite spectrum. We believe that a sizeable area of the broad spectral component is lost in this double-integration method due to the fact that integration is limited to the area appearing in a limited spectral window and the broad spectral component does not approach the spectral baseline as fast as the well-resolved spectrum.[32]

No broad spectral component was observed for $BQ^{\cdot -}$ in the presence of all types of lipids used in this work up to 210 mM. Thus, the association

TABLE I Hyperfine coupling constants for semiquinones under study here in nitrogen-saturated cacodylate buffer (pH 7.4) and the corresponding pK_a values of the protonated semiquinones

Semiquinone	a_H* (Gauss)	a_H† (Gauss)	pK_a
$BQ^{\cdot -}$	2.38 (4H's)	2.35 (4H's)	4.1‡
$NQ^{\cdot -}$	3.18 (2H's), 0.63 (4H's)	3.11 (2H's), 0.63 (2H's), 0.55 (2H's)	4.1¶
$JQ^{\cdot -}$	3.50 (1H), 2.83 (1H), 1.37 (2H's), 0.66 (1H), 0.34 (1H)	3.30 (1H), 3.05 (1H), 0.30 (1H), 1.27 (2H's), 0.68 (1H's)	3.65§
$NZQ^{\cdot -}$	2.34 (4H's), 0.59 (2H's)	2.356 (4H's), 0.587 (2H's)	2.7§
$ONQ^{\cdot -}$	0.64 (1H's), 4.62 (1 H's), 0.22 (1H's), 1.44 (1H's), 0.19 (1H's), 1.48 (1H's)	0.56 (1H's), 4.61 (1H's), 0.27 (1H's), 1.45 (1H's), 0.17 (1H's), 1.36 (1H's)	#

*This work.
†Ref. [22] (in alkaline aqueous solutions).
‡Ref. [49].
¶Ref. [41].
§Ref. [33].
pK_a values reported for ortho-semiquinones are in the range from 3.6 to 5.2.[36,37]

of $BQ^{\cdot -}$ with the membranes under study here, if any, is too weak to be detected utilizing this technique. However, an upper limit of $K_2 = 0.8$ was estimated by permitting Winsim to assume that a broad spectral component due to a membrane-bound semiquinone was present (Fig. 2(d)). This low affinity of $BQ^{\cdot -}$ for these membranes, even in the presence of 210 mM PC:CTAB, is indicative of the high free energy of hydration of this anion by water. Since $BQ^{\cdot -}$ is the smallest of the anions under study here, its charge density is larger than that corresponding to the other semiquinones and, thus, it is more difficult for $BQ^{\cdot -}$ to lose solvating water molecules to bind PC membranes.[17]

TABLE II Semiquinone membrane–buffer partition coefficients (samples contained 400 µl of the semiquinone solution in 20 mM cacodylate (pH 7.4) and from 1 to 100 mg of PC or mixtures of PC:PA or PC:CTAB, in equimolar ratios, in the form of MLVs)

$Q^{\cdot -}$	*MLVs composition*	K_2
$NQ^{\cdot -}$	PC	300 ± 70
	PC:PA (pH 9)	25 ± 4
	PC:CTAB	1200 ± 300
$NZQ^{\cdot -}$	PC	200 ± 10
	PC:PA (pH 9)	40 ± 10
	PC:CTAB	700 ± 40
$JQ^{\cdot -}$	PC	170 ± 70
	PC:PA (pH 9)	9 ± 3
	PC:CTAB	940 ± 40
$ONQ^{\cdot -}$	PC	16 ± 8
	PC:PA (pH 9)	4.5 ± 8
	PC:CTAB	140 ± 30
$BQ^{\cdot -}$	PC	Less than 0.8*
	PC:PA (pH 9)	Less than 0.8*
	PC:CTAB	Less than 0.8*

*A maximum value of K_2 was estimated for $BQ^{\cdot -}$ by assuming the existence of a broad component in $BQ^{\cdot -}$ EPR spectra and using Winsim to determine the I_m/I_{aq} ratio after optimization of the "composite spectra".

Not including $BQ^{\cdot -}$, the partition coefficients follow the order PC:PA<PC<PC:CTAB for all the semiquinones studied here. Since these semiquinones are anions at pH 7.4 (pK_a of $QH^{\cdot}$ for *para*-semiquinones ≤4[33–35] and for *ortho*-semiquinones ≤5,[36,37] Table I), this is the expected result. Furthermore, it is interesting to notice in the case of PC:PA that there is still a small fraction of semiquinone species bound to the membrane even though the membrane charge is negative. Distribution coefficients using PC/PA mixtures were determined at pH 9 due to the very low signal to noise obtained for the composite spectra of $NQ^{\cdot -}$ and $ONQ^{\cdot -}$, which induced large irreproducibility. Intense EPR signals were observed for $JQ^{\cdot -}$ and $NZQ^{\cdot -}$ at both pH 7.4 and 9. It was thought that semiquinones under study here bind to the

TABLE III Hydroquinone, quinone and semiquinone concentrations at pH 7.4 in the absence of lipids

Semiquinone	K_1	$[Q^{\cdot -}]$ (10^{-6} M)	$[Q]$ (10^{-4} M)	$[QH_2]$ (10^{-3} M)
$NZQ^{\cdot -}$	2.1×10^{19}*	3.5	2.4†	1.6
$NQ^{\cdot -}$	1.5×10^{20}*	2	9.5†	1
$JQ^{\cdot -}$	$(8.5\pm0.6)\times10^{19}$‡	1.6	3.1†	1
$ONQ^{\cdot -}$	$(1.1\pm0.7)\times10^{21}$‡	1.6	10	4.5
$BQ^{\cdot -}$	6×10^{20}*	9.2	100	8

* From Ref. [26].
† Solubility of these quinones in the buffer used at pH 7.4.
‡ This work.

negative PC/PA membranes in their protonated forms, $QH^{\cdot}$. Thus, it was expected that a decrease in pH from 9 to 7.4 should increase the semiquinone membrane–buffer distribution constant due to a shift to the semiquinone conjugated acid of its acid ionization equilibria (Eq. (4)). However, the same value for the $NZQ^{\cdot -}$ distribution constant was obtained at both of these pH conditions ($K_2 = 40$).

$$QH^{\cdot} \rightarrow Q^{\cdot -} + H^{+} \qquad (4)$$

Thus, either this pH difference is not large enough to produce a measurable change in K_2 or these semiquinones behave like hydrophobic ions, characterized by their ability to overwhelmingly partition from aqueous media into neutral or anionic phospholipid membranes.[38–40]

Interestingly, $ONQ^{\cdot -}$ have the smaller set of K_2 values among the naphthosemiquinone derivatives. This implies better water solvation for this semiquinone as compared to the 1,4-naphthosemiquinone derivatives under study here. This marked difference in water solvation could be caused by a more concentrated negative charge at the oxygen atoms in $ONQ^{\cdot -}$, favored by oxygen atoms proximity, which hydrogen bonds more tightly to water molecules. This observation is also consistent with a less pronounced effect of positively charged membranes on $ONQ^{\cdot -}$ disproportionation equilibrium (see below).

Effect of Membrane Charge on Semiquinone Disproportionation

Semiquinone generation in the presence of neutral or charged membranes induces a shift in semiquinone disproportionation equilibria (Eq. (1), Fig. 3) as indicated by the variations in the semiquinone concentrations with increasing lipid concentrations. These shifts in semiquinone disproportionation equilibria are not limited by the concentrations of quinones and hydroquinones available for all the semiquinones under study here. There concentrations can be calculated from the reported semiquinone disproportionation constants in the absence of lipids,[26] the aqueous pH and the observed semiquinone concentration in the absence of lipids, Table III. As stated above, semiquinones are all anionic species at this pH. The hydroquinones of BQ and NQ are neutral species at pH 7.4 ($pK_a = 11.9$ and 11.2, respectively[41]). However, NZQ and $NZQH_2$ have first ionization constants of 7.85[33] and 7.6,[42] respectively. Thus, around 35 and 63% of the NZQ and $NZQH_2$ molecules, respectively, are ionized at pH 7.4. To our best knowledge, the pK_a values of $ONQH_2$ are not reported, although the pK_a value of 2,3-dihydroxynaphthalene is 8.68[43] and in the range of 8.6–10.4 for several catechols.[44] Thus, it is estimated that at most 10% of $ONQH_2$ is anionic. The acid ionization constant of JQ has been determined ($pK_a = 8.85$[33]), although that of JQH_2 is not reported. Thus, appreciable proportions of the quinones NZQ and JQ and

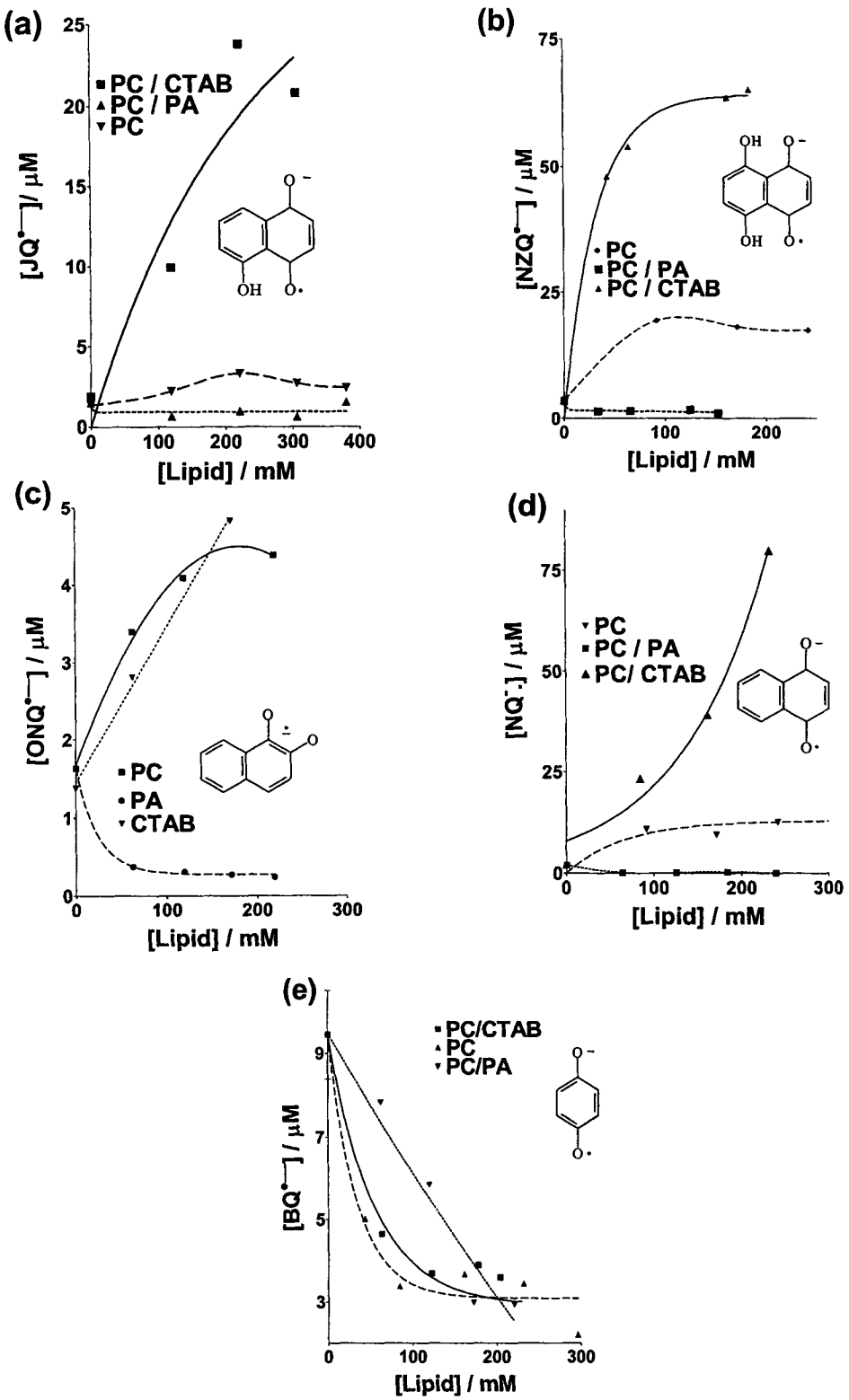
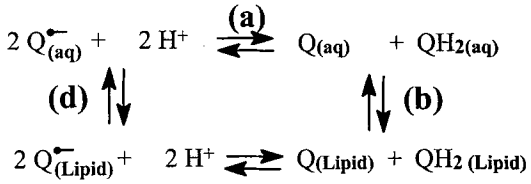


FIGURE 3 Effect of lipid membrane presence on semiquinone disproportionation corresponding to (a) $JQ^{\bullet-}$, (b) $NZQ^{\bullet-}$, (c) $ONQ^{\bullet-}$, (d) $NQ^{\bullet-}$, and (e) $BQ^{\bullet-}$. Curves correspond to PC:PA 1:1 mol ratio (· · ·), PC:CTAB 1:1 mol ratio (—) and PC (- - -).

their hydroquinones are expected to be ionized at the aqueous phase. However, according to Fig. 3, all naphthosemiquinone derivatives are stabilized, i.e. disproportionation equilibria shifted to the left, relative to the absence of lipids, upon interacting with positive and neutral membranes. In contrast, all of the semiquinones under study here are destabilized in the presence of negative membranes. The observed stabilization is more strongly pronounced for the most hydrophobic naphthosemiquinone derivatives, i.e. $NQ^{\cdot -}$, $JQ^{\cdot -}$ and $NZQ^{\cdot -}$. These observations seem, at first glance, paradoxical since a considerable fraction of the quinones NZQ and JQ as well as their hydroquinones are negatively charged and, thus, the anionic quinones and hydroquinones NZQ and JQ are expected also to be stabilized by positive (and destabilized by negative) membranes in the same fashion as the semiquinones. The explanation to this apparent paradox resides, most probably, in the relative population of the species quinone, hydroquinone and semiquinone, which is ionized. Assuming the same strength in the interaction between the quinone, semiquinone and hydroquinone anions and the charged membranes, a larger fraction of the semiquinone anions should be attracted or repelled by the charge at the membrane surface as compared to the fraction of the quinone and hydroquinone anions interacting with the membrane charge due to the fact that only a fraction of the total population of quinones and hydroquinones are ionized while the semiquinone species are 100% anions. Thus, a relatively larger change is expected in the denominator of the expression of the semiquinone disproportionation constant (Eq. (3)) as compared to the numerator. In addition, the semiquinone disproportionation constant depends on the inverse square of the semiquinone concentration while it depends directly on both the quinone and hydroquinone concentrations. Thus, a large dependence of the disproportionation constant on semiquinone environmental variations is expected.

The relationship between the semiquinone disproportionation equilibria and the partition of semiquinones and their corresponding quinones and hydroquinones can be better understood using Scheme 1, initially excluding quinone systems where appreciable ionization of either quinone and hydroquinone exists (NZQ and JQ). The amount of non-charged molecules, i.e. quinone and neutral hydroquinones, are expected to increase in the lipid phase as the lipid concentration increased (reaction (b) in Scheme 1), being this process essentially independent of the membrane charge. This partition of neutral species will decrease the semiquinone concentration at the aqueous phase due to a shift of the semiquinone disproportionation equilibrium in the aqueous phase to the right (reaction (a) in Scheme 1). An increase in the observed total semiquinone concentration should then be ascribed to shifts in the semiquinone disproportionation equilibrium occurring at the lipid phase (reaction (c) in Scheme 1). Thus, positively charged membranes will be shifting the semiquinone disproportionation equilibrium at the lipid phase to the left overcoming the increase in disproportionation induced by quinone and hydroquinone partition into the lipid phase. Negatively charged membranes will induce the opposite due to semiquinone–membrane electrostatic interactions, thus increasing the disproportionation provoked by quinone and hydroquinone partition into the membrane. Scheme 1 could also be used in explaining shifts in $JQ^{\cdot -}$ and $NZQ^{\cdot -}$ with membrane charge if it is assumed that the disproportionation equilibrium is more sensitive to semiquinone–membrane interactions than charged-quinone–membrane



SCHEME 1

or charged-hydroquinone–membrane interactions, as explained in the previous paragraph. Since these partition and disproportionation equilibria are mutually related, as depicted in Scheme 1, it is observed that semiquinone stabilization correlated with K_2 values. Therefore, for the most hydrophilic semiquinones, $BQ^{\cdot -}$ and $ONQ^{\cdot -}$, smaller changes in semiquinone concentration were observed with lipid concentration increase, even in the presence of PC/CTAB membranes, as compared to the more hydrophobic quinones. Since semiquinone disproportionation shifts to the production of hydroquinones and hydroquinones are known to act as antioxidants[45–47], negatively charged membranes could be better protected from autoxidation than neutral membranes if reduced quinone species are present. A shift in semiquinone disproportionation equilibrium could imply a change in the semiquinone disproportionation and comproportionation rate constants. Since oxygen consumption rates catalyzed by quinones in the presence of ascorbate are known to be the result of a competition between semiquinone disproportionation and semiquinone-mediated oxygen reduction rates,[8] it is expected that membrane–semiquinone interaction will also affect oxygen consumption rates by changing semiquinone disproportionation or comproportionation rates.

The concentration of $BQ^{\cdot -}$ decreases with an increase in lipid concentration and independently of membrane charge. As stated above, this semiquinone is largely located in the aqueous phase even in the presence of positive MLVs. Thus, the partition of neutral quinone-derived species to the lipid phase and the consequent shift of equilibrium (a) of Scheme 1 to the right supersedes a shift of equilibrium (c) to the left, due to the weak interaction of $BQ^{\cdot -}$ with all of the membranes used here. Consequently, a decrease in total semiquinone concentration with increase in lipid concentration is observed.

In a previous work reported by us, lipid peroxidation inhibition was observed upon inclusion of several simple *p*-quinones in linolenate-enriched vesicles containing 1 mM ascorbate and 50 μM Fe^{2+}.[48] In the absence of ascorbate and in the presence of Fe^{2+}, lipid peroxidation enhancements were observed for some of the quinones studies. Thus, lipid peroxidation inhibition in the presence of 1 mM ascorbate was ascribed to hydroquinone formation, and the consequent antioxidant activity of this species, since at the larger reducing agent/quinone mol ratio more of the hydroquinone is produced. However, samples in this work were first incubated for several minutes under nitrogen before being exposed to air, thus facilitating the reduction of quinones to the hydroquinones. In the work reported by Roginsky *et al.*, quinones were added to air-saturated ascorbate-containing solutions. Thus, for quinones below a redox potential threshold (known in that work as "standard behaving" quinones), a constant rate of oxygen consumption was observed, thus implying a constant catalytic activity of the quinone and consuming much more oxygen after several minutes than quinones above the redox potential threshold for which hydroquinone formation is favored. Thus, an interesting question to answer is whether or not lipid peroxidation could be enhanced by the addition of "standard behaving" quinones to air-saturated lipid suspensions (thus, avoiding or limiting hydroquinone formation) and whether or not lipid peroxidation is less enhanced, or diminished, if negative PC membranes are used. Preliminary results in our laboratory show that oxygen consumption rates catalyzed by NQ in the presence of ascorbate are enhanced upon addition of neutral and positively charged lipid membranes, which is consistent with predictions and observations made here.

In summary, semiquinone partition into lipid membranes follows the order PC/PA<PC<PC/CTAB. Semiquinone disproportionation is enhanced in negatively charged membranes and diminished in positively charged membranes. Larger semiquinone mem-

brane–buffer partition constants are roughly associated with larger decreases in semiquinone disproportionation. This increased or decreased semiquinone stabilization could correlate with larger or smaller rates of oxygen consumption, respectively, and probably with lipid peroxidation extents.

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