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HKOH-1: A Highly Sensitive and Selective Fluorescent Probe for Detecting Endogenous Hydroxyl Radicals in Living Cells

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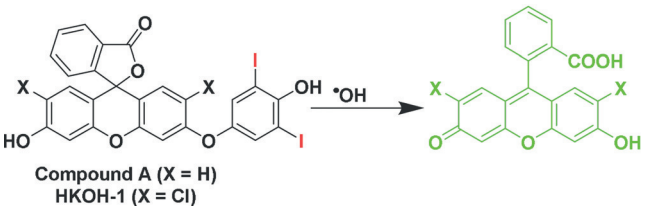
Abstract: *The hydroxyl radical (·OH), one of the most reactive and deleterious reactive oxygen species (ROS), has been suggested to play an essential role in many physiological and pathological scenarios. However, a reliable and robust method to detect endogenous ·OH is currently lacking owing to its extremely high reactivity and short lifetime. Herein we report a fluorescent probe HKOH-1 with superior in vitro selectivity and sensitivity towards ·OH. With this probe, we have calibrated and quantified the scavenging capacities of a wide range of reported ·OH scavengers. Furthermore, HKOH-1r, which was designed for better cellular uptake and retention, has performed robustly in detection of endogenous ·OH generation by both confocal imaging and flow cytometry. Furthermore, this probe has been applied to monitor ·OH generation in HeLa cells in response to UV light irradiation. Therefore, HKOH-1 could be used for elucidating ·OH related biological functions.*

Reactive oxygen species (ROS), including the hydroxyl radical (·OH), hypochlorous acid (HOCl), peroxynitrite ($ONOO^-$), superoxide ($O_2^{\cdot-}$), nitric oxide (NO), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2), play essential roles in various physiological and pathological processes.[1] Among all ROS, ·OH is considered to be the most reactive and harmful species. It has a short lifetime (approximately 10^{-9} s) and can react with numerous biomolecules such as DNA bases, lipids, and proteins at a diffusion-controlled rate.[2] Its excessive production leads to cell damage and has been implicated in various diseases.[3] On the other hand, more evidence suggests that generation of ·OH and other ROS can be applied to cancer treatment.[4] Therefore, monitoring intracellular ·OH is of paramount importance in understanding its biological impact and further investigating its therapeutic utilization. However, selective detection of endogenous ·OH is highly challenging, given its short lifetime and low concentration in cells. Over the past decades, several methods to detect ·OH have been reported, for example, those based on electron spin resonance (ESR) spectroscopy[5] and aromatic hydroxylation reactions with salicylate,[6] respectively. However, these methods are limited as they are not sensitive enough towards ·OH and cannot selectively distinguish it from other radicals. Furthermore, such methods are not suitable for detecting ·OH in live cells.

Fluorescent probes are useful tools for detecting ROS in live cells and tissues with extraordinary temporal and spatial resolution. Indeed, there have been several reported fluorescent or luminescent probes for ·OH detection.[7] However, the use of these probes has been doomed by their indirect detection method, weak sensitivity or poor selectivity. Among all of the reported ·OH probes, commercially available 2-[6-(4′-hydroxy)phenoxy-3*H*-xanthen-3-on-9-yl]benzoic acid (HPF) is most widely used.[7a] It relies on an *O*-dearylation of the masked fluorescein by an electron-rich phenol moiety. However, HPF demonstrated poor selectivity over other ROS, especially $ONOO^-$ (730-fold increase in fluorescence intensity towards 10 equiv of Fenton's reagent vs. a 120-fold increase towards 0.3 equiv of $ONOO^-$). Considering the low concentration and high reactivity of ·OH in cells, it would be difficult to confirm the real origin of fluorescence response in live cell imaging. Some controversial results were obtained when HPF was used as a ·OH probe and thiourea as a ·OH scavenger.[8] Therefore the major challenges in developing a desired ·OH fluorescence probe are to efficiently differentiate ·OH from other ROS, especially strong oxidants like HOCl and $ONOO^-$, and competently capture intracellular ·OH in the presence of various endogenous ·OH scavengers. Herein we report the development of fluorescent probes Compound A and HKOH-1 with excellent sensitivity, selectivity, and extremely rapid turn-on response toward ·OH in live cells in both confocal imaging and flow cytometry experiments.

Among the various highly reactive oxygen species (hROS), the hydroxyl radical stands out from the rest, namely HOCl and $ONOO^-$, as a reactive radical species. Based on previous reports, ·OH favors an electron transfer reaction when reacts with a bulky phenol such as diiodophenol to form a phenoxyl radical, which finally leads to decomposition of aromatic compounds.[9] Thus, we designed two novel probes, Compound A and HKOH-1 (Scheme 1), by introducing two iodine atoms at the *ortho* position of the phenolic hydroxyl group. While the diiodophenol would act as a quencher of fluorescein or 2′,7′-dichlorofluorescein, the



Scheme 1. Design of Compound A and HKOH-1.

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steric hindrance of the two iodine atoms would block oxidation by other strong oxidant like HOCl or $ONOO^-$, thus avoiding the selectivity problem of HPF. After oxidation with ·OH, fluorescein or 2′,7′-dichlorofluorescein would be released, leading to a "turn-on" of fluorescence.

Compound A and HKOH-1 were prepared in good yield in three steps from commercially available starting materials (Supporting Information, Schemes S1 and S2). The Fenton reaction is the most common used method for ·OH generation in vitro. Zhu and co-workers also reported that ·OH could also be produced by reaction of tetrachloro-1,4-benzoquinone (TCBQ) and H_2O_2.[10] For chemical characterization, we applied both methods to investigate the reactivity of Compound A and HKOH-1 towards ·OH. Compound A and HKOH-1 showed absorption peaks at 490 nm and 500 nm, respectively (Supporting Information, Figure S1). In absence of ·OH, both probes were nonfluorescent; upon treatment with 1 equiv of Fenton reagent ($[H_2O_2]$:$[FeCl_2]$ = 10:1) or TCBQ/H_2O_2 ($[H_2O_2]$:[TCBQ] = 10:1), extremely rapid time-dependent enhancement in fluorescence intensity was observed (Figure 1 a,b; Supporting Information, Figure S2 a,b). HKOH-1 showed >30-fold and >180-fold increases in fluorescence intensity upon treatment with Fenton reagent and TCBQ/H_2O_2, respectively, while Compound A showed >40-fold and >200-fold increases in fluorescence intensity upon treatment with Fenton reagent and TCBQ/H_2O_2, respectively. Both Compound A and HKOH-1 completed the oxidation by Fenton reagent within 10 s, while the oxidation with TCBQ/H_2O_2 completed within 3 min yet with even stronger enhancement in fluorescence intensity, illustrating the remarkable sensitivity and fast response of these two probes.

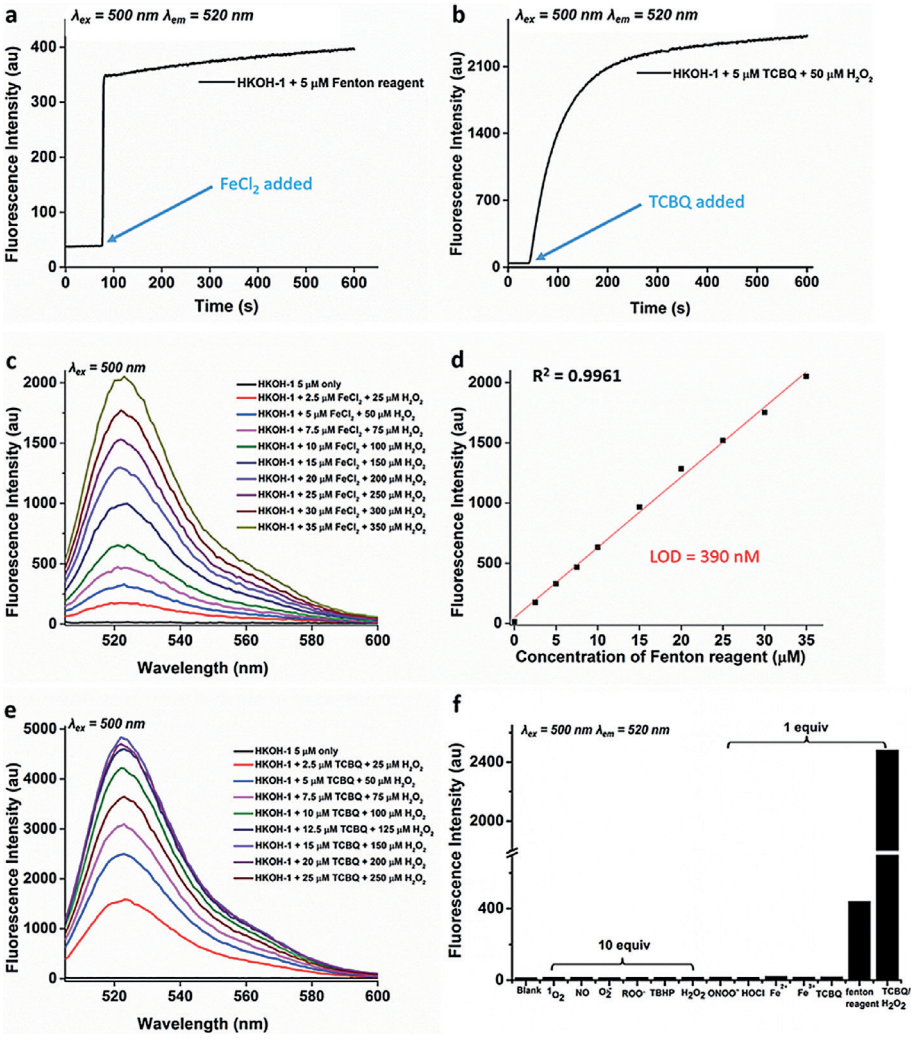

Figure 1. Characterization of HKOH-1 performance in chemical systems. Fluorescence spectra of HKOH-1 (5 μM) in 0.1 M potassium phosphate buffer (pH 7.4, 0.1% DMF) at 25 °C for 30 min. a),b) Time course of the fluorescence response of HKOH-1 towards ·OH: a) Fenton reagent; b) TCBQ/H_2O_2. c) Fluorescence emission spectra of HKOH-1 upon treatment with different amounts of Fenton reagent. d) Fluorescence intensity of HKOH-1 as a function of concentration of Fenton reagent (at 0.5–7 equiv). e) Fluorescence emission spectra of HKOH-1 upon treatment with different amounts of TCBQ/H_2O_2. f) Fluorescence responses of the probe HKOH-1 toward various ROS/RNS. The fluorescence spectra were measured at 520 nm with an excitation at 500 nm.

Next, different equivalents of Fenton reagent and TCBQ/H_2O_2 were added to the testing solution of Compound A (Supporting Information, Figure S2) and HKOH-1 (Figure 1 c–e). The fluorescence intensity was found to increase linearly with the concentration of Fenton reagent (Supporting Information, Figure S2; Figure 1 d) and the detection limit was as low as 490 nM and 390 nM ($3\sigma/k$), respectively, for Compound A and HKOH-1. However, no such relationship was observed between fluorescence intensity enhancement and TCBQ/H_2O_2 concentration as the generation mechanism of ·OH from TCBQ/H_2O_2 is still not clear. In spite of this, the results confirm that our probes are highly sensitive towards ·OH.

The selectivity of Compound A and HKOH-1 was examined by measuring the response upon treatment against various analytes. As shown in Figure 1 f, Compound A displayed >7-fold and >40-fold increases in fluorescence intensity when treated with Fenton reagent and TCBQ/H_2O_2, respectively, over other ROS/RNS (HOCl, ONOO-, $O_2^{\cdot-}$, NO, $^1O_2$, ROO·, TBHP, and H_2O_2), $FeCl_2$, $FeCl_3$, and TCBQ alone (Supporting Information, Figure S2). HKOH-1 exhibited >20-fold and >100-fold increases in fluorescence intensity when treated with Fenton reagent and TCBQ/H_2O_2, respectively, over other ROS/RNS and analysts (Figure 1 f). Collectively our probes, especially HKOH-1, showed significantly

improved selectivity for ·OH over other ROS/RNS compared with previously reported ·OH probes.

For the reactions of Compound A and HKOH-1 with ·OH, the fluorescent products were confirmed to be fluorescein and dichlorofluorescein, respectively, by ESI-MS analysis (Supporting Information, Figure S3). Furthermore, the stability of HKOH-1 toward pH change was examined. As shown in the Supporting Information, Figure S4, HKOH-1 exhibited significant response across physiological pH range (6.0–8.5) when treated with Fenton reagent. This result implies the possibility of using HKOH-1 in detecting ·OH in biological systems.

A wide range of chemical entities possess ·OH scavenging abilities, for instance, DMSO,[11] thiourea,[12] glutathione (GSH),[13] *N*-acetylcysteine (NAC),[14] 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO)[15] and thyroid hormones,[16] such as triiodothyronine (T_3) and its prohormone, thyroxine (T_4), among which a great proportion are biologically relevant. However, a reliable and robust method to quantify their reactivity is currently lacking, impeding the understanding of various fundamental processes in chemical and biological systems. With our probe HKOH-1 in hand, calibration and quantification of the scavenging efficiency of reported scavengers would therefore be possible, allowing further investigation of the antioxidant capacity of cells. Considering that the Fenton reaction is widely accepted to be responsible for endogenous ·OH generation, we detected the response of HKOH-1 towards Fenton reagent in the presence of different concentrations of scavengers. The results shown in Figure 2 implicated that the order of scavenging efficiency could be described as follows: $T_4 > T_3 >$ TEMPO $>$ GSH $\approx$ thiourea $>$ NAC $\gg$ DMSO. The high efficiency of T_3 and T_4 was attributed to their direct reaction with ·OH as Compound A and HKOH-1 do. Thus we have successfully confirmed and compared the antioxidant activities of several representative ·OH scavengers in vitro with our highly sensitive probe HKOH-1.

The excellent performance of our probes, especially the HKOH-1, in chemical system prompted us to explore its biological applications. HKOH-1r was thus synthesized for better cellular uptake and retention by introducing a dimethyl diester group, which could be hydrolyzed in live cells by various esterases. Cell viability study revealed that HKOH-1r was not toxic even at 50 μM in both RAW264.7 mouse macrophages and HeLa cells (Supporting Information, Figure S5).

To explore the potential of HKOH-1r for ·OH detection in live cells, we first examined its performance in RAW264.7 mouse macrophages. PMA (phorbol myristate acetate), a protein kinase C (PKC) activator, was applied to induce endogenous ·OH. Cells were co-incubated with HKOH-1r (5 μM) and PMA (500 ng mL^{-1}) for 30 min. In contrast to the control group, a robust fluorescence signal enhancement was observed upon treatment with PMA (Figure 3a; Supporting Information, Figure S6). To confirm the fluorescence increase was a result of endogenous ·OH generation, we preloaded cells with 10 mM thiourea (a recognized hydroxyl radical scavenger; see above) for 30 min before treated with PMA. A remarkable decrease in fluorescence intensity was observed (Figure 3a; Supporting Information, Figure S6), which indicates that the alteration of fluorescence is a reflection of intracellular ·OH concentration change. Rapid time-lapse monitoring of intracellular ·OH with HKOH-1r confirmed that there was no sign of photoactivating and photobleaching (Supporting Information, Figure S7).

To detect endogenous ·OH quantitatively, we explored the application of HKOH-1r in flow cytometry. RAW264.7 cells were co-incubated with HKOH-1r (5 μM) with or without PMA (500 ng mL^{-1}) for 30 min, followed by flow cytometry analysis (Figure 3b,c). PMA treatment induced a significant fluorescence intensity

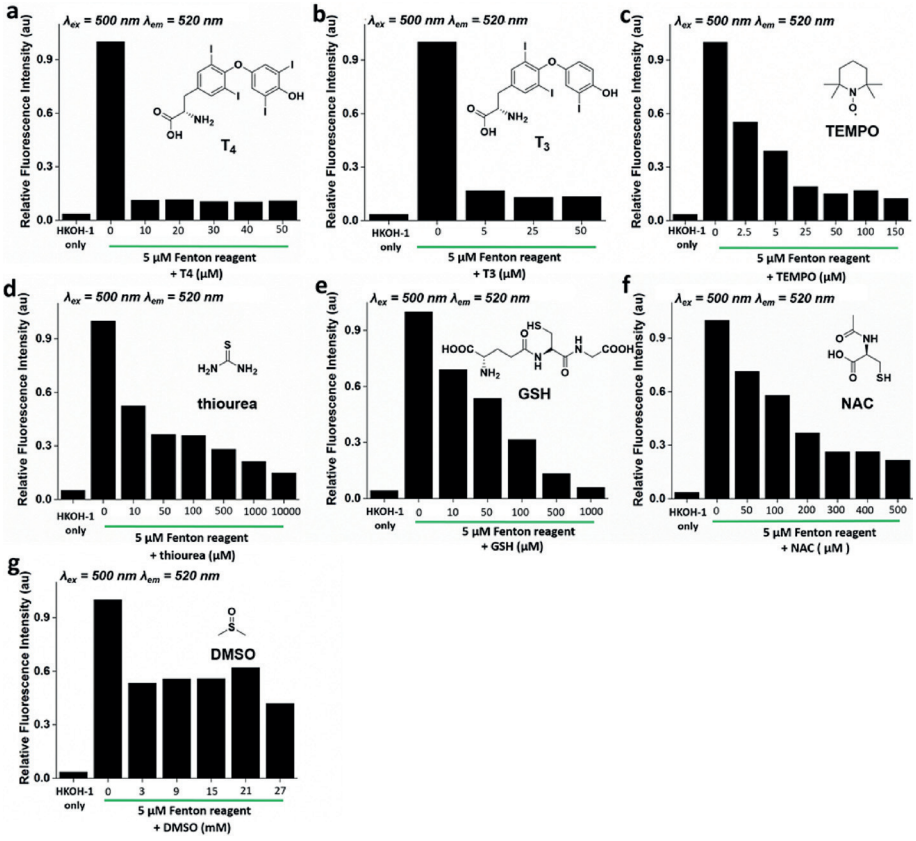

Figure 2. Investigating the ·OH scavenging ability of a) T_4; b) T_3; c) TEMPO; d) thiourea; e) GSH; f) NAC and g) DMSO with HKOH-1 (5 μM) in 0.1 M potassium phosphate buffer at pH 7.4. Hydroxyl radical source: 5 μM Fenton reagent. The fluorescence spectra were recorded at 30 min with an excitation at 500 nm and emission at 520 nm.

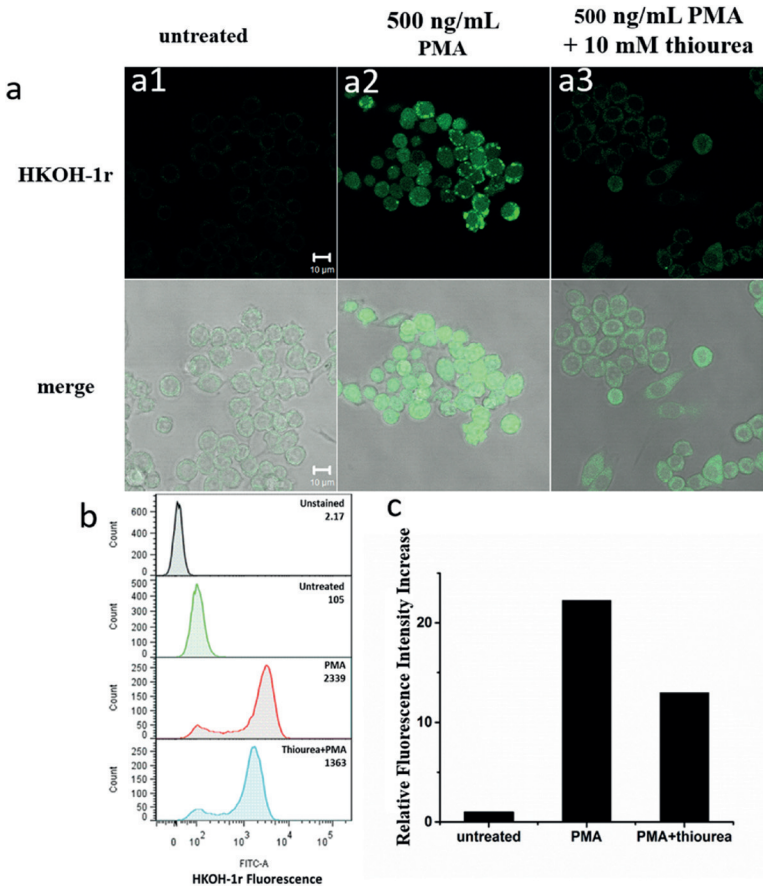

Figure 3. a) Detection of endogenous ·OH by HKOH-1r in RAW264.7 mouse macrophages in confocal imaging: a1) cells were incubated with HKOH-1r (5 μM) alone; a2) cells were co-incubated with HKOH-1r (5 μM) and PMA (500 ng mL^{-1}); a3) cells were pre-treated with thiourea (10 mM) for 30 min before PMA (500 ng mL^{-1}) stimulation. b),c) Detection of endogenous ·OH in flow cytometry. RAW264.7 cells were co-incubated with HKOH-1r (5 μM) in the presence or absence of thiourea for 30 min. The unstained and untreated group were taken as control. b) The histogram of fluorescence intensity changes towards PMA (500 ng mL^{-1}) stimulation and thiourea treatment. c) Relative fluorescence intensity increase calculated based on the left histogram. Results are representative of at least three independent experiments. Scale bar: 10 μm.

enhancement (> 20 fold) when compared with the untreated group. Another group of cells were preloaded with thiourea (10 mM) for 30 min, followed by PMA stimulation. The result showed that thiourea efficiently blunted the fluorescence increase. Overall, the FACS result showed that HKOH-1r could be employed to detect endogenous ·OH in the flow cytometry platform.

It is well-documented that UV irradiation from the sunlight generates ROS, especially highly reactive ·OH and ONOO$^-$ which induce skin damage and even skin cancers. According to previous reports, UVA (320–400 nm) radiation is more damaging than UVB (290–320 nm) to cells through generation of ROS and RNS as delayed biological processes.[17,18] Furthermore, the ·OH generated contributes to DNA degradation through both DNA base oxidation and DNA single strand breaks. However, the mechanism of ROS generation and downstream processes for each cell type is different. Combined with the use of our established ONOO$^-$ probe HKYellow-AM,[19] we successfully profiled simultaneous ·OH and ONOO$^-$ generation in HeLa cells. HKOH-1r and HKYellow-AM were preloaded in HeLa cells for 30 min and then the cells were exposed to UVA irradiation (365 nm) for different periods. Compared to the untreated control, robust fluorescence intensity enhancements were observed in both channels of the UV treatment group, and the fluorescence intensity increased with UV irradiation time, which suggests that both ·OH and ONOO$^-$ are generated in HeLa cells upon UVA exposure (Figure 4; Supporting Information, Figure S10). Meanwhile, ·OH scavenger thiourea and ONOO$^-$ scavenger urate obviously inhibited the formation of ·OH and ONOO$^-$, respectively. Hence, with HKOH-1r, endogenous ·OH generation in UV-irradiated cancer cells can be readily detected, and together with other probes like HKYellow-AM, we can further investigate UVA-related biological processes.

In summary, based on a selective O-dearylation reaction of 2,6-diiodophenol toward ·OH, we have developed a highly sensitive and selective probe HKOH-1 for monitoring ·OH formation in live cells, with > 30-fold and > 75-fold increase in fluorescence intensity upon treatment with Fenton reagent or TCBQ/H_2O_2, respectively. We have also successfully established the utility of HKOH-1r in confocal imaging and flow cytometry by detecting endogenous ·OH generated in PMA treated RAW264.7 cells. Moreover, HKOH-1r could be robustly applied in detecting ·OH generation in UVA irradiated HeLa cells. Therefore, our probe HKOH-1 is a powerful tool for the discovery of ·OH related biological processes.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: bioimaging · flow cytometry · fluorescent probes · hydroxyl radicals · live cells

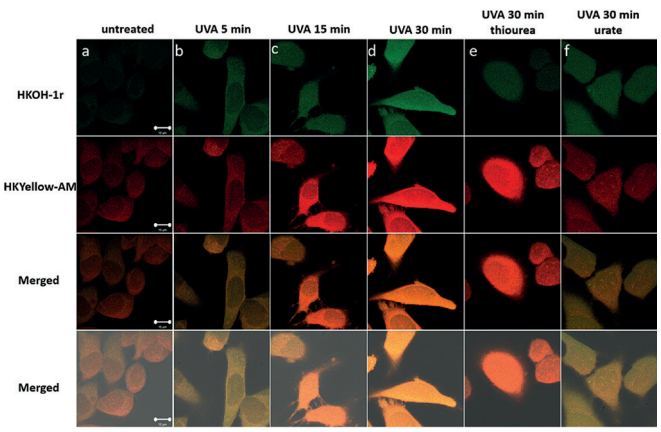

Figure 4. Detection of endogenous $^{\bullet}OH$ and $ONOO^{-}$ generation upon UVA irradiation by HKOH-1r and HKYellow-AM in HeLa cells. a) Cells were co-incubated with HKOH-1r (10 μM) and HKYellow-AM (10 μM) without further treatment. b)–d) Cells were incubated with HKOH-1r (10 μM) and HKYellow-AM (10 μM) for 30 min first and then washed with HBSS buffer, followed by exposure to UVA (365 nm) for different time periods: b) 5 min; c) 15 min; d) 30 min. e),f) Cells were pre-treated with thiourea (10 mM) or urate (100 μM) for 30 min before exposure to UVA. Scale bar: 10 μm. Results are representative of at least three independent experiments.

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