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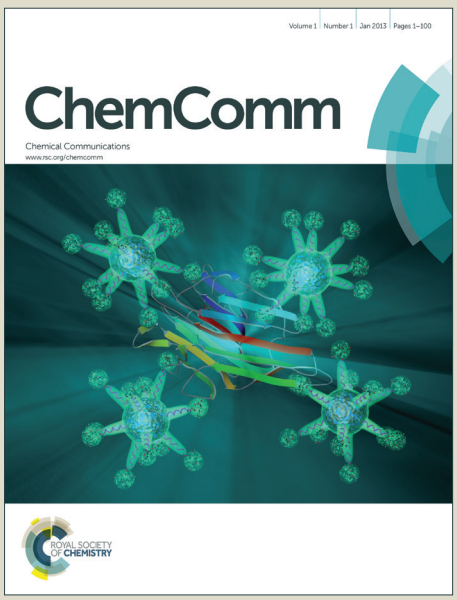

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ARTICLE TYPE

Fluorescence Imaging for Selenol in HepG2 Cell Apoptosis Induced by Na_2SeO_3

Fanpeng Kong[a], Bo Hu[a], Yan Gao[a] , Kehua Xu*[a], Xiaohong Pan[a], Fang Huang[a], Qiuling Zheng[b], Hao Chen[b] and Bo Tang*[a]

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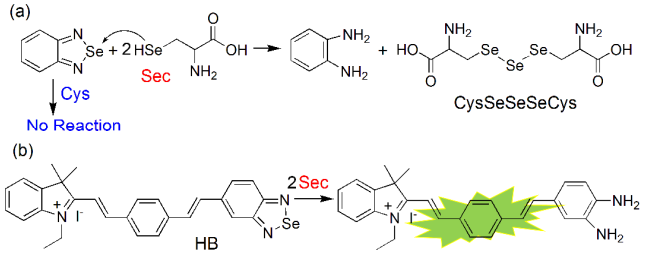
A novel fluorescence probe (HB) has been designed and synthesized to image selenol in living cells and *in vivo* for the first time, and been used to investigate the Na_2SeO_3 anticancer mechanism in HepG2 cells.

Selenium (Se) is an essential trace element involved in different physiological functions of the human body.[1] More importantly, the protective ability of Se against cancer was suggested in 1969 with the discovery of an inverse relationship between cancer occurrence and Se content of local crop in the USA.[2] There are several proposed mechanisms to explain the effect of Se in cancer prevention and treatment, such as apoptosis, autophagy, necrosis, mitotic catastrophe or combinations thereof.[3,4] But the exactly mechanism of Se compounds for anticancer property remains still unclear. It is suggested that the efficacy of Se in cancer prevention and treatment is dependent on the chemical speciation of Se.[2,5] Thus there is a clear need to identify different Se species *in vivo* in order to investigate their biological functions. The low molecular weight selenium compounds, such as methylselenol[6a,6b] and selenocysteine (Sec) are the key metabolites and found to be important in cancer prevention.[6c-6d] Especially, Sec as an important low molecular weight selennium-containing amino acid, is located in the active sites of selenoproteins (SePs) and encoded by a UGA stop codon.[7] Sec is also the metabolic product of Na_2SeO_3 and plays an important role in cancer treatment. In order to investigate the physiological function of Sec and clarify the anticancer mechanism of selenium, there is a pressing need to develop a method to monitor Sec in living cells and *in vivo*.

Fluorescence probes with high sensitivity, good selectivity, short response time, and advantage of direct observation, are powerful tool for the identification of substances in biologial systems.[8-11] Up to now, very few fluorescence probes for detecting the Sec or selenoprotein[12] have been reported. In 2006, Maeda *et al*. reported a fluorescent probe BESThio to discriminate between Sec and Cys, based on the difference that the selenol group of Sec exists in the more-reactive, ionized form, whereas the thiol group of Cys is protonated and less reactive at pH 5.8.[13] Unfortunately, it was not suitable for detecting or imaging Sec at physiologic conditions. So, it is still a challenge to develop a fluorescence method to detect Sec in living cells and tissues to gain insight into the physiological functions of Sec and the inhibitory mechanism of Se to tumor cells.

In our previous work, we have found that the Se-N bond involved in ebselen can be cleaved by sulfhydryl group with high selectivity, and developed some fluorescence methods to monitor intracellular thiols and the changes of redox state.[14-16] 2,1,3-benzoselenadiazole (BS) is also a containing Se-N compound, but the Se-N bond should be stronger than that of ebselen, which has been confirmed by calculating the Wiberg bond indices (WBI) of BS and ebselen in Figure S1. The result shows that the WBI of Se-N in BS is larger than that of ebselen (1.145 vs 0.832), indicating that BS has less reaction activity than ebselen in the presence of nucleophilic reagent. Thus we can assume that it needs a stronger nucleophilic reagent to cleave the Se-N bond in BS. Maybe, BS could discriminate between selenol and thiol, because selenol group (-SeH) has higher nucleophilicity than thiol group (-SH) does.[17,18] Soon afterwards, we tried the idea and found that BS can indeed react rapidly with Sec, but not with Cys, in 0.01 M, pH 7.4 phosphate buffer solution (PBS), as shown in Figure S3-S4. Based on this, we designed a novel fluorescent probe, 2-(benzoselenadiazol-5-yl)vinyl)styryl)-1-ethyl-3,3-dimethyl-indolium iodide (HB), for probing intracellular Sec in living cells and *in vivo* (Scheme 1). The probe shows highly selective and sensitive to Sec in physiological conditions and is able to monitor the changes of selenol level in living cells and *in vivo*. Furthermore, we have observed that selenol concentrations increase gradually in HepG2 cells induced by Na_2SeO_3 in a time- and dose-dependent manner, with accompanying tumor cell apoptosis, cell cycle arrest and depletion of mitochondrial membrane potential.



Scheme 1. (a) A novel reporter group for Sec; (b) Design strategy of fluorescent probe (HB)

The fluorescence probe (HB) was synthesized by integrating the BS moiety into a hemicyanine dye via Knoevenagel condensation and Wittig reaction in moderate yields (Scheme S1), and the

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structure of HB was confirmed by ^{1}H NMR, ^{13}C NMR and HRMS (see ESI). Here, a hemicyanine dye was chosen, not only because its positive charge can improve the reaction of HB with Sec and increase the water solubility of HB, but also because an obvious change in fluorescence intensity before and after addition of Sec into the probe solution may be observed. That is, when the BS moiety in HB is transformed into diamine product, the double electron-acceptor structure of HB will become an electron-donor and electron-acceptor form of product, which would be very beneficial to emit a bright fluorescent signal. The reaction products of HB and Sec were fully characterized by mass spectrometry and the collision-induced dissociation (CID) MS^2 spectrum. (Figure S5).

The interaction between HB and Sec was first studied in phosphate buffer (10 mM, pH 7.4, 1% DMSO) at room temperature by emission spectroscopy. Excitation and emission spectra were recorded during the reaction of HB with Sec (Figure S6). HB shows an emission of 580 nm with a large Stokes shift of 120 nm. Optimal conditions of the response of HB to Sec were worked out as shown in Figure S7, including pH and concentration of HB. In order to apply in biological system, physiological pH (7.4) was selected for the following determination system. A gradual increase in the fluorescence intensity was observed with adding different concentrations of Sec (The details of preparing the stock solution of Sec see ESI part 3) into 10 μM of probe solution under optimal conditions (Figure 1a), and the fluorescence quantum yield increased from 0.019 to 0.110 upon addition of Sec to HB. There was a good linearity between the fluorescence intensities and Sec concentrations in the range 0-20 μM (Figure 1b). The regression equation was F = 104.9 + 14.7 [Sec] μM with a linear coefficient of 0.9942. The limit of detection was 7.0 nM (standard deviation 3.5%, n = 11).

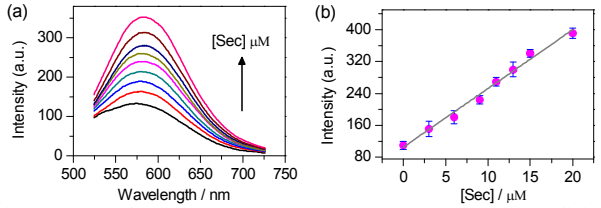

Fig. 1 (a) Fluorescence responses of 10 μM HB to different concentrations of Sec (0, 3, 6, 9, 11, 13, 15, 20 μM). (b) A linear correlation between emission intensities and concentrations of Sec. All spectra were acquired in 10 mM PBS buffer with pH 7.4 at 37°C (λ_{ex} /λ_{em} = 460/ 580 nm).

Furthermore, a fast response of HB to Sec was confirmed by a kinetics experiment, in which the fluorescence intensity instantly increased by adding Sec into the probe solution and the intensity was kept on a relative stable level for at least 30 min, as shown in Figure 2a.

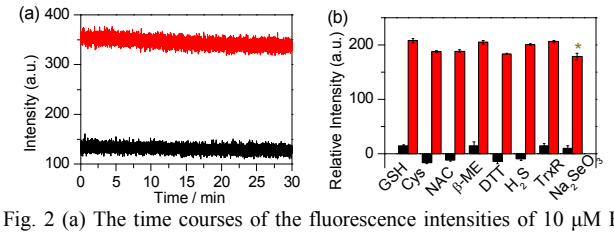

Fig. 2 (a) The time courses of the fluorescence intensities of 10 μM HB with 20 μM Sec (red line) and without Sec (black line). Sec was generated from 10 μM of $(CysSe)_2$ and 20 μM of Cys in H_2O for 30 min. (b) The relative fluorescence responses of 10 μM HB to thiols and other sulfur compounds (10 mM for GSH, 2.5 mM for Cys and NAC, 1.0 mM for β-ME, 20 μM for DTT, 50 μM for H_2S, 10 μM for Na_2SeO_3, and 2.5 μg/mL for TrxR. Black bars represent the addition of one of these interferences to a 10 μM solution of HB. Red bars represent the addition of Sec plus one of these interferences to the probe solution (*Sec was replaced by 60 μM of GSH). All data were acquired in 10 mM PBS buffer with pH 7.4 at 37°C (λ_{ex} /λ_{em} = 460/ 580 nm).

To study the selectivity of HB toward Sec, we tested its responses toward thiols, thioredoxin reductase (TrxR), N-acetyl-L-cysteine (NAC), hydrogen sulfide (H_2S), Na_2SeO_3 and a mixture of Na_2SeO_3 and GSH. The reducing reagent beta-mercaptoethanol (β-ME), dithiothreitol (DTT), Cys and glutathione (GSH) were examined as the thiols. The addition of Cys is essential not only because its content is rich in living cells but also because Sec is generated from $(CysSe)_2$ and Cys. As expected, HB exhibited an obvious increase in fluorescence intensity upon reaction with Sec or a mixture of Na_2SeO_3 and GSH (GSeH was generated[19]), whereas the responses toward thiols and others, or even TrxR were negligible (Figure 2b). One probable reason for no response of HB to TrxR is that the Sec residues in the SePs are buried too deeply to react with HB (Figure S8). In addition, the interference from metal ions, reactive oxygen species (ROS), amino acids and vitamin C were also studied, as shown in Figure S8. HB displayed no fluorescent response toward the above-mentioned bioanalysts (An error of < ± 5.0% in the relative fluorescence intensity was considered tolerable).

We applied HB to visualize the intracellular Sec. Cells were divided into four groups. One group was chosen as control, and the other three groups were treated with Cys, selenocystine $(CysSe)_2$, and Sec generated by $(CysSe)_2$ plus Cys, respectively. Then the in situ changes of fluorescence were real-timely observed. Figure 3a-b showed that the Sec-treated cells emitted immediately bright fluorescence, while the fluorescence signal derived from the $(CysSe)_2$-treated cells increased gradually until 1.5 h. The result reveals that the probe could monitor the dynamic changes of Sec level, and $(CysSe)_2$ could be transformed into Sec in living cells. We next assessed the ability of HB to image Sec in a mouse model[20] (Figure S12), and the results indicated that the probe was capable of detecting Sec *in vivo*.

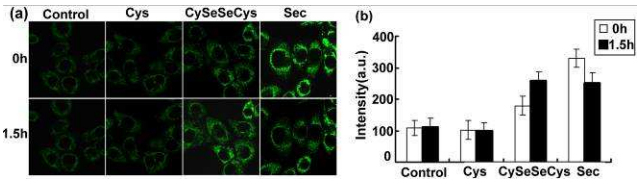

Fig. 3 (a) The in situ fluorescence changes of HB (10 μM) response to Sec with time in HepG2 cells. HepG2 cells were treated with 20 μM of Cys, 20 μM of $(CysSe)_2$, and Sec derived from 10 μM of $(CysSe)_2$ and 20 μM of Cys for 10 min, respectively. The fluorescence changes of cells were detected in 0 h and 1.5 h under confocal microscopy. (b) The fluorescence intensity was quantified based on the results of the relative fluorescence intensity of per cell in the scanned area.

Sodium selenite (Na_2SeO_3) is often used as anticancer reagents in cancer treatment.[21,22] However, how Na_2SeO_3 intersects with cancer development and therapeutic response is controversial[3,4]. Because Sec is the important metabolic product of Na_2SeO_3, it

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would be beneficial to reveal the exactly anticancer mechanism of Na_2SeO_3 by investigating the change of selenol level in vivo and the relationship between Sec and apoptosis or cell cycle. Firstly, we have real-timely observed the difference of selenol concentrations between normal cells and cancer cells response to sodium selenite, as shown in Figure 4a. HepG2 and HL-7702 cells were exposed to 5 μM of sodium selenite for different time (0-24 h) and then were cultured with the probe for 15 min. The nuclei were stained by DAPI to show apoptosis process. A further experiment was carried out with different concentrations of sodium selenite (0-10 μM) for 12 h in Figure 4b. These results show that the concentrations of selenol in HepG2 cells are higher than those of HL-7702 cells under the same experimental conditions, and the apoptosis appears in HepG2 cells induced by 5 μM of sodium selenite after 24 h or 10 μM of sodium selenite after 12 h (see the nuclei-stained blue images and AnnexinV-FITC/PI assay in Figure S13).

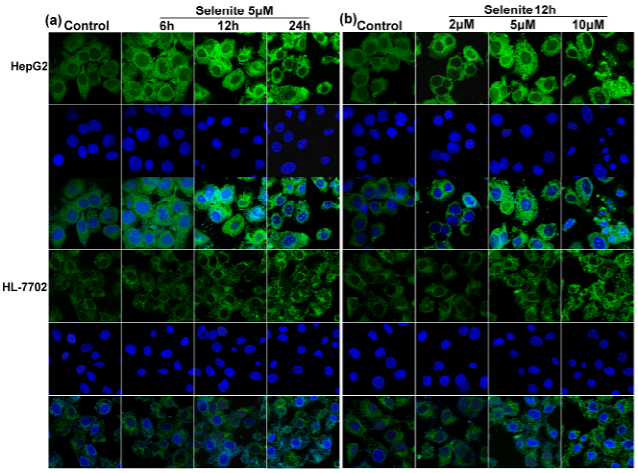

Fig. 4 (a) The in situ fluorescence changes of HepG2 and HL-7702 cells exposed to 5 μM sodium selenite for different time (0-24 h) and then incubated by HB (10 μM). The nuclei were stained by DAPI. (b) The in situ fluorescence changes of HepG2 and HL-7702 cells exposed to different concentrations of sodium selenite (0-10 μM) for 12 h. The nuclei were stained by DAPI.

The relation between cell cycle distribution and selenol level was studied. HepG2 cells were exposed to 5 μM of sodium selenite up to different time (6-24 h), then the cells were harvested and divided into two equal groups. One group was stained with 10 μM of HB for 15 min and the fluorescence intensity of cells was detected using flow cytometer. The other group was fixed with 70% ethanol overnight at 4°C for cell cycle distribution analysis. The results show that the number of G1 phase cells increased gradually and the S phase cells reduced correspondingly (Figure 5a), which is accompanied by a higher selenol concentration (Figure 5c). Besides, we observed that the depletion of mitochondrial membrane potential (MMP) induced by sodium selenite had a close relationship with selenol level. HepG2 cells were exposed to different concentrations of sodium selenite (2-10 μM) up to 12 h. One group was stained with 10 μM of HB for 15 min and the fluorescence intensity was detected using flow cytometer. The other group was incubated with 10 μM of JC-1 (a commercial probe for mitochondrial membrane potential) for 30 min at 37°C in the dark for MMP. The results were displayed in Figure 5b and Figure 5d. Mitochondrial membrane potential decreases significantly with the increasing concentration of sodium selenite (Figure 5b), and selenol level rises gradually and reaches a maximum at 5 μM of sodium selenite for 12 h (Figure 5d). Consequently, according to the above data, a potential mechanism of Se anticancer could be suggested that sodium selenite can be rapidly converted into high active selenol in HepG2 cells, accompanying tumor cell cycle arrest and mitochondrial membrane potential collapse.

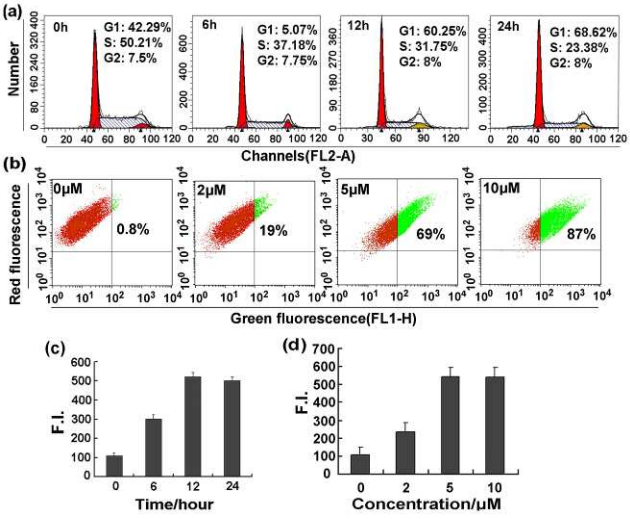

Fig. 5 Relationships between cell cycle distribution/ depletion of mitochondrial membrane potential and Sec level. (a) HepG2 cell cycle distribution, exposed to 5 μM of selenite for 0-24 h, was detected by flow cytometry. (b) HepG2 cells treated with different concentrations of selenite for 12 h and then stained with the mitochondria-selective JC-1 dye were analyzed by flow cytometry. The values in the top right quadrant of each dot plot represent the percentage of cells that emit green fluorescence due to the JC-1 monomers. (c) The corresponding changes of fluorescence in (a) were detected by flow cytometer with CellQuest analysis software. (d) The corresponding changes of fluorescence of (b) were obtained by flow cytometer with CellQuest analysis software.

Conclusions

In summary, in order to investigate the Na_2SeO_3 anticancer mechanism, we have designed and synthesized a novel fluorescence probe (HB) by integrating the BS moiety into a hemicyanine dye, for the first time. The probe is highly sensitive and selective for Sec over thiols and other bioactive species such as essential ions and amino acids under physiological conditions. HB has been successfully applied to image the concentration changes of Sec in living cells and *in vivo*. Furthermore, combined with the analysis of cell cycle and mitochondrial membrane potential, we found that the HepG2 cells apoptosis induced by Na_2SeO_3 was closely related with the selenol levels. The results would be beneficial to improve the efficacy and expend the application generality of Se compounds in cancer treatment and prevention.

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Notes and references

[a] *College of Chemistry, Chemical Engineering and Materials Science, Collaborative Innovation Center of Functionalized Probes for Chemical Imaging in Universities of Shandong, Key Laboratory of Molecular and Nano Probes, Ministry of Education, Shandong Provincial Key Laboratory of Clean Production of Fine Chemicals, Shandong Normal University, Jinan 250014, P. R. China*
[b] *Department of Chemistry and Biochemistry, Ohio University, Athens, Ohio 45701, USA*

† Electronic Supplementary Information (ESI) available: [Details of general experimental section; MTT assay; Cell cycle analysis; Mitochondrial membrane potential analysis; ^{1}H NMR, ^{13}C NMR and HRMS spectrum of intermediate products and HB]. See DOI: 10.1039/b000000x/

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