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Interaction of Retinoic Acid Radical Cation with Lysozyme and Antioxidants: Laser Flash Photolysis Study in Microemulsion

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ABSTRACT

All-trans retinoic acid (ATRA) plays essential roles in the normal biological processes and the treatment of cancer and skin diseases. Considering its photosensitive property, many studies have been focused on the photochemistry of ATRA. In this study, we investigated the transient phenomena in the laser flash photolysis (LFP) of ATRA in microemulsion to further understand the photochemistry of ATRA. Results show that 355 nm LFP of ATRA in both acidic and alkaline conditions leads to the generation of retinoic acid cation radicals ($ATRA^{\bullet+}$) *via* biphotonic processes. The employment of microemulsion system allows us to investigate the reaction of hydrophobic $ATRA^{\bullet+}$ with molecules of different polarity. Therefore, we studied the reaction activity of $ATRA^{\bullet+}$ to many hydrophobic and hydrophilic molecules. Results show that $ATRA^{\bullet+}$ can efficiently interact with lysozyme, tyrosine, tryptophan and many antioxidants, such as curcumin (Cur), vitamin C (VC) and gallic acid (GA). The apparent rate constants of these reactions were measured and compared. These findings suggest that $ATRA^{\bullet+}$ is a reactive transient product which may pose damage to lysozyme, and antioxidants, such as Cur, VC and GA, may inactivate $ATRA^{\bullet+}$ by efficient quenching reactions.

INTRODUCTION

All-trans retinoic acid (ATRA) is one kind of retinoids and plays an important role in different biological processes, such as embryonic development, cellular differentiation (1,2). Besides, it was widely used in the treatment of cancer and some kinds of skin diseases (3–7). However, the use of retinoids, in particular topical, can cause skin photosensitization or phototoxicity (8–11).

Many studies have been made to investigate the transient phenomena in the laser flash photolysis and pulse radiolysis studies of retinoids (12–28). Previous studies on ATRA in methanol showed that 347 nm laser flash photolysis (LFP) of ATRA led to the production of triplet state of ATRA (^{3}ATRA*) *via* photoexcitation and retinoic acid cation radicals ($ATRA^{\bullet+}$) *via* photoionization (16). Pulse radiolysis study by Różanowska *et al.* showed that $ATRA^{\bullet+}$ could also be generated *via* oxidation of ATRA by peroxyl radicals and $ATRA^{\bullet+}$ had efficient reaction activity to ascorbate (17). The interaction of $ATRA^{\bullet+}$ with ascorbate implies that $ATRA^{\bullet+}$ is a reactive substance which may pose oxidative damage to other biomolecules such as proteins. Therefore, further studies are needed to investigate the reactivity of $ATRA^{\bullet+}$ with other biomolecules.

ATRA is an amphiphilic molecule and its amphiphilic property makes biological membranes one of the most likely sites for the distribution of ATRA. The molecule of ATRA consists of two parts, the hydrophobic part which binds efficiently to the hydrocarbon chain region of the lipid membranes, and the other hydrophilic carboxylic group in the head group region which is anchored in the surface of the lipid bilayer *via* nonspecific electrostatic interactions as well as hydrogen bonding with lipid head groups (29,30). The distribution of ATRA in biomembrane actually is heterogeneous. Therefore, heterogeneous system is more suitable than homogenous system to mimic membrane structure. In Różanowska's studies, a heterogeneous system, Triton X-100 micelle was used as reaction system which facilitated the interaction of hydrophobic $ATRA^{\bullet+}$ with water-soluble ascorbate (17). Meanwhile, micelle system was also employed in the LFP study of retinol and pulse radiolysis study of retinyl polyenes (12,14,20). Their works inspired us to refer to another heterogeneous system, microemulsion which is a thermodynamically and mechanically stable and optically transparent system, and can be self-assembly to form after simple mixing of oil phase, water phase, surfactant and cosurfactant in term of appropriate proportion. The oil and water phases of microemulsions facilitate simultaneous dissolution of hydrophilic and hydrophobic molecules in one transparent system which provides an easily preparing system for laser flash photolysis study to investigate the reactions between hydrophilic molecules and hydrophobic molecules. This study aimed to investigate the photochemical and photophysical properties of ATRA in microemulsions by 355 nm LFP. Meanwhile, the interaction of $ATRA^{\bullet+}$ with lysozyme and many antioxidants was also investigated to evaluate the reaction activity of $ATRA^{\bullet+}$ to biomolecules. In this study, the employment of microemulsion system facilitates our LFP studies on the reactions between hydrophobic $ATRA^{\bullet+}$ and molecules with different polarity.

MATERIALS AND METHODS

ATRA, lysozyme (Lyso), tert-butyl alcohol (t-BuOH) and β-carotene (β-car) were purchased from Sigma. Diphenylamine (DPA), *N*, *N*-dime-

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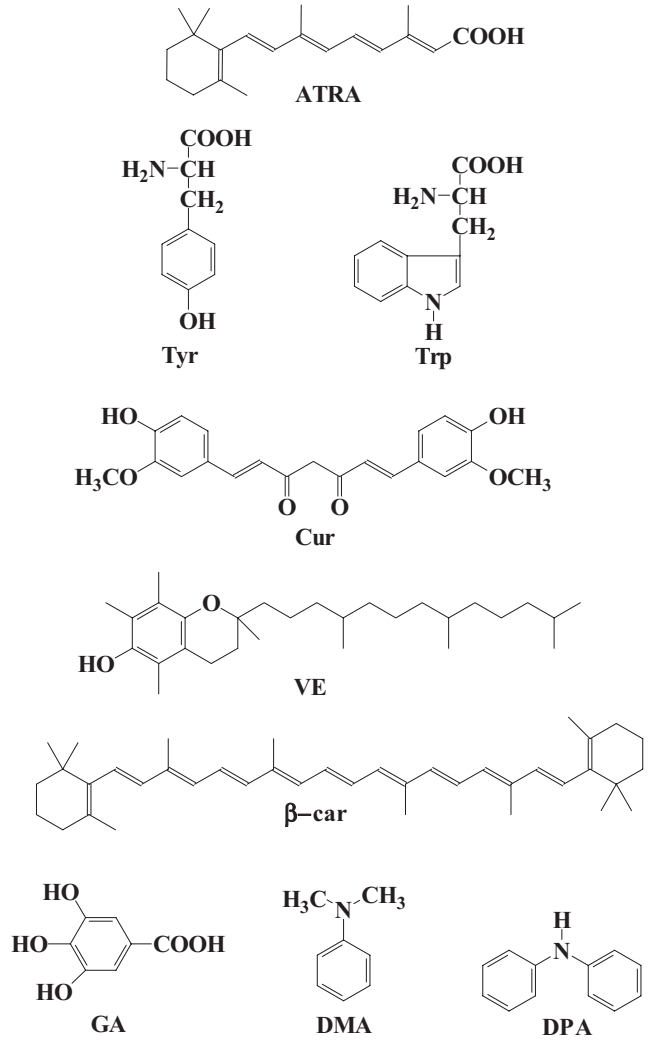
thylaniline (DMA), curcumin (Cur), gallic acid (GA), cyclohexane, 1-butanol, H_3PO_4, NaH_2PO_4, Na_2HPO_4 and Na_3PO_4 were purchased from Sinopharm Chemical Reagent Co. Ltd (China). Sodium dodecyl sulfonate (SDS), vitamin E (VE), tyrosine (Tyr), tryptophan (Trp) and ascorbate (VC) were purchased from Sangon Biotech (Shanghai) Co. Ltd. (China). All reagents are analytic grade reagent and used without further purification. Water was purified in a Millipore Milli-Q unit. The pH value of solution was adjusted by phosphate solution (0.04 M) with different pH values. The solutions were saturated with high-purity N_2 (≥99.99%), N_2O, or O_2 (≥99.5%) for different purposes by bubbling for at least for 20 min prior to the experiments. All experiments were performed in a 1 cm quartz cuvette at room temperature. The structures of some substances used in this study are presented in Scheme 1.

Preparation of microemulsion. The microemulsion of this study is oil in water (o/w) which was composed of emulsifier 30% (v/v), cyclohexane (oil) 10% (v/v) and water 60% (v/v). The emulsifier was prepared by mixing SDS (surfactant) 25% (m/m), 1-butanol (cosurfactant) 50% (m/m) and water 25% (m/m). ATRA was dissolved in the oil phase (cyclohexane) of microemulsion. The preparation of microemulsion used in this study has been described in our previous laser flash photolysis study of Coenzyme Q10 in microemulsion (31).

Absorption spectroscopy. Absorption spectra of ATRA in microemulsion were measured as a function of pH using a UV–Vis spectrometer (Varian Cary 50 Probe). The solvent pH was varied by 0.04 M phosphate solution with different pH, and the solution pH was measured with a calibrated pH meter (Mettler).



Scheme 1. The structures of molecules used in this study.

Evaluation of the degree of ionization of ATRA in microemulsion. According to the Lambert–Beer law, the optical density (OD) of ATRA at a fixed wavelength where both $ATRA^-$ and ATRA have absorbance can be presented as follows:

$$\begin{aligned} OD &= \varepsilon_1 L[ATRA^-] + \varepsilon_2 L[ATRA] \\ &= L(\varepsilon_1 - \varepsilon_2)[ATRA^-] + a\varepsilon_2 L \quad ([ATRA] + [ATRA] = a) \\ &= aL(\varepsilon_1 - \varepsilon_2)\frac{[ATRA^-]}{[ATRA^-] + [ATRA]} + a\varepsilon_2 L \end{aligned} \tag{1}$$

In Eq. (1), $[ATRA^-]$ is the concentration of deprotonated form of ATRA ($ATRA^-$) and [ATRA] is the concentration of protonated form of ATRA. ε_1 and ε_2 are the molar absorption coefficients of $ATRA^-$ and ATRA at the fixed wavelength respectively. L, a, ε_1 and ε_2 are constants in the condition of this study. This equation indicates that OD is linearly correlated with the fraction of $ATRA^-$ in different pH values. The isosbestic point for $ATRA^-$ and ATRA is the wavelength where ε_1 is equal to ε_2 and the OD is theoretically not influenced by pH. Then, Eq. (2) can be derived from Henderson–Hasselbalch equation:

$$\frac{OD - a\varepsilon_2 L}{aL(\varepsilon_1 - \varepsilon_2)} = \frac{[ATRA^-]}{[ATRA^-] + [ATRA]} = \frac{1}{1 + 10^{(pK_a - pH)}} \tag{2}$$

In Eq. (2), ε_1 is not equal to ε_2. By fitting OD values at the fixed wavelength (except for the isosbestic point) in different pH conditions and corresponding pH values using Eq. (2), pK_a for ATRA in microemulsion was obtained.

Laser flash photolysis. Laser flash photolysis (LFP) experiments were carried out using Nd:YAG laser of 355 nm light pulses with a duration of 5 ns and a laser energy range 0–65 mJ per pulse used as the pump light source. A xenon lamp was employed as analyzing light source. The laser and analyzing light beam passed perpendicularly through a quartz cell with an optical path length of 10 mm. The transmitted light entered a monochromator equipped with an R955 photomultiplier. The output signal from the Agilent 54830B digital oscilloscope was transferred to a personal computer for data treatment. The intensity of laser pulse was measured with a laser energy meter (Coherent EPM1000).

ATRA is an amphiphilic molecule and it cannot be guaranteed that ATRA only distribute in the interface of heterogeneous microemulsion. Therefore, it is hard to confirm the accurate ATRA concentrations in different phases and which phase the excited ATRA may distribute in, which makes it difficult for us to measure the accurate reaction rate constants of the reactions of $ATRA^{\bullet+}$ with the chosen compounds. Meanwhile, unlike in homogeneous system, the free diffusion ability of $ATRA^{\bullet+}$ may be limited because of its limited distribution in heterogeneous microemulsion. Herein, "apparent rate constant" was used to represent the apparent and average reactivity of $ATRA^{\bullet+}$ to the chosen compounds in the condition of this study. Apparent bimolecular rate constant (k_R) for the reaction of $ATRA^{\bullet+}$ with the chosen compound in microemulsion was roughly estimated from the plot of the observed pseudo-first-order rate constant (k_{obs}) for the decay of $ATRA^{\bullet+}$ at 590 nm *vs* reductant concentration ([*R*]) using the following equation:

$$k_{obs} = k_0 + k_R[R]$$

Here, k_0 (intercept) is the apparent rate constant for the decay of $ATRA^{\bullet+}$ in the absence of reductant.

RESULTS AND DISCUSSION

Influence of pH on the absorption of ATRA in microemulsion

As shown in Fig. 1B, the maximum absorption wavelength (λ_{max}) of ATRA varies between the constant values 355 and 335 nm, as a function of the pH values from 2 to 13. The pH-dependent λ_{max} of ATRA in microemulsion agrees well with the case for ATRA in lipid bilayers and membrane (29,30,32).

The blueshift of λ_{max} observed when the pH was adjusted from low to high values is attributed to the deprotonation process of ATRA. From Fig. 1A, the isosbestic point for $ATRA^-$ and ATRA can be roughly evaluated at 352 nm. Eq. (2) indicates that OD is linearly correlated with the percentage of $ATRA^-$ at different pH values in the condition of this study. And the pK_a for ATRA in microemulsion was evaluated as 6.98.

Characterization of photoionization of ATRA in microemulsion

The UV–Vis spectra show that microemulsion system itself has negligible absorption at 355 nm compared with that of ATRA (Fig. 1A). Therefore, 355 nm laser pulses are mainly absorbed by ATRA in microemulsion.

355 nm LFP of N_2-saturated ATRA in microemulsion gives a broad absorption band above 630 nm, a strong absorption band centered at 590 nm and a strong bleaching around 350 nm (Fig. 2A). The kinetic decay profile observed at 720 nm can be efficiently removed by typical hydrated electrons (e_{aq}^-) scavengers (Inset of Fig. 2A), such as O_2 and N_2O with addition of t-BuOH Eqs. (3–5), which suggests that e_{aq}^- is generated immediately after 355 nm laser pulse (33). Therefore, photoionization of ATRA in microemulsion can be confirmed by the appearance of e_{aq}^-. The appearance of bleaching at 350 nm suggests the participation of ATRA in photoreaction. The kinetic decay at 590 nm is not influenced by N_2O and O_2. The insensitivity of this intermediate to N_2O and O_2 agrees with retinyl polyene radical cations obtained by pulse radiolysis (13). Moreover, the generation of $ATRA^{\bullet+}$ with maximum absorption around 590 nm was confirmed previously by laser flash photolysis study and pulse radiolysis study (13,16,17). Therefore, the absorption band centered at 590 nm herein is assigned as the absorption of $ATRA^{\bullet+}$.

$$e_{aq}^- + O_2 \rightarrow O_2^{\bullet-} \quad (3)$$

$$e_{aq}^- + N_2O + H_2O \rightarrow HO^\bullet + OH^- + N_2 \quad (4)$$

$$HO^\bullet + t\text{-}BuOH \rightarrow t\text{-}BuOH(-H)^\bullet + H_2O \quad (5)$$

The kinetic curve at 420 nm includes a generation process which can be eliminated by e_{aq}^- scavengers N_2O (Fig. 2B), indicating that the subsequent reaction of e_{aq}^- accounts for the generation. The decay process at 420 nm which is insensitive to N_2O can be accelerated by O_2, suggesting that formation of triplet state of ATRA (^{3}ATRA*) occurs after 355 nm laser pulses, for O_2 is not only an e_{aq}^- scavenger but also a triplet state quencher. It is reasonable because the characteristic absorption of ^{3}ATRA* was reported around 440 nm (16,23).

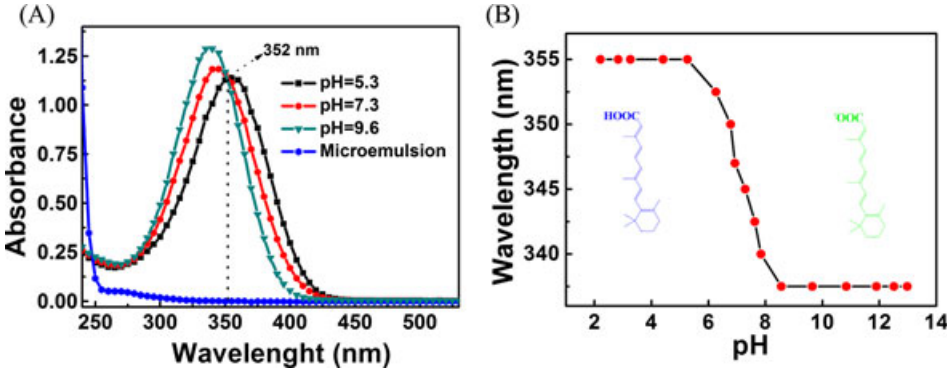


Figure 1. (A) UV–Vis absorption spectra of ATRA in microemulsion at pH 5.3, 7.3 and 9.6 respectively. The isosbestic point for $ATRA^-$ and ATRA was roughly evaluated from the intersection of the three absorption spectra. (B) λ_{max} for ATRA as a function of pH in microemulsion (●).

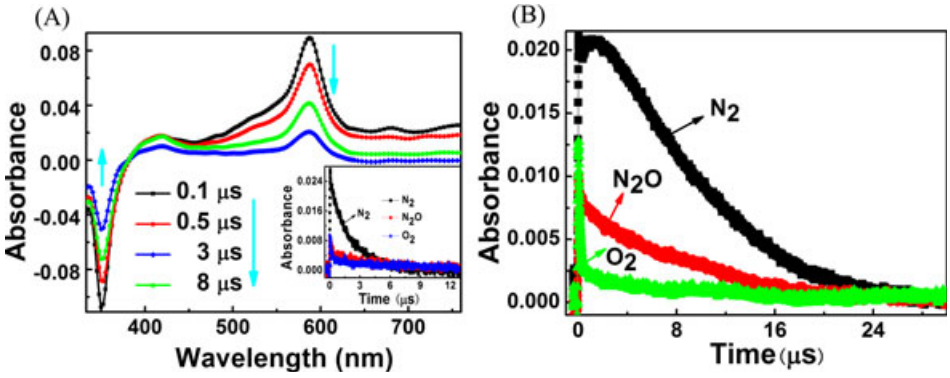


Figure 2. (A) Transient absorption spectra obtained by 355 nm LFP of 0.06 mM ATRA in N_2-saturated microemulsion at pH 7.4, recorded at 0.1, 0.5, 3 and 8 μs after laser pulse. Inset: Kinetic decay curves recorded at 720 nm obtained from 355 nm LFP of 0.06 mM ATRA at pH 7.4 in N_2-saturated, N_2O-saturated and O_2-saturated microemulsion. (B) Kinetic decay curves recorded at 420 nm obtained from 355 nm LFP of 0.06 mM ATRA at pH 7.4 in N_2-saturated, N_2O-saturated and O_2-saturated microemulsion.

Influence of pH on the formation of ATRA$^{\bullet+}$ in microemulsion

It was found that ΔOD (the maximum optical density of decay curves) at 590 nm, *i.e.* the yield of ATRA$^{\bullet+}$, is very sensitive to the pH of microemulsion (Fig. 3A). Therefore, we investigated the influence of pH on ΔOD at 590 nm. Figure 3B shows that ΔOD and the fraction of ATRA$^-$ have the same variation tendency as the pH was changed, which indicated that the yield of ATRA$^{\bullet+}$ is positively dependent on the degree of deprotonation of ATRA in microemulsion.

To determine whether the formation of ATRA$^{\bullet+}$ by 355 nm irradiation is a one- or a two-photon process, the ΔOD of ATRA$^{\bullet+}$ at 590 nm in both alkaline and acidic microemulsions was measured as a function of relative laser intensity (16,34). The results suggest that formation of ATRA$^{\bullet+}$ is biphotonic processes in both alkaline and acidic microemulsion (Fig. 4). Previous study showed that LFP (347 nm) of ATRA in methanol led to biphotonic photoionization with the formation of ATRA$^{\bullet+}$ (16). In alkaline and neutral conditions, e_{aq}^- was observed, and thus photoionization of ATRA$^-$ can be confirmed to be responsible for the formation of ATRA$^\bullet$. However, photoionization of ATRA in microemulsion was not confirmed because e_{aq}^- cannot be observed in acidic condition.

Reactions of ATRA$^{\bullet+}$ with lysozyme and amino acids

Previous study demonstrated that ATRA$^{\bullet+}$ had efficient reaction activity to ascorbate (17). It indicates that ATRA$^{\bullet+}$ has oxidative ability and may pose oxidative damage to other biomolecules. To evaluate the potential of ATRA$^{\bullet+}$ to damage biomolecules, we herein investigated the interactions of ATRA$^{\bullet+}$ with Lyso and two amino acids. Lyso was chosen as a model protein because of its extensive use in the studies of photodamage of proteins (35–40). Result shows that the decay of ATRA$^{\bullet+}$ at 590 nm is accelerated by the presence of Lyso at pH 7.4, indicating that ATRA$^{\bullet+}$ can interact with Lyso (Fig. 5). The apparent rate constant was measured (Table 1).

Tyr and Trp, the aromatic amino acid residues of proteins, are prime targets for oxidation by various forms of reactive oxidative species (ROS) (41). Previous reports indicated that Lyso could be attacked by ROS *via* the interaction of ROS with its Tyr and Trp residues (35,36). The ability of ATRA$^{\bullet+}$ to react with Tyr and Trp was evaluated in this study. Results showed that ATRA$^{\bullet+}$ can also react with Tyr and Trp. The apparent rate constants for the reactions of ATRA$^{\bullet+}$ with Tyr and Trp were also measured, which were found to be close to the apparent rate constant for the reaction of ATRA$^{\bullet+}$ with Lyso (Table 1). Therefore, it is inferred that ATRA$^{\bullet+}$ may interact with Lyso by reacting with the Tyr and Trp residues.

Reactions of ATRA$^{\bullet+}$ with antioxidants

Pulse radiolysis study on the interaction of ATRA$^{\bullet+}$ with VC showed that VC reacts effectively with ATRA$^{\bullet+}$ (17). VC has a higher reaction rate than Lyso to react with ATRA$^{\bullet+}$ (Table 1). And thus it may competitively protect Lyso from the attack of

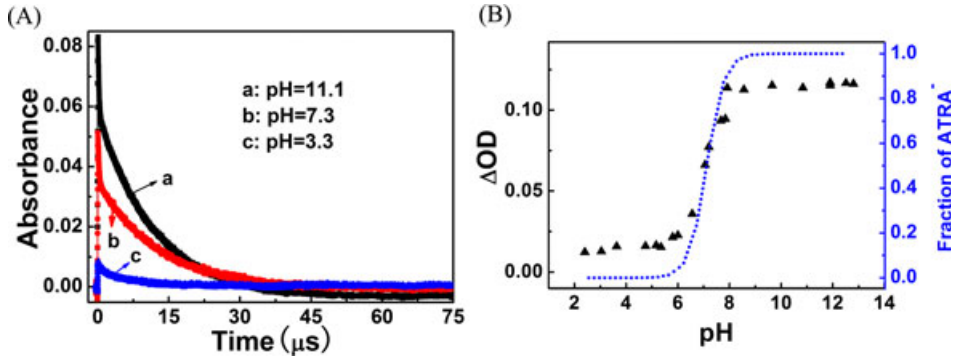


Figure 3. (A) Influence of pH on kinetic decay curves recorded at 590 nm obtained from 355 nm LFP of 0.06 mM ATRA in O_2-saturated microemulsion. (B) Dependence of ΔOD at 590 nm for ATRA$^{\bullet+}$ on pH values in O_2-saturated microemulsion (▲). The short dot line (…) is the theoretical pH titration curve derived from Eq. (2) when pK_a was set as 6.98.

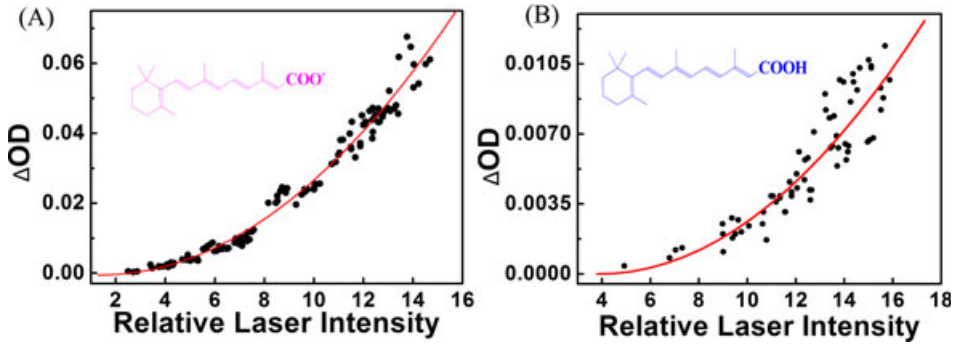


Figure 4. (A) Variation of ΔOD on relative laser intensity immediately after 355 nm LFP of 0.06 mM ATRA at pH 9.26 in O_2-saturated microemulsion. (B) Variation of ΔOD on laser intensity immediately after 355 nm LFP of O_2-saturated 0.06 mM ATRA in microemulsion at pH 4.53.

$ATRA^{\bullet+}$. To find efficient antioxidants to scavenge $ATRA^{\bullet+}$, we then investigated the reactions of $ATRA^{\bullet+}$ with several hydrophilic and hydrophobic antioxidants, including VC, GA, Cur, VE and β-car, and corresponding apparent rate constants were measured and listed in Table 1. Results show that Cur is the most efficient antioxidant to interact with $ATRA^{\bullet+}$ in the investigated antioxidants. Generally, hydrophilic antioxidants are more favorable than hydrophobic antioxidants to quench $ATRA^{\bullet+}$ in microemulsion.

$ATRA^{\bullet+}$ was found to be quenched by β-car with bimolecular rate constants of 1×10^{10} $M^{-1}s^{-1}$ in methanol and 2.1×10^{10} $M^{-1}s^{-1}$ in hexane *via* electron transfer (16). However, the interaction of $ATRA^{\bullet+}$ with β-car (0.05 mM) in microemulsion was not confirmed by our obtained data. The effect of solvent polarity on the λ_{max} of radical cation of retinyl polyenes suggested that there existed a strong ion–dipole interaction between the ground state of radical ions and polar solvent (13). In microemulsion, the polar part of $ATRA^{\bullet+}$ may be anchored *via* ion–dipole interaction in the oil/water interface where it is difficult for highly hydrophobic molecule to reach. It may explain why the interaction of β-car with $ATRA^{\bullet+}$ cannot be observed in microemulsion. VE has a comparatively polar phenolic hydroxyl group that can reach the oil/water interface, and thus shows a higher reaction activity to $ATRA^{\bullet+}$ than β-car.

Table 1. Apparent rate constants for reactions of $ATRA^{\bullet+}$ with lysozyme and amino acids and reductants at neutral pH in microemulsion. (Unit: $M^{-1}s^{-1}$).

	Apparent rate constants ($M^{-1}s^{-1}$)
Lyso	$(1.85 \pm 0.09) \times 10^8$
Tyrosine	$(2.73 \pm 0.65) \times 10^8$
Tryptophan	$(1.04 \pm 0.03) \times 10^8$
VC	$(3.42 \pm 0.14) \times 10^8$
GA	$(1.72 \pm 0.09) \times 10^8$
Cur	$(1.39 \pm 0.17) \times 10^9$
DMA	$(2.42 \pm 0.13) \times 10^9$
DPA	$(1.67 \pm 0.02) \times 10^9$
VE	$(3.94 \pm 0.29) \times 10^6$
β-car	—

These apparent rate constants were roughly evaluated and only used as a reference to compare the reactivity of $ATRA^{\bullet+}$ to the compounds used in this study.

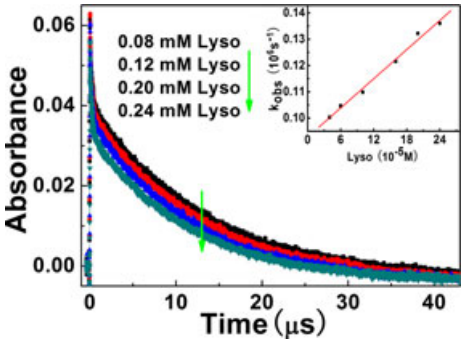


Figure 5. Kinetic decay profiles observed at 590 nm from 355 nm LFP of 0.05 mM ATRA in microemulsion at pH 7.4 in the presence of Lyso (0.08, 0.12, 0.20 and 0.24 mM). Inset: Dependence of k_{obs} for $ATRA^{\bullet+}$ at 590 nm on Lyso concentrations.

It is well known that $ATRA^{\bullet+}$ can be generated *via* not only photoinduced processes but also oxidation of ATRA by oxidative transient species, such as peroxyl radicals and hydroxyl radicals (16,17). Generation of $ATRA^{\bullet+}$ is unavoidable in its application as drugs because peroxyl radicals and hydroxyl radicals are also generated in the biological systems. Although the interactions of $ATRA^{\bullet+}$ with Lyso and amino acids are suggestive of the potential damage of $ATRA^{\bullet+}$ to proteins, our studies on the reactions of $ATRA^{\bullet+}$ with antioxidants indicated rapid deactivation of $ATRA^{\bullet+}$ by antioxidants is possible.

Reactions of $ATRA^{\bullet+}$ with organic amines

Organic amines, strong nucleophilic agents, were proved to be effective quenchers for radical cations of retinyl polyene and retinoids (12,13). The most possible mechanism for the quenching reaction was proposed to be nucleophilic attack or electron transfer (12). In this study, two organic amines, DPA and DMA were used to quench $ATRA^{\bullet+}$ and the apparent rate constants are nearly diffusion-controlled rate (Table 1). The characteristic absorption bands for radical cation of DPA ($DPA^{\bullet+}$) and radical cation of DMA ($DMA^{\bullet+}$) were reported around 680 and 465 nm respectively (42,43). In spite of the effective interaction of $ATRA^{\bullet+}$ with DMA, the formation process of $DMA^{\bullet+}$ cannot be observed in its corresponding characteristic absorption spectra most because of the strong absorption of $ATRA^{\bullet+}$. However, the characteristic absorption band around 465 nm can be observed after the absorption of $ATRA^{\bullet+}$ decayed back to the baseline (Fig. 6A). The kinetic decay curve at 470 nm evidently included two decay processes: a fast decay process and a slow decay process (Fig. 6B). By subtracting the decay curve at 590 nm from

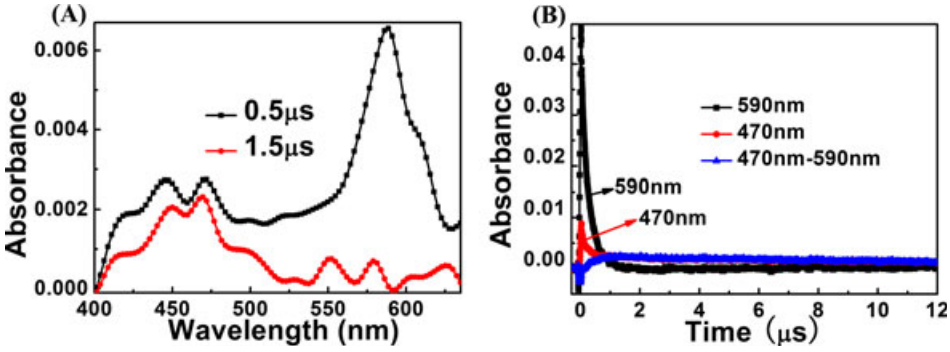


Figure 6. (A) Transient absorption spectra obtained by 355 nm LFP of 0.06 mM ATRA in O_2-saturated microemulsion at pH 7.4 with 4 mM DMA, recorded at 0.5 μs (-■-) and 1.5 μs (-●-) after laser pulse. (B) Kinetic decay profiles observed at 470 nm (-■-) and 590 nm (-●-) obtained by 355 nm LFP of 0.06 mM ATRA in O_2-saturated microemulsion at pH 7.4 with 1 mM DMA, and the decay curve (-▲-) obtained by subtracting the decay curve at 590 nm from 470 nm.

470 nm, a formation process can be observed which should be assigned to the formation of $DMA^{\bullet+}$ (Fig. 6B). The similar phenomena were also observed for the reaction of $ATRA^{\bullet+}$ with DPA (data not shown). Therefore, it can be inferred that electron transfer should be responsible for the efficient quenching reactions of $ATRA^{\bullet+}$ by DMA and DPA.

CONCLUSION

We investigated the photochemistry of ATRA in microemulsion and characterized the properties of reaction products. 355 nm LFP of ATRA in both acidic and alkalic condition leads to the generation of $ATRA^{\bullet+}$ *via* biphotonic processes. It was found that $ATRA^{\bullet+}$ can efficiently react with Lyso and Tyr and Trp, which is suggestive of the possibility of $ATRA^{\bullet+}$ to damage proteins. Many antioxidants, such as VC, GA and Cur, were proved to be efficient quenchers for $ATRA^{\bullet+}$.

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