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Original article

Quenching effect of deferoxamine on free radical-mediated photon production in luminol and *ortho*-phenanthroline-dependent chemiluminescence

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ABSTRACT

Removing excessive free radicals (FRs) by a synthetic chemical might give a clue for treatment of many iron-mediated diseases. Deferoxamine (DFO) can be one of the chemicals of choice for the clue. To investigate photoredox properties of DFO, its quenching effect on superoxide radical ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^\bullet$) was examined using luminol and *ortho*-phenanthroline (*o*-phen) chemiluminescence (CL) systems and UV–vis spectrophotometry. Stern–Volmer equation was also used for the CL kinetics. The observed quenching effect of DFO on CL/photon production in luminol and *o*-phen CL systems strongly confirmed the static arm of quenching properties of DFO on $OH^\bullet$ and H_2O_2, but much less pronounced on $O_2^{-\bullet}$; the quenching property was maximal when iron was involved in the reaction systems. The Stern–Volmer plots in the designed photochemical reaction systems also confirmed a potent quenching effect of DFO on FR-mediated CL. Our study highlights strong photoreducing and antioxidant properties of DFO with huge quenching capacity on excessive FRs, and thus implies its promising prospects for therapeutic applications.

1. Introduction

Free radicals (FRs) such as $O_2^{-\bullet}$, H_2O_2, $OH^\bullet$, $^1O_2$ and $ONOO^-$ are normally generated *in vivo* [1–4]. Formed by one electron reduction of O_2, in the body, the $O_2^{-\bullet}$ is produced mostly in inflamed sites [1,2], dismuted to H_2O_2 [5,6] and further converted to $OH^\bullet$, mainly by Fe^{2+} and Cu^+, initiating Fenton's-like reactions and extensive oxidative damage to vital biomolecules like nucleic acids, proteins and lipids [7–9]. Among the FRs, $OH^\bullet$ highly reacts with functional groups of biomolecules and destroys them [10–12] (Eqs. (A) and (B)). Also, oxidation of Fe^{2+} by H_2O_2 produces $OH^\bullet$ [13]; the $OH^\bullet$ is an intermediate product of reactions in many biochemical systems such as, (A) $H_2O_2 + Fe^{2+} \rightarrow OH^- + OH^\bullet + Fe^{3+}$, (B) $OH^\bullet + RH \rightarrow R^\bullet + H_2O$, (C) $R^\bullet + Fe^{3+} \rightarrow R^+ + Fe^{2+}$ and (D) $Fe^{2+} + OH^\bullet \rightarrow Fe^{3+} + OH^-$.

To combat the destructive effects of FRs, the body utilizes elaborate enzymatic/endogenous and non-enzymatic/exogenous antioxidant defenses [14] to quench or remove excessive FRs. Many synthetic chemicals also possess redox properties, eliminating oxidants–antioxidants imbalances *in vivo*. Among several available synthetic antioxidants, deferoxamine (DFO; Desferal®) can be a photochemical of choice for therapeutic purposes, and its clinical application in human and animal is promising [15–19].

As a siderophore, DFO is naturally produced by *Streptomyces pilosus*; it has been purified and synthesized since 1960 (Scheme 1A) [20]. As a specific iron chelator and by forming water soluble complex with iron, DFO effectively removes and eliminates excessive iron (Scheme 1B) [19,21], thereby balancing redox system in blood. Though to a much less extent than Fe^{3+}, DFO also exhibits affinity toward Al^{3+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Ga^3 and other metal ions [22].

Despite its promising implication in medicinal chemistry, little studies have been done on photochemical properties of DFO in FRs producing chemical systems. This study aimed to pinpoint the luminescent properties of DFO to which how it behaves and interacts in the photochemical reactions systems using Fenton's

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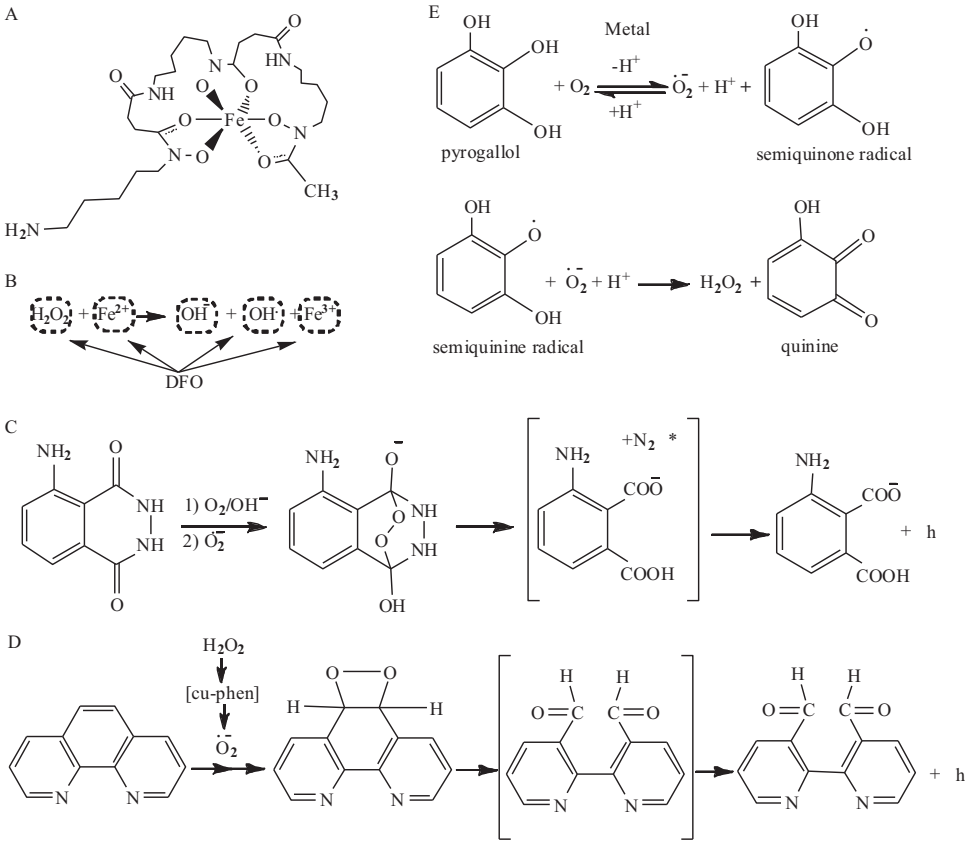

Scheme 1. Chemical structure of deferoxamine (DFO) in complex with iron (A) and its interaction with some free radicals in the Fenton's reaction system (B). Chemiluminescence (CL) mechanisms for luminol (C) and *ortho*-phenanthroline (D) and a generally accepted pyrogallol autoxidation pathway (E).

reaction and Fenton's-like reaction. To investigate photochemical properties of DFO, we tested the quenching and scavenging capacities of DFO on $OH^{\bullet}$, H_2O_2 and $O_2^{\bullet-}$ using luminol and *ortho*-phenanthroline (*o*-phen)-enhanced CL systems, UV–vis absorption spectroscopy and Stern–Volmer equation model.

2. Experimental

All chemicals and reagents were analytical grade. DFO, as mesylate salt (Desferal®), and *o*-phen were purchased from Sigma Chemical Co., St. Louis, MO, USA. Other chemical reagents were purchased from Merck, Darmstadt, Germany. Stock solutions of DFO (0.3 and 0.01 mmol/L in ddH_2O), luminol (0.1 mmol/L in DMSO, dimethylsulfoxide), *o*-phen (0.01 mmol/L in ddH_2O), $CuSO_4$ (0.01 mmol/L in ddH_2O), $FeSO_4$ (0.01 mmol/L in ddH_2O), were freshly prepared and appropriately protected from light for further use. Main buffers used in the study were phosphate-buffered saline solution (PBS), Tris–HCl, at pH 7.4 and 8, Tris–HCl at pH 8, carbonate at pH 10.2 and acetate at pH 5.5.

To test the effects of DFO on FR, various CL assays, in which the FR, especially $OH^{\bullet}$, H_2O_2 and $O_2^{\bullet-}$ that are central photo reactants *in situ*, were used. Photochemically, decrease of CL intensity in our method with DFO load always attributes to scavenging capacity/quenching ability of DFO on FR.

To examine the quenching effect of DFO on $OH^{\bullet}$-induced luminol CL, $OH^{\bullet}$ was generated by a Fenton's-type reaction [23] containing 100 μL $FeSO_4$ (0.4 mmol/L) and 100 μL of H_2O_2 1.5%. This mixture was incubated for 2 min at 37 °C and then 100 μL of PBS with and without different concentrations of DFO was added to the reaction mixture (solution 1). Luminol solution (600 μL of 0.15 mmol/L) was added into the luminometer cell (solution 2), and background of photon production was recorded on a FB12/Sirius Berthold ultra weak luminometer. Finally, 150 μL of solution 1 was added to the solution 2 and CL/photon production was counted (counts/10 s) and total CL count was integrated. Further, Stern–Volmer plot was drawn from equation $I_0/I = 1 + K_Q[Q]$ [24], where K_Q is the Stern–Volmer quenching constant, I_0 and I are CL intensity without and with DFO, respectively, and $[Q]$ is concentrations of DFO. Also % of scavenging capacity (SC) was calculated using: $SC = [(CL_{control} - CL_0) - (CL_{sample} - CL_0)]/(CL_{control} - CL_0)$, where $CL_{control}$ is the photon production of the control, CL_0 is the photon production of the background and CL_{sample} is the photon production of DFO mixed samples.

The inhibitory effect of DFO on Fenton's generated $OH^{\bullet}$ was performed using Cu^{2+} and ascorbic acid instead of Fe^{2+}, and *o*-phen was used as CL probe [25,26]. Briefly, 100 μL of 2×10^{-4} mmol/L $CuSO_4$, 100 μL of 10^{-3} mmol/L ascorbic acid, 100 μL of 10^{-3} mmol/L *o*-phen, 400 μL of 0.1 mmol/L acetate buffer and 100 μL of PBS with different concentrations of DFO. After recording the background CL (CL_0), the reaction was started after addition of 200 μL of 1 mmol/L H_2O_2. The CL intensity was counted once every 20 s at 37 °C. The Stern–Volmer quenching constant (K_Q) and SC were obtained as aforementioned procedure.

To evaluate the SC of DFO on H_2O_2, 600 μL of 50 mmol/L PBS, pH 8.0, with and without 200 μL of DFO in PBS and 200 μL of H_2O_2 1% were mixed for 10 min at 37 °C. Then 150 μL luminol (15 mmol/L) was added to the mixture; CL was quantified every 4 s, and KQ and SC were eventually obtained.

To examine the scavenging effect of DFO on $O_2^{-\bullet}$, the $O_2^{-\bullet}$ was generated from a pyrogallol autoxidation system accordingly [27]; the SC was determined with UV–vis spectrophotometer (cecill model 5000, Cambridge, England). Briefly, 500 μL of 100 mmol/L

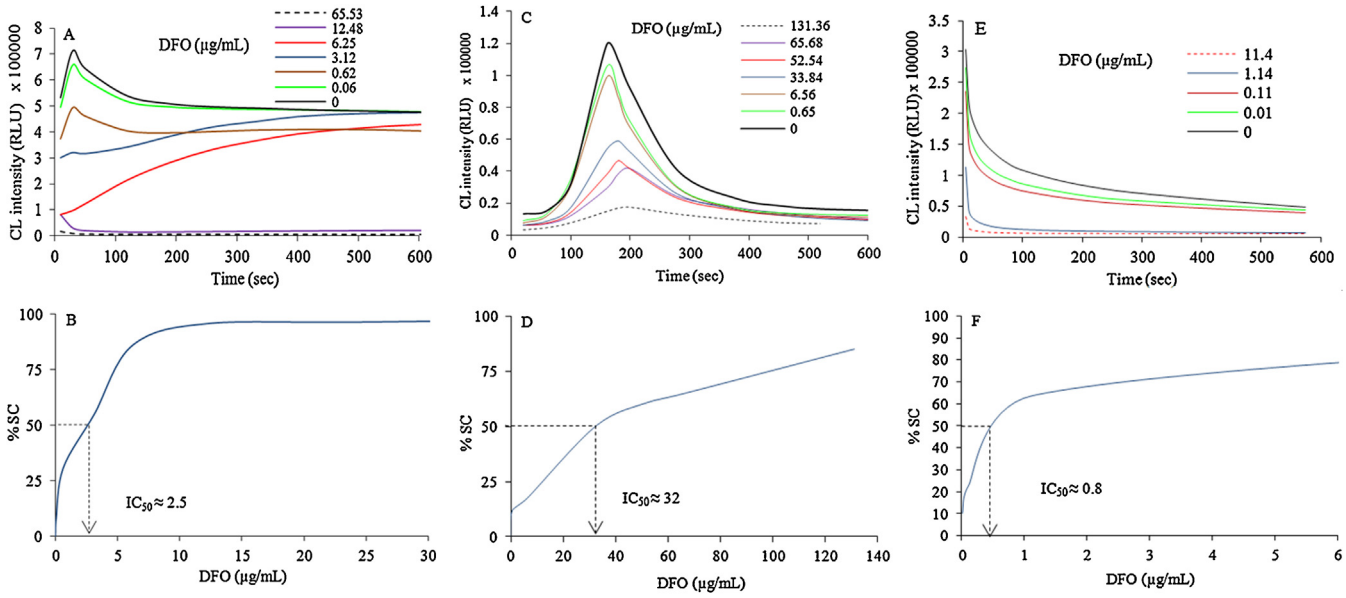

Fig. 1. Typical pattern of deferoxamine (DFO) effect on FRs-induced chemiluminescence (CL), Fenton's reaction: Fe^{2+}-H_2O_2-luminol, A and B; quenching/scavenging capacity (%SC), Fenton's-like reaction: Cu^{2+}-ascorbic acid-H_2O_2-*o*-phen, C and D, %SC, and H_2O_2-induced luminol CL, E and F, %SC.

Tris–HCl, pH 8.2, $(1500 - X)$ μL ddH_2O, X μL of 300 mmol/L DFO plus 100 μL of 0.33 mmol/L pyrogallol, were carefully mixed. The absorbance was measured every 10 s at λ = 310 nm. The autoxidation rate of pyrogallol was calculated and controlled using the slope of the absorbance fluctuations at λ 310 nm in function of time (s) by adjusting the concentration of pyrogallol. The autoxidation rate of pyrogallol was recorded every second, and was linearly correlated with the absorbance. The scavenging rate, expressed as %SR, of DFO for $O_2^{-\bullet}$ was calculated using, $SR = (k_0 - k_1)/k_0 \times 100\%$, where k_0 and k_1 are autoxidation rates of pyrogallol without and with DFO, respectively.

3. Results and discussion

As confirmed in our study, DFO inhibited Fenton's reaction *via* its ability to: (1) scavenge $OH^{\bullet}$, (2) form complexes with iron and (3) scavenge H_2O_2 and to a lesser extent $O_2^{-\bullet}$. Based on the recorded Fenton's reaction, two CL systems (luminol and *o*-phen) were used to pinpoint the effect of DFO on $OH^{\bullet}$ in the CL systems mainly *via* complexation of DFO with catalysts, Fe^{2+} and Cu^{2+}. Kinetics of $OH^{\bullet}$-induced photon production with and without different concentrations of DFO on luminol and *o*-phen CL systems are representatively shown in Fig. 1A and C. In both systems, photon production intensity dose-dependently decreased with increasing of DFO concentration. The CL intensity peaked at 30 s (T_m) then decreased slowly with increasing reaction time (Fig. 1A). DFO inhibited $OH^{\bullet}$-induced photons from the start of the CL reaction; in this system the $OH^{\bullet}$ generated mainly from a Fenton's reaction (Eq. (A)). DFO load at 8 μg/mL forms strong complex with Fe^{2+}, thus inhibiting $OH^{\bullet}$ production (half inhibition concentration (IC_{50}) ≈ 2.5 μg/mL, Table 1 and Fig. 1B).

Since the affinity of DFO to form complexes with Cu^{2+} is much less than with Fe^{2+}, we further examined the effect of DFO on $OH^{\bullet}$ without iron, using Fenton's-like reaction, in which the contribution of Cu^{2+}, instead of Fe^{2+}, to *o*-phen as a CL probe to generate $^{\bullet}OH$-induced CL is central. Also, in this CL system ascorbic acid played key role in the reaction mechanism (Eqs. (E)–(H)): (E) Ascorbic acid + $2Cu^{2+} \rightarrow$ Dehydroascorbic acid + $2Cu^{+} + 2H^{+}$, (F) $2Cu^{+} + 2O_2(aq) \rightarrow 2Cu^{2+} + 2O_2^{-}$, (G) $2O_2^{-} + 2H^{+} \rightarrow H_2O_2 + O_2$ and (H) $Cu^{+} + H_2O_2 \rightarrow {}^{\bullet}OH + OH^{-} + Cu^{2+}$.

The kinetics of the *o*-phen-mediated $OH^{\bullet}$-induced CL showed the CL intensity reached to its T_m at about 160 s and more slowly decreased afterwards Fig. 1C; this was remarkably different from what observed in luminol-mediated $OH^{\bullet}$-induced CL (Fig. 1A). Indeed, DFO instantly inhibited $OH^{\bullet}$-induced CL from the start of the reaction and remained inhibited until the endpoint. In this system increasing of DFO concentration to140 μg/mL resulted in quenching of more than 90% of CL signal ($IC_{50} \approx 32$ μg/mL, Fig. 1D); this might be due to far less affinity of DFO to form complexes with Cu^{2+}.

Part of the quenching effect of DFO on Fenton's reaction-induced CL may be ascribed to the scavenging ability of DFO on H_2O_2. We, therefore, examined the effect of DFO on H_2O_2 *via* its quenching effect on H_2O_2-induced luminol-dependent CL/photon production with no interference of Fe^{2+}, $Fe^{3+}$and Cu^{2+}. The kinetics curve of H_2O_2-induced luminol-dependent CL (Fig. 1C) differed from that of $OH^{\bullet}$-induced CL (Fig. 1E). CL intensity instantly reached to its maximal value then rapidly decreased to half of the initial value at ~40 s (Fig. 1E), clearly indicating that H_2O_2 was

Table 1
Quenching parameters of deferoxamine (DFO) from Stern–Volmer plot in different CL systems.

CL system	K_Q (mL/μg)	Stern–Volmer equation ($y = I_0/I$, x = [DFO])	r^2	N^a	$IC_{90}{}^b$ (μg/mL)	$IC_{50}{}^c$ (μg/mL)
Fe^{2+}-H_2O_2-luminol	1.302	$y = -0.510 + 1.302x$	0.994	6	8	2.5
Cu^{2+}-ascorbic acid-H_2O_2-*o*-phen	0.042	$y = 0.783 + 0.042x$	0.955	7	140	32
H_2O_2-luminol	0.693	$y = 1.282 + 0.693x$	0.990	5	12	0.8

[a] Number of points.
[b] 90% inhibition concentration.
[c] 50% inhibition concentration.

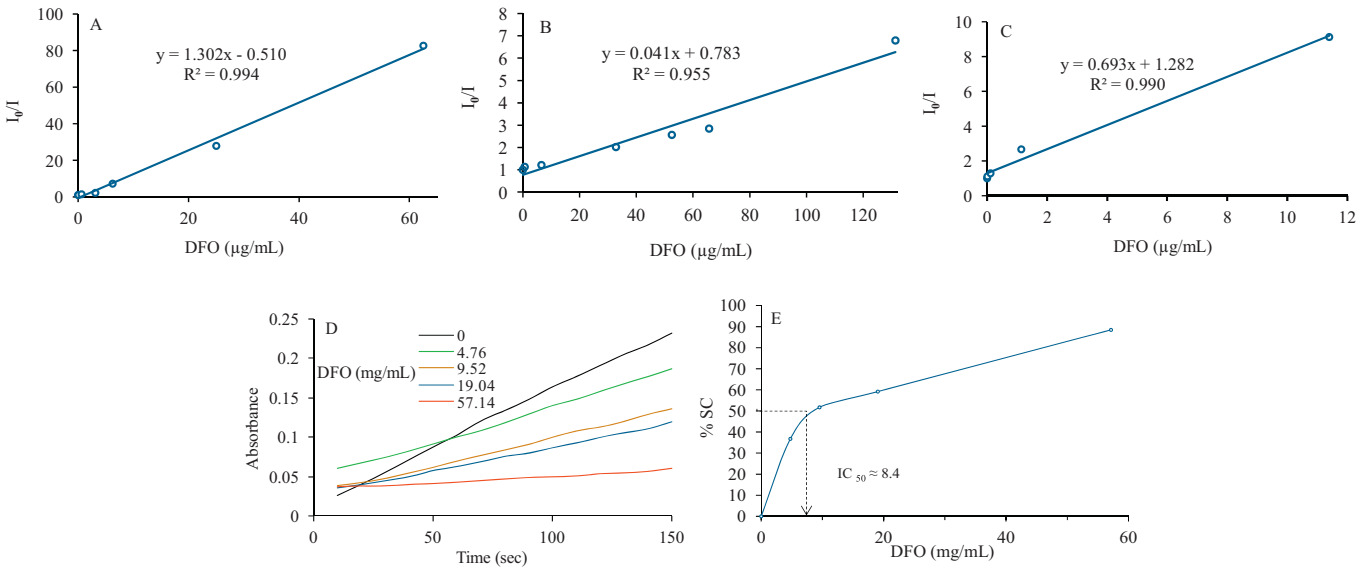

Fig. 2. Stern–Volmer plots for quenching effect of deferoxamine (DFO) on (A) Fe^{2+}-H_2O_2-luminol CL, (B) Cu^{2+}-ascorbic acid-H_2O_2-*o*-phen CL and (C) H_2O_2-luminol C systems. (D) Kinetics plot of autoxidation of pyrogallol by UV–vis spectrometry at λ 310 nm and (E) scavenging rate of pyrogallol with different concentrations of DFO.

rapidly consumed by DFO in luminol-CL system. As clearly observed, low range of [DFO] acted as strong scavenger of H_2O_2 in concentration-dependent manner. At 12 μg/mL, DFO quenched more than 90% of CL signal ($IC_{50} \approx 0.8$ μg/mL, Fig. 1F).

The photon production decay curves for all CL systems evaluated with and without DFO are shown in Figs. 1 and 2A–C. Nevertheless, in all above-mentioned CL systems DFO was found to markedly quench the designed photoreaction systems. In the presence of DFO, the CL intensity of photon production reduced from I_0 to I, the ratio is directly proportional to the DFO load [Q] according to Stern–Volmer equation, in which a plot of I_0/I versus [Q] yielded a linear graph with an intercept of 1 and a slope of K_Q. For a measurement system based on quenching and interpretation, the K_Q is central; the larger the K_Q in our CL systems with DFO, the higher quenching capacity of DFO; indeed, the K_Q is directly proportional to the effect of DFO on the photochemical reaction. A plot of I_0/I versus [DFO] for the photochemical system for some key parameters of the plots are given in Table 1 and Fig. 2A–C. Interestingly, the quantum of K_Q for the three different CL systems clearly reveals the fact that quenching capacity of DFO on Fenton's reaction behaved somewhat differently. The Stern–Volmer constant in the presence of Fe^{2+} was more than that of the Cu^{2+}, further revealing the fact that DFO can far better form complex with Fe^{2+} than Cu^{2+}.

Indeed, FRs are key compartments of both luminol and *o*-phen CL systems [28], see Scheme 1C and D. Both Fe^{2+} and Cu^{2+} have catalytic role in the CL system. Low K_Q with Cu^{2+} is mainly due to the presence of *o*-phen in the CL system, because Cu^{2+} can perform strong complex with *o*-phen. The *o*-phen possesses two nitrogen atoms in the heterocyclic system. These aromatic molecules appropriately interact with Cu^{2+} and produce relatively tight complexes with copper. DFO in the presence of *o*-phen cannot appropriately breakdown the Cu-*o*-phen complexes and, thus K_Q for *o*-phen CL system is far less than that of luminol CL system. Indeed, DFO, an easily oxidizable antioxidant and as a strong iron lowering chemical in our photo reactive mixture and therefore functioned as a static quencher in our luminol-dependent CL system. The most probable mechanism for the quenching of CL by DFO could be *via* electron transferring pathway. Mechanistically, there would be two forms of quenching pathways for DFO in our examined CL systems, static and dynamic [24]. The static one results from formation of DFO-photoreactant complexes. In contrast, the dynamic one is the result of collision of DFO with the photoreactants, accelerating energy loss in the reaction mixture [24]. Indeed, both the static and the dynamic pathways of quenching can be predicted in the applied Stern–Volmer equation model. To us, the observed quenching effect of DFO in our study belongs mainly to the static part of quenching pathway, and DFO-iron complexation in our CL systems clearly exemplifies the static arm of the quencher, DFO.

In the presence of iron the maximal quenching capacity of DFO on Fenton's reaction systems were observed; in contrast, the minimal quenching capacity of DFO were observed in the Fenton's-like reaction system, further supporting far lower affinity of DFO to react with Cu^{2+}, compared with Fe^{2+}; very well correlation between K_Q and IC_{90}, further confirms our point on the affinity of DFO in different photochemical reactive systems designed in our study.

We applied the autoxidation properties of widely used pyrogallol [29,30] to pinpoint photoactive capacity of DFO against FR, especially $O_2^{-\bullet}$ in our CL system. The presence of oxygen can be detected or measured by absorbance of the oxidized-colored product of pyrogallol with spectrophotometry; their mechanism of action is given in Scheme 1E. production of end product of the reaction, quinine, directly links to $O_2^{-\bullet}$ [29,30]. So, any $O_2^{-\bullet}$ scavenger weakens the rate of quinine production in our designed CL system. These changes can be easily monitored by a time-driven UV–vis detector. Effect of DFO on kinetics of autoxidation of pyrogallol [29,30] by UV–vis spectrophotometry at λ 310 nm (Fig. 2D) further confirmed that the decreased slope of the association lines inextricably linked to the DFO load in the reaction mixture. The concentration-dependent manner of scavenging effect of DFO on $O_2^{-\bullet}$ increased with increase of DFO load (Fig. 2E). At the concentration of 8400 μg/mL SC of 50% was achieved for $O_2^{-\bullet}$, *i.e.*, IC_{50} ~8.4 μg/mL. In this photoreactive system, IC_{50} was also measured for some other well-known

antioxidants such as ascorbic acid and vitamins B_6 and B_9; the IC_{50} for these well-known reducing agents was 5.5, 79 and 115 μg/mL, respectively (data not shown). Compared to other FR, scavenging effects of DFO on $O_2^{-\bullet}$ was far less than those of $OH^\bullet$ and H_2O_2; biologically, this can be pivotal especially *in vivo* while using DFO as anti-inflammatory chemical for pharmaceutical formulations.

4. Conclusion

The photoanalytical and plotting assays in our CL systems reveal a promising photoredox properties of DFO with huge quenching capacity mainly on $OH^\bullet$ and H_2O_2 with much less pronounced on $O_2^{-\bullet}$. This quenching is mainly derived from the complexation of DFO with catalyst, Fe^{2+}, and thus Fe^{2+} removal from the oxidation reaction; this complexation process in the presence of $OH^\bullet$ and/or H_2O_2 might be faster than of $O_2^{-\bullet}$. Further study is needed for the detailed mechanism of metal ions catalyzing the CL reaction. DFO would be a chemical of choice in biological system to remove excessive FRs, especially $OH^\bullet$ in the body for therapeutic purposes. As such, application of DFO in pharmaceutical formulations is highly encouraged.

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