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# Double-ratiometric fluorescence imaging of H<sub>2</sub>Se and O<sub>2</sub><sup>•−</sup> under hypoxia for exploring Na<sub>2</sub>SeO<sub>3</sub>-induced HepG2 cells' apoptosis†

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Sodium selenite (Na<sub>2</sub>SeO<sub>3</sub>), as an anti-tumor drug for inducing tumor cells' apoptosis, has been widely studied under normoxic conditions and has been shown to exhibit oxidative stress process-induced apoptosis. However, since the real tumor environment is hypoxic, the actual mechanism is still unclear. Hence, considering the main metabolite of Na<sub>2</sub>SeO<sub>3</sub> in the metabolic process to be hydrogen selenide (H<sub>2</sub>Se) and also that it can be converted to superoxide anion (O<sub>2</sub><sup>•−</sup>) instantaneously in the presence of oxygen, a dual-ratiometric fluorescence imaging system for simultaneous monitoring of the changes of H<sub>2</sub>Se and O<sub>2</sub><sup>•−</sup> induced by Na<sub>2</sub>SeO<sub>3</sub>-guided tumor cell apoptosis under hypoxic conditions was constructed. Two molecular probes NIR-H<sub>2</sub>Se and dihydroethidium were used to detect H<sub>2</sub>Se and O<sub>2</sub><sup>•−</sup>, respectively, whereas Rhodamine 110 was used as the reference fluorophore. Notably, H<sub>2</sub>Se levels significantly increased under hypoxic conditions, but there was no change in the level of O<sub>2</sub><sup>•−</sup>, which is inconsistent with the results of the previous researches. Therefore, we hypothesize that the mechanism of Na<sub>2</sub>SeO<sub>3</sub>-induced apoptosis for tumor cells is caused by reductive stress; also, this method can be applied for the future study of other anti-cancer selenium compounds.

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## Introduction

Selenium,<sup>1,2</sup> as a common protective agent against cancer, can effectively reduce the morbidity and mortality of tumors including lung cancer, colorectal cancer, lymphoma, and prostate cancer.<sup>3–5</sup> The evidence from latent periods, clinical stages and epidemiology supports the chemopreventive role of selenium.<sup>6–8</sup> Therefore, sodium selenite (Na<sub>2</sub>SeO<sub>3</sub>) has recently become the research hotspot as an anti-cancer medicine due to its high efficiency, low toxicity and cost-effectiveness. However, the precise anti-cancer mechanism of how Na<sub>2</sub>SeO<sub>3</sub> can induce tumor cell apoptosis remains poorly understood. To date, research has been done on tumor cell cultures with Na<sub>2</sub>SeO<sub>3</sub> under normoxic conditions. It shows that the most generally accepted mechanism for Na<sub>2</sub>SeO<sub>3</sub> treatment is by cell apoptosis induced by elevating the reactive oxygen species (ROS) levels and the involvement of oxidative stress.<sup>9–12</sup> Specifically, hydrogen selenide (H<sub>2</sub>Se) is the key small molecule metabolite

of Na<sub>2</sub>SeO<sub>3</sub> *in vivo*, and it reacts with O<sub>2</sub> to produce and accumulate a large amount of ROS (mainly the superoxide anion, O<sub>2</sub><sup>•−</sup>), resulting in tumor cell oxidative stress, which leads to apoptosis.<sup>13–16</sup> Nevertheless, since the tumor microenvironment is hypoxic (0.7–1.8% O<sub>2</sub>)<sup>17</sup> due to the rapid proliferation of cancer cells and imbalance of new blood vessel formation,<sup>18–20</sup> it is still a great challenge to understand the anti-cancer mechanism in hypoxic conditions, which needs to be solved urgently.

To clarify the mechanism, fluorescent probes, which are promising detection tools<sup>21</sup> due to their non-invasiveness, high sensitivity, selectivity, rapid response, and high spatial resolution,<sup>22–28</sup> are the primary option. Unfortunately, these fluorescent probes are limited by environmental conditions, probe distribution, and instrument performance, thereby only showing changes in emission intensities.<sup>29,30</sup> In contrast, ratio-metric probes, using the ratio of two different emission wavelengths as the detection signal, provide a built-in correction to the above factors for a more accurate analysis.<sup>31–34</sup>

In this regard, a double-ratiometric method for simultaneous fluorescence imaging analysis to monitor the changes in H<sub>2</sub>Se and O<sub>2</sub><sup>•−</sup> levels in tumor cells was developed. The method studies the apoptosis process induced by pharmacological doses of Na<sub>2</sub>SeO<sub>3</sub> in hypoxic conditions. Herein, we chose the fluorescence probe (NIR-H<sub>2</sub>Se, λ<sub>ex</sub>/λ<sub>em</sub> = 688/735 nm) designed by our group<sup>35</sup> and the commercial fluorescence probe (dihydroethidium, λ<sub>ex</sub>/λ<sub>em</sub> = 370/420 nm)<sup>36,37</sup> to monitor the changes in intracellular H<sub>2</sub>Se and O<sub>2</sub><sup>•−</sup> concentrations in both normoxic

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conditions and hypoxic conditions, respectively. Meanwhile, Rhodamine 110 ( $\lambda_{\text{ex}}/\lambda_{\text{em}} = 496/532 \text{ nm}$ ) was chosen as a reference fluorophore. This method could potentially avoid the influences of certain external factors on detection accuracy including environmental conditions, light scattering and probe distribution, therefore improving the detection accuracy of the target molecules *in vivo*.<sup>38,39</sup>

## Experimental

### Materials

All chemicals were analytical-reagent grade and all solvents were purified by a conventional method before use. Rhodamine 110 was purchased from J&K (China). The NIR-H<sub>2</sub>Se probe was synthesized in our laboratory following a previous protocol.<sup>35</sup> Dihydroethidium (DHE) and KO<sub>2</sub> were purchased from Aladdin (China). Stock solutions (1 mM) for probes were prepared by dissolving probe in DMSO (Sigma-Aldrich). Sartorius ultrapure water (18.2 M $\Omega$  cm) was used throughout the experiments. H<sub>2</sub>Se was prepared by reacting Al<sub>2</sub>Se<sub>3</sub> with H<sub>2</sub>O under an N<sub>2</sub> atmosphere for 30 min at room temperature before use every time.<sup>40,41</sup> O<sub>2</sub><sup>•−</sup> was generated from KO<sub>2</sub> in DMSO or xanthine oxidase. Na<sub>2</sub>SeO<sub>3</sub> was purchased from Sigma-Aldrich. Dulbecco's Modified Eagle Medium (DMEM) was obtained from British Biotechnology (China). HepG2 cells were purchased from the Committee on Type Culture Collection of the Chinese Academy of Sciences.

### Instruments

The fluorescence spectra measurements were performed with an FLS-920 Fluorescence Spectrometer (Edinburgh, UK). The fluorescence imaging of cells was recorded on a TCS SP5 confocal laser scanning microscope with an objective lens ( $\times 40$ ) (Leica, Germany). The cells were cultured in a 5% CO<sub>2</sub>/95% air incubator MCO-15 AC (Sanyo, Japan).

### Fluorescence spectra measurements

Ten  $\mu\text{M}$  probe NIR-H<sub>2</sub>Se was mixed with 5  $\mu\text{M}$  probe DHE, 1  $\mu\text{M}$  Rhodamine 110 and 10 mM PBS (pH = 7.4); then, various amounts of H<sub>2</sub>Se (0, 1, 2, 4, 6, 8, 9, 10, 11  $\mu\text{M}$ ) were added. The fluorescence intensities were measured at  $\lambda_{\text{ex}}/\lambda_{\text{em}} = 496/532 \text{ nm}$  and  $\lambda_{\text{ex}}/\lambda_{\text{em}} = 688/735 \text{ nm}$ . For a parallel study, 5  $\mu\text{M}$  probe DHE was mixed with 10  $\mu\text{M}$  probe NIR-H<sub>2</sub>Se, 1  $\mu\text{M}$  Rhodamine 110 and 10 mM PBS (pH = 7.4) and then, various amounts of O<sub>2</sub><sup>•−</sup> (0, 1, 2, 3, 4, 5, 6, 7, 8  $\mu\text{M}$ ) were added for fluorescence measurements at  $\lambda_{\text{ex}}/\lambda_{\text{em}} = 496/532 \text{ nm}$  and  $\lambda_{\text{ex}}/\lambda_{\text{em}} = 488/638 \text{ nm}$ .

### Cell culture

HepG2 cells in the exponential phase of growth were used for the cell experiments. The concentrations of the counted cells were adapted to  $1 \times 10^6$  cells per mL for confocal fluorescence imaging with a culture medium. The cells were cultured with DMEM and supplemented with 1% antibiotics (100 U mL<sup>−1</sup> penicillin and 100 mg mL<sup>−1</sup> streptomycin) and 10% fetal bovine serum (FBS) at 37 °C under a humidified atmosphere of 5%

CO<sub>2</sub>/95% air (20% O<sub>2</sub>), 75% CO<sub>2</sub>/25% air (5% O<sub>2</sub>) and 95% CO<sub>2</sub>/5% air (1% O<sub>2</sub>) for normoxic and hypoxic conditions in an MCO-15AC incubator, respectively.

### Confocal fluorescence imaging

HepG2 cells were prepared in glass-bottom culture dishes and cultured for 24 h. One group of cells without treatment of Na<sub>2</sub>SeO<sub>3</sub> served as the control group and the other groups were treated with various amounts of Na<sub>2</sub>SeO<sub>3</sub> (2, 5, 10  $\mu\text{M}$ ) for 12 h under either normoxic or hypoxic conditions. All groups were further incubated in an FBS-free culture medium mixed with 10  $\mu\text{M}$  probe NIR-H<sub>2</sub>Se, 5  $\mu\text{M}$  probe DHE and 1  $\mu\text{M}$  Rhodamine 110 at 37 °C for 15 min and then washed with PBS (pH = 7.4, 10 mM) three times. Afterwards, the cells were imaged immediately using a confocal microscope with an objective lens ( $\times 40$ ) and examined by CLSM with different laser transmitters. The emitted light intensities were collected with an META detector from 490 to 550 nm, 590 to 670 nm and 690 to 760 nm.

## Results and discussion

### Spectroscopic properties of NIR-H<sub>2</sub>Se with H<sub>2</sub>Se, DHE with O<sub>2</sub><sup>•−</sup> and Rhodamine 110

The excitation and emission spectra of NIR-H<sub>2</sub>Se with H<sub>2</sub>Se, DHE with O<sub>2</sub><sup>•−</sup> and Rhodamine 110 were recorded (Fig. S1†). Interestingly, the emission spectra of the reaction products of NIR-H<sub>2</sub>Se with H<sub>2</sub>Se, DHE plus O<sub>2</sub><sup>•−</sup>, and Rhodamine 110 are located at 735, 638 and 532 nm, respectively. Therefore, the emission spectra of Rhodamine 110 and the two reaction products exhibit a fairly ideal separation effect with approximately 100 nm between each peak, making their simultaneous detection possible (Fig. 1).

### Ratiometric detection

The fluorescence responses of DHE to H<sub>2</sub>Se and NIR-H<sub>2</sub>Se to O<sub>2</sub><sup>•−</sup> were tested by using the mixture of NIR-H<sub>2</sub>Se, DHE and Rhodamine 110 to simultaneously monitor the levels of H<sub>2</sub>Se and O<sub>2</sub><sup>•−</sup>. As shown in Fig. 2a, O<sub>2</sub><sup>•−</sup> (5  $\mu\text{M}$ ) yielded a strong fluorescence signal with DHE (5  $\mu\text{M}$ ), but high concentrations

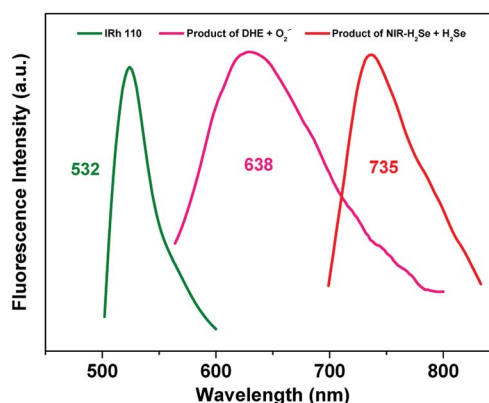


Fig. 1 Fluorescence emission spectra of (red) NIR-H<sub>2</sub>Se with H<sub>2</sub>Se, (pink) DHE with O<sub>2</sub><sup>•−</sup> and (green) Rhodamine 110.



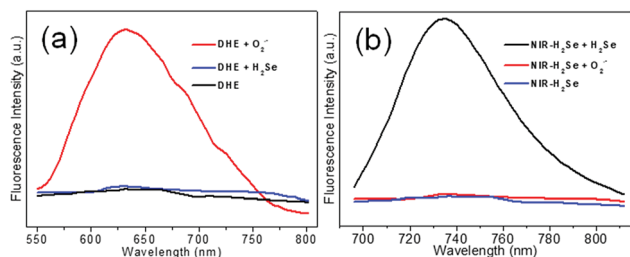


Fig. 2 Interference measurement of (a) DHE to H<sub>2</sub>Se and (b) NIR-H<sub>2</sub>Se to O<sub>2</sub><sup>•-</sup>. All of the spectra were acquired at room temperature in 10 mM PBS (pH = 7.4) with (a)  $\lambda_{\text{ex}}/\lambda_{\text{em}} = 488/638$  nm or (b)  $\lambda_{\text{ex}}/\lambda_{\text{em}} = 688/735$  nm.

of H<sub>2</sub>Se (15  $\mu\text{M}$ ) did not exhibit a fluorescence response. Similarly, the fluorescence response of NIR-H<sub>2</sub>Se (10  $\mu\text{M}$ ) to O<sub>2</sub><sup>•-</sup> (10  $\mu\text{M}$ ) was negligible, but a strong fluorescence signal was obtained with 10  $\mu\text{M}$  H<sub>2</sub>Se (Fig. 2b). Then, the optical properties of the probe mixtures with Rhodamine 110 were recorded with various concentrations of H<sub>2</sub>Se in simulated physiological conditions (10 mM PBS, pH = 7.4). The fluorescence intensities increased with increasing concentrations of H<sub>2</sub>Se at 735 nm (Fig. 3a) but hardly fluctuated at 532 nm (Fig. 3b, black curve, right Y scale). Interestingly, there is a good linear correlation between the fluorescence intensity ratio  $F_{735}/F_{532}$  and H<sub>2</sub>Se concentrations from 0 to 11.0  $\mu\text{M}$  (Fig. 3b, red curve, left Y scale). The equation of linear regression is  $F_{735}/F_{532} = 0.23 + 0.17 [\text{H}_2\text{Se}] \mu\text{M}$  with the  $R$  value of 0.9937, indicating that the fluorescence intensity ratio  $F_{735}/F_{532}$  increased with increasing concentrations of H<sub>2</sub>Se linearly. Simultaneously, similar

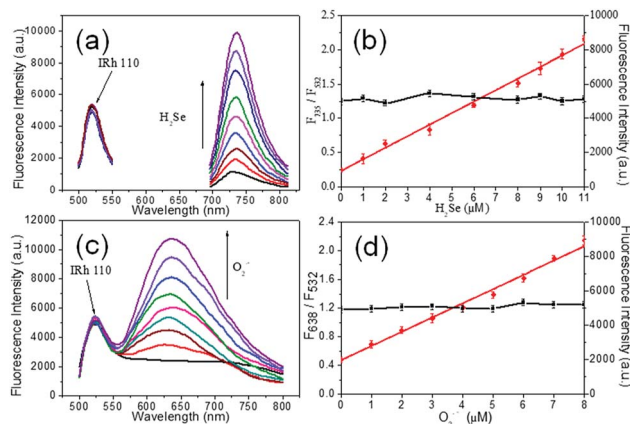


Fig. 3 Ratiometric detection with the mixture of 10  $\mu\text{M}$  NIR-H<sub>2</sub>Se, 5  $\mu\text{M}$  DHE and 1  $\mu\text{M}$  Rhodamine 110 toward H<sub>2</sub>Se and O<sub>2</sub><sup>•-</sup>. (a) Fluorescence response of the mixture to various concentrations (0 to 11.0  $\mu\text{M}$ ) of H<sub>2</sub>Se. (b) The linear correlation between the fluorescence intensity ratio  $F_{735}/F_{532}$  and H<sub>2</sub>Se concentrations (red) and fluorescence intensities changes of Rhodamine 110 to H<sub>2</sub>Se concentrations (black). (c) Fluorescence response of the mixture to various concentrations (0 to 8.0  $\mu\text{M}$ ) of O<sub>2</sub><sup>•-</sup>. (d) The linear correlation between the fluorescence intensity ratio  $F_{638}/F_{532}$  and O<sub>2</sub><sup>•-</sup> concentrations (red) and changes in the fluorescence intensity of Rhodamine 110 to O<sub>2</sub><sup>•-</sup> concentrations (black). The data shown are representative of repeating experiments ( $n = 3$ ).

fluorescence responses of the mixture towards O<sub>2</sub><sup>•-</sup> under various concentrations of O<sub>2</sub><sup>•-</sup> in simulated physiological conditions (10 mM PBS, pH = 7.4) were also obtained (Fig. 3c); the fluorescence intensity was significantly enhanced *via* increasing O<sub>2</sub><sup>•-</sup> concentrations at 638 nm but was maintained at 532 nm (Fig. 3d, black curve, right Y scale). Also, a linear correlation between the fluorescence intensity ratio  $F_{638}/F_{532}$  and O<sub>2</sub><sup>•-</sup> concentrations from 0 to 8.0  $\mu\text{M}$  (Fig. 3d, red circles, left Y scale) was observed. The equation of linear regression is  $F_{638}/F_{532} = 0.47 + 0.20 [\text{O}_2^{\bullet-}] \mu\text{M}$  with the  $R$  value of 0.9923, indicating that the fluorescence intensity ratio of  $F_{638}/F_{532}$  increased linearly with increasing concentrations of O<sub>2</sub><sup>•-</sup>. Overall, the results demonstrated that it is entirely feasible to use NIR-H<sub>2</sub>Se and DHE for simultaneously monitoring the changes in H<sub>2</sub>Se and O<sub>2</sub><sup>•-</sup> in a complex system.

### Co-staining imaging in living cells

To further test the simultaneous imaging of H<sub>2</sub>Se and O<sub>2</sub><sup>•-</sup> *in vitro*, co-staining experiments were carried out. Since Na<sub>2</sub>SeO<sub>3</sub> metabolite H<sub>2</sub>Se is mainly accumulated in the liver, the cell model HepG2 was selected for the following study. HepG2 cells were first incubated in FBS-free culture medium mixed with 10  $\mu\text{M}$  NIR-H<sub>2</sub>Se, 5  $\mu\text{M}$  DHE and 1  $\mu\text{M}$  Rhodamine 110 at 37  $^{\circ}\text{C}$  for 15 min and then, confocal fluorescence images were obtained. Fig. 4 displays the distribution of Rhodamine 110 (green), the product of DHE with native O<sub>2</sub><sup>•-</sup> of HepG2 cells (yellow), the product of NIR-H<sub>2</sub>Se with H<sub>2</sub>Se (red), and the merged image. The two probes and the reference achieved excellent spectral separation in the cell as well as great biocompatibility. The two probes' reaction products and the reference molecular structure all contain two amino groups, making the degree of impact by the intracellular complex environment almost the same, which further improved the sensitivity of the detection. Hence, the mixture of NIR-H<sub>2</sub>Se, DHE and Rhodamine 110 can be used to simultaneously recognize H<sub>2</sub>Se and O<sub>2</sub><sup>•-</sup> in living cells.

### Double-ratiometric simultaneous detection under normoxia and hypoxia

For the next step, the probe mixture was applied to explore its application for double-ratiometric, simultaneous imaging of endogenous H<sub>2</sub>Se and O<sub>2</sub><sup>•-</sup> in HepG2 cells pre-treated with Na<sub>2</sub>SeO<sub>3</sub> under normoxic (20% O<sub>2</sub>) and hypoxic (5% O<sub>2</sub>, 1% O<sub>2</sub>)

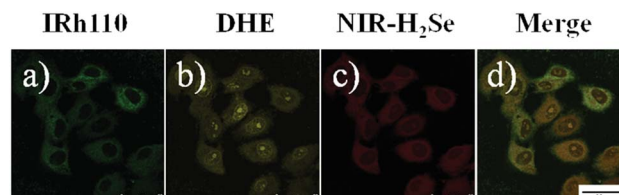


Fig. 4 Co-staining images of HepG2 cells incubated with FBS-free culture medium mixed with 1  $\mu\text{M}$  Rhodamine 110, 5  $\mu\text{M}$  DHE and 10  $\mu\text{M}$  NIR-H<sub>2</sub>Se at 37  $^{\circ}\text{C}$  for 15 min. The emitted light was collected with an META detector from 490 to 550 nm, 590 to 670 nm and 690 to 760 nm. (a) Green channel. (b) Yellow channel. (c) Red channel. (d) Merge of (a), (b) and (c). Scale bar = 50  $\mu\text{m}$ .





conditions. One group of HepG2 cells without treatment of  $\text{Na}_2\text{SeO}_3$  served as the control group, and the other groups were treated with  $\text{Na}_2\text{SeO}_3$  (2, 5, 10  $\mu\text{M}$ ) for 12 h under either normoxia (20%  $\text{O}_2$ ) or hypoxia (5%  $\text{O}_2$ , 1%  $\text{O}_2$ ). Afterwards, the cells were incubated in an FBS-free culture medium mixed with 10  $\mu\text{M}$  NIR- $\text{H}_2\text{Se}$ , 5  $\mu\text{M}$  probe DHE and 1  $\mu\text{M}$  Rhodamine 110 at 37  $^\circ\text{C}$  for 15 min. The ratiometric images of  $\text{H}_2\text{Se}$  were achieved by combining the images obtained in the band path of 490–550 nm with the corresponding images in the band path of 690–760 nm. Meanwhile, the concentration of  $\text{O}_2^{\cdot-}$  was also obtained by combining the images obtained in the band path of 490–550 nm with the corresponding images in the band path of 590–670 nm (Fig. 5 and S2–S4†). Fig. 5a shows that under different oxygen concentrations, the concentration of  $\text{H}_2\text{Se}$  in HepG2 cells increased by the  $\text{Na}_2\text{SeO}_3$  concentration. From the quantitative map, it can be seen that with the decrease in oxygen concentration and increase in  $\text{Na}_2\text{SeO}_3$  concentration, the  $\text{H}_2\text{Se}$  content gradually increased. At 1%  $\text{O}_2$ , the concentration of  $\text{H}_2\text{Se}$  in the HepG2 cells was relatively high with 10  $\mu\text{M}$   $\text{Na}_2\text{SeO}_3$  (Fig. 5b). Consequently, under different oxygen concentrations, the content of  $\text{O}_2^{\cdot-}$  in the HepG2 cells increased while increasing  $\text{Na}_2\text{SeO}_3$  since the increase of oxygen and  $\text{Na}_2\text{SeO}_3$  led to gradually rising  $\text{O}_2^{\cdot-}$  levels (Fig. 5c). At 20%  $\text{O}_2$ , the concentration of  $\text{O}_2^{\cdot-}$  in the HepG2 cells was relatively high with 10  $\mu\text{M}$   $\text{Na}_2\text{SeO}_3$  incubation, but the content of  $\text{O}_2^{\cdot-}$  in the HepG2 cells remained unchanged under the condition of 1%  $\text{O}_2$  (Fig. 5d).

In this regard, higher  $\text{H}_2\text{Se}$  contents were observed in hypoxic environments rather than in normoxic conditions in parallel experiments. Correspondingly, lower  $\text{O}_2^{\cdot-}$  contents were maintained and they hardly changed under hypoxic environments, whereas  $\text{O}_2^{\cdot-}$  contents gradually increased in the

HepG2 cells with  $\text{Na}_2\text{SeO}_3$  in a time- and dose-dependent manner under normoxic conditions. These results indicate that the  $\text{Na}_2\text{SeO}_3$  anticancer mechanism is not an ROS-induced apoptosis process under hypoxic conditions. Thus, the anti-cancer effect of  $\text{Na}_2\text{SeO}_3$  in solid tumors should be reductive stress to trigger cell death of cancer cells.

## Conclusions

In summary, we developed a novel double-ratiometric fluorescence probe system for simultaneous fluorescence imaging analysis of the changes in  $\text{H}_2\text{Se}$  and  $\text{O}_2^{\cdot-}$  levels in the tumor cell HepG2's apoptosis process induced by pharmacological doses of  $\text{Na}_2\text{SeO}_3$ . The system verifies that the content of  $\text{O}_2^{\cdot-}$  is clearly higher in the tumor cell apoptosis process induced by  $\text{Na}_2\text{SeO}_3$  in normoxic conditions, and  $\text{H}_2\text{Se}$  is almost completely oxidized by  $\text{O}_2$  to produce  $\text{O}_2^{\cdot-}$ . In contrast, under hypoxic conditions, the  $\text{O}_2^{\cdot-}$  content is extremely low, but the  $\text{H}_2\text{Se}$  content increases dramatically. Therefore, in solid tumors, the anti-cancer mechanism of  $\text{Na}_2\text{SeO}_3$  can be ascribed to reductive stress induced by  $\text{H}_2\text{Se}$  but not oxidative stress. This finding will significantly improve the investigation of the anti-cancer mechanism of  $\text{Na}_2\text{SeO}_3$ , and the double-ratiometric fluorescence method can be used as a suitable tool for the study of other selenium compounds in the future.

## Conflicts of interest

There are no conflicts to declare.

## Acknowledgements

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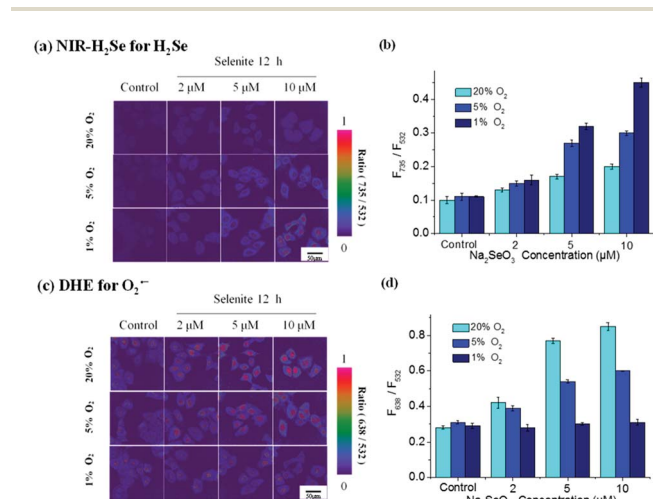


Fig. 5 Confocal fluorescence double-ratiometric images of endogenous  $\text{H}_2\text{Se}$  and  $\text{O}_2^{\cdot-}$  in living HepG2 cells treated with various concentrations of  $\text{Na}_2\text{SeO}_3$  under normoxic and hypoxic conditions. (a) The fluorescence ratio image ( $F_{735}/F_{532}$ ). (b) The corresponding statistic fluorescence ratio ( $F_{735}/F_{532}$ ) for (a). (c) The fluorescence ratio image ( $F_{638}/F_{532}$ ). (d) The corresponding statistic fluorescence ratio ( $F_{638}/F_{532}$ ) for (c). The images shown are representative of repeated experiments ( $n = 3$ ). Scale bar = 50  $\mu\text{m}$ .



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