Targetable Mesoporous Silica Nanoprobes for Mapping the Subcellular Distribution of H$_2$Se in Cancer Cells

Bo Hu,$^\dagger$ Ranran Cheng,$^\dagger$ Xiaonan Gao, Xiaohong Pan, Fanpeng Kong, Xiaojun Liu, Kehua Xu,* and Bo Tang* $^\diamond$

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

$^\dagger$ Supporting Information

ABSTRACT: Hydrogen selenide, a highly active reductant, is believed as a key molecule in the cytotoxicity of inorganic selenium compounds. However, the detail mechanism has hardly been studied because the distribution of H$_2$Se in the subcellular organelles remains unclear. Herein, we exploited a series of novel targetable mesoporous silica nanoplatforms to map the distribution of H$_2$Se in cytoplasm, lysosome, and mitochondria of cancer cells. The subcellular targeting moiety-conjugated mesoporous silica nanoparticles were assembled with a near-infrared fluorescent probe (NIR-H$_2$Se) for detecting endogenous H$_2$Se in the corresponding organelles. The confocal fluorescence imaging of cancer cells induced by Na$_2$SeO$_3$ found out a higher concentration of H$_2$Se accumulated only in mitochondria. Consequently, the H$_2$Se burst in mitochondria-triggered mitochondrial collapse that led to cell apoptosis. Hence, the selenite-induced cytotoxicity in cancer cells associates with the alteration in mitochondrial function caused by high level of H$_2$Se. These findings provide a new way to explore the tumor cell apoptosis signaling pathways induced by Na$_2$SeO$_3$, meanwhile, we propose a research strategy for tracking the biomolecules in the subcellular organelles and the correlative cellular function and related disease diagnosis.

KEYWORDS: H$_2$Se, mesoporous silica, subcellular organelle, Na$_2$SeO$_3$, hypoxic conditions

INTRODUCTION

The study of selenium has become research hotspot in recent years due to its excellent anticarcinogenic properties through induction of cancer cell death by different mechanisms, including DNA damage, glutathione (GSH) depletion, and protein inactivation. $^1$ Although these mechanisms remain elusive, recent reports demonstrated that hydrogen selenide (H$_2$Se), a common intermediate of dietary selenium metabolism, $^2$ is considered to influence the toxicity induced by Se compounds. $^{1,5}$ Typically, endogenous H$_2$Se is produced by reducing selenite in the presence of in vivo thiols. As a precursor for selenol-protein synthesis or undergoes methylating to generate methylated selenium compounds, the H$_2$Se might be involved in various biological functions in tissues and cell. $^{1,5}$ Therefore, the real-time mapping of endogenous H$_2$Se in cancer cells is significant to understand its biological and pathological behaviors.

Moreover, the targeting and imaging subcellular organelles attract great attention in clinical applications for their crucial contribution in physiological functions and monitoring diseases. $^6$ Recently, to track the distribution of specific biomolecules in subcellular organelles, many novel fluorescent probes have been designed. $^7$ However, due to the strong reduction ability and short life time of H$_2$Se, it is difficult to detect the H$_2$Se in vivo alone in the subcellular organelles. Wu presented a functionalized cadmium sulfide quantum dots nanosensor to detect HSe$^-$ in aqueous solution. $^{8}$ Afterward, our group designed a fluorescent probe (NIR-H$_2$Se) to detect H$_2$Se in living cells. $^9$ To date, there is lack of efficient tools for real-time revealing H$_2$Se at subcellular levels as well as mapping the distribution of H$_2$Se in the subcellular organelles.

To fully elucidate biological functions of H$_2$Se in cellular signaling pathways, we choose mesoporous silica nanoparticles (MSNs) as the carrier of NIR-H$_2$Se because of their easy functionality, great biocompatibility, and high loading capacity. $^{10-14}$ Meanwhile, as the polyethylenimine (PEI), morpholines, and triphenylphosphines can target the cytoplasm, lysosome, and mitochondria respectively, $^{15}$ three targetable nanoprobes to monitor the distribution of H$_2$Se at subcellular levels are cytoplasm (Cyto-NIR-H$_2$Se-MSN), lysosome (Lyso-NIR-H$_2$Se-MSN), and mitochondria (Miton-NIR-H$_2$Se-MSN). The PEI-, morpholine-, and triphenylphos-
phine-modified MSNs nanoplatforms could specifically target
the cytoplasm, lysosomes, and mitochondria, respectively, and
accurately screen the changes of H2Se in cancer cells induced by
Na2SeO3 under hypoxic conditions with high selectivity and
sensitivity. More significantly, in vitro study exhibits higher
H2Se concentration, accumulated only in mitochondria but not
in cytoplasm or lysosomes (Scheme 1). These findings offer
new perspectives for future subcellular organelle studies and
also greatly help the cancer therapy investigation of Na2SeO3.

Scheme 1. Schematic Illustration of the Three Targetable
Nanoprobes To Map the Subcellular Distribution of H2Se in
Tumor Cells

EXPERIMENTAL SECTION

Synthesis of the Three Nanoprobes. MSNs (40 nm) were
synthesized according to the reported method.26 Cetyltrimethylam-
moniumbromide (CTAB) (0.7657 g) and tetraethylammonium (154.2
μL) were mixed in 50 mL of water, followed by intensive stirring
for 30 min at 80 °C. Later tetraethoxy silane (7 mL) was added and stirred
for 1 h. To remove the residual reactants, the products were washed
with ethanol and methanol for several times. Finally, the products were
calcined at 450 °C for 10 h to completely remove the CTAB.

NIR-H2Se (0.06 mg) was mixed with MSNs solution (1 mg/mL)
and stirred at room temperature for 12 h. Excess NIR-H2Se was
removed by washing the nanoparticles with water for several times.
Then, PEI (0.2 mg/mL) was added to the mixture and stirred for
another 24 h at room temperature in dark to activate the amino group
of MSN-NH2. Afterward, the solution was centrifuged for 10 min (12 000
rpm) and dispersed in the 2-(N-morpholino)ethanesulfonic acid
(MES) (10.0 mM, pH = 6.0), resulting in the assembly of the Cyto-
NIR-H2Se-MSN to target cytoplasm. The as-synthesized Cyto-NIR-
H2Se-MSN solution (1 mL) was added to 5 μL MPP (0.1 M) and 10 μL
1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (0.1 M) solution
to activate carboxylic groups, stirred at room temperature for 30 min
in dark, and then N-hydroxysuccinimide was added (5 μL, 0.1 M). The
mixture reacted at room temperature for another 24 h. The products
were centrifuged and dispersed in 10.0 mM MES (pH 6.0), which
finished the assembly of the Cyto-NIR-H2Se-MSN to target lysosome.
According to the synthetic method of Cyto-NIR-H2Se-MSN above,
the same method was applied, but just changed 5 μL MPP (0.1 M) with
5 μL triphenylphosphonium (0.1 M), to finally obtain the Mito-NIR-
H2Se-MSN to target mitochondria.

Quantitation of NIR-H2Se Loaded on the Nanoprobes. Nanoprobe (1 mL, 0.1 mg/mL) were heated at 70 °C for 1 h. The
solution was centrifuged, and the supernatant solution was collected.
Then, the precipitates were redispersed in 1 mL of phosphate-buffered
saline (PBS) buffer (10.0 mM, pH 7.4) and repeated the above
procedure. The fluorescence of the supernatant was excited at 688 nm
and measured at 735 nm. The concentrations of NIR-H2Se were
determined according to the standard linear calibration curve of NIR-
H2Se.

Fluorescence Intensity (FI) Measurements. To test the stability of the three nanoprobes under various pH values, 50 μM H2Se
was added to the three nanoprobes (0.1 mg/mL) in PBS buffer (10.0 mM,
pH = 9). The fluorescence spectra were collected with λex/λem = 688/
735 nm.

H2Se (0–50 μM) was added to react with the three nanoprobes
(0.1 mg/mL) to detect the fluorescent response under different pH
buffers (Cyto-NIR-H2Se-MSN for 7.2, Lyso-NIR-H2Se-MSN for 4.5,
and Mito-NIR-H2Se-MSN for 7.8). The fluorescence spectra were collected with λex/λem = 688/735 nm.

To evaluate the selectivity of the three nanoprobes for H2Se, metal
ions, oxidative-stress-associated chemicals, and amino acids were
tested.17 The concentrations applied were as follows: 50 μM for H2Se,
500 μM for NO; 5 mM for NO for other interfering substances.

The responses of the three nanoprobes to H2Se were evaluated via a
kinetics experiment in different pH buffers (Cyto-NIR-H2Se-MSN for
The time course for the fluorescence intensity of the nanoprobes with
25 μM H2Se was 15 min, followed by another addition of 25 μM H2Se,
compared with only PBS buffer (10.0 mM) at 37 °C.

Cellular Uptake Pathways and Colocalization. In the
subcellular targeting moiety-modified nanoplatforms, a bright
and acid resistance NIR fluorescent dye CF-640 was loaded, instead of
NIR-H2Se, to improve the velocity in the colocalization experiments.

To test the targeting time of the three nanoprobes, HepG2 cells
were incubated with each nanoprobe (0.1 mg/mL) for 2, 4, 6, 8, and
10 h, then the cells were washed with PBS (pH 7.4) for three times,
at last the confocal fluorescence imaging was obtained with an excitation
at 633 nm.

To test the availability of the three nanoprobes for subcellular
location, the colocalization imaging experiments in cancer cells (HepG2, HeLa, and MCF-7 cells) were further performed. In
the cytoplasm colocalization study, Cyto-CF-640-MSN (0.1 mg/mL) was
incubated with the cells for 6 h, and then incubated with commercial
Calcine, AM, Ultrapure Grade (CAUG, 50 nM) for 15 min. Finally,
the solution was washed with PBS and examined by confocal laser
scanning microscopy (CLSM). The fluorescence images were
recorded in two channels. The green channel was excited at 488 nm
(CAUG), the red channel was excited at 633 nm (CF-640). In the
lysosomes and mitochondria colocalization studies, 0.1 mg/mL of
Lyso-CF-640-MSN or Mito-CF-640-MSN, respectively, was incubated
with cells for 6 h, then washed, and incubated with Lyso-Tracker
DND-26 (LTD, 100 nM) or Mito-Tracker Green, respectively, (MTG,
100 nM) for 15 min. Similarly, the solution was washed with PBS for
three times, and the CLSM image of stained cells was also captured
using two channels.

The cellular uptake pathways were investigated: inhibit
macropinocytosis by cytochalasin D (30 μM); inhibit caveolae-mediated
endocytosis by genistin (1 mg/mL); inhibit clathrin-mediated uptake
by chlorpromazine (10 mg/mL); and inhibit the above two
endocytosis processes by dynasore (80 μM).26,27 The cells were incubated with the inhibitors for 30 min, then incubated with 0.1 mg/mL
of the three nanoprobes for 6 h at 37 °C. The fluorescence images
were excited at 633 nm and collected between 650 and 750 nm. The
fluorescence intensity (FI) was an average value for the cells area from
the confocal fluorescence images.

Fluorescence Imaging of H2Se at the Subcellular Level
Induced by Na2SeO3. Na2SeO3 could induce the apoptosis of cancer
and cell, and then it was chosen to be the H2Se precursor.21,22 Three types of
tumor cells, HepG2, HeLa, and MCF-7, were chosen in the
following experiments. The cells were incubated with 0–10 μM
Na2SeO3 for 12 h or 5 μM Na2SeO3 for 0–12 h, and then incubated with
each nanoprobe (0.1 mg/mL) in hypoxic environments.28 The
fluorescence images were excited at 633 nm and collected between 650
and 750 nm.

Mitochondrial Membrane Potential (MMP) Analysis. HepG2,
HeLa, and MCF-7 cells were chosen in the following experiments.
Mitochondrial membrane potential (∆Ψm) was detected via staining
The tumor cells were first incubated with 0−10 μM Na2SeO3 for 12 h or 10 μM Na2SeO3 for 0−12 h under hypoxic condition, and then washed and incubated with Mito-NIR-H2Se-MSN for 6 h. Afterward, the cells were stained with rhodamine 123 for 10 min, and washed with PBS. The CLSM image was captured using two channels with the same set as previous.

**RESULTS AND DISCUSSION**

First, the MSNs and the H2Se-responded small-molecule fluorescent probe (NIR-H2Se) were synthesized according to previous literature with certain modifications (Figures S1 and S2). Then, NIR-H2Se, PEI, and targeting moiety were modified onto the MSNs in sequence to form the nanoprobes.
The sizes of the three nanoprobes (Cyto-NIR-H\textsubscript{2}Se-MSN, Lyso-NIR-H\textsubscript{2}Se-MSN, and Mito-NIR-H\textsubscript{2}Se-MSN) are approximately 40 nm and their morphologies stay spherical (Figure 1). 0.6 mg/g NIR-H\textsubscript{2}Se was loaded into the pore of the MSNs (Figure S3). Moreover, ζ potential changes in Figure S4 display a successful assembly of the nanoprobe step by step.

Fluorescence responses of the subcellular targeting nanoprobes for H\textsubscript{2}Se were tested. Considering the different pH values in each organelle, the cytoplasm for 7.2,30−32 the lysosome for 4.5,33−35 and the mitochondria for 7.8,36,37 the stabilities of the three nanoprobes in different PBS buffers (pH ranges from 4 to 9) were investigated, which almost cover the physiological pH ranges (Figure 2). The increasing fluorescence intensity of the nanoprobes could be observed after addition of H\textsubscript{2}Se, without significant variances at different pH values. These results demonstrate that the three nanoprobes could respond toward H\textsubscript{2}Se without pH interference in different organelles.

Fluorescence responses of the subcellular targeting nanoprobes for H\textsubscript{2}Se were tested. Considering the different pH values in each organelle, the cytoplasm for 7.2,30−32 the lysosome for 4.5,33−35 and the mitochondria for 7.8,36,37 the stabilities of the three nanoprobes in different PBS buffers (pH ranges from 4 to 9) were investigated, which almost cover the physiological pH ranges (Figure 2). The increasing fluorescence intensity of the nanoprobes could be observed after addition of H\textsubscript{2}Se, without significant variances at different pH values. These results demonstrate that the three nanoprobes could respond toward H\textsubscript{2}Se without pH interference in different organelles.

The fluorescence responses of the three nanoprobes for different concentrations of H\textsubscript{2}Se were studied in different pH buffers (Cyto-NIR-H\textsubscript{2}Se-MSN is 7.2, Lyso-NIR-H\textsubscript{2}Se-MSN is 4.5, and Mito-NIR-H\textsubscript{2}Se-MSN is 7.8), which were consistent with corresponding organelles (Figure 3). Each nanoprobe exhibited remarkably fluorescence enhancement when H\textsubscript{2}Se was added and a positive linearity between the H\textsubscript{2}Se concentration and fluorescence intensity. In addition, the kinetics experiment revealed that the nanoprobes could real-time monitor H\textsubscript{2}Se because the rapid responds to H\textsubscript{2}Se can be completed in corresponding pH buffer (Figure S5).

The selectivity of the nanoprobes toward H\textsubscript{2}Se was studied by comparing with the background fluorescence. In Figure S6, the fluorescence change of the nanoprobes was not obviously in the presence of all other substances, demonstrating that every nanoprobe could selectively recognize H\textsubscript{2}Se under corresponding physiological conditions.

The cytotoxicity of the nanoprobes was also verified in HepG2 cells and HL-7702 liver cell line via MTT assay, which confirmed the low toxicity of the three nanoprobes for living cells detection (Figures S7 and S8). First, the incubating time of the nanoprobes to HepG2 cells was optimized, and the fluorescence intensity of HepG2 cells incubated with the nanoprobes reached a plateau after 6 h (Figure S9). Afterward, the uptake pathways of the nanoprobes were explored in HepG2 cells. The results suggested that endocytosis was the main uptake pathway of the nanoprobes in HepG2 cells (Figure S10).

The cellular localization experiments were performed with CLSM to confirm the intracellular location of the three nanoprobes. Because H\textsubscript{2}Se is an intermediate of selenite metabolism, for the cancer cells without treatment of Na\textsubscript{2}SeO\textsubscript{3}, the background fluorescence intensity of the nanoprobes is

![Figure 4. Intracellular localizations of (A) Cyto-CF-640-MSN, (B) Lyso-CF-640-MSN, (C) Mito-CF-640-MSN in HepG2 cells. The green channels of (a), (e), and (i) correspond to CA, LTG, and MTG respectively. The red channels of (b), (f), and (j) are for Cyto-CF-640-MSN, Lyso-CF-640-MSN, and Mito-CF-640-MSN respectively. (c), (g), and (k) are the corresponding merged images. (d), (h), and (l) are the fluorescence intensity profiles of regions of interest across the line.](image-url)
quite weak. However, in the subcellular targeting moiety-modified nanoplatforms, CF-640, a bright and acid resistance NIR fluorescent dye instead of NIR-H$_2$Se, was loaded to improve the veracity in the colocalization experiments. Calcein (AC), Lyso-Tracker Green (LTG), and Mito-Tracker Green (MTG) are commercially available targeting dyes for cytoplasm, lysosomes, and mitochondria, respectively. The Cyto-CF-640-MSN, Lyso-CF-640-MSN, and Mito-CF-640-

Figure 5. CLSM images of endogenous H$_2$Se in cytoplasm, lysosome, and mitochondria treated with Na$_2$SeO$_3$ under hypoxic (1% pO$_2$) conditions. (a) The fluorescence changes for HepG2 cells incubated with 0−10 μM Na$_2$SeO$_3$ for 12 h and incubated with the three nanoprobes (0.1 mg/mL). (b) The fluorescence changes of HepG2 cells incubated with 5 μM of Na$_2$SeO$_3$ for 0−12 h and incubated with the three nanoprobes (0.1 mg/mL). (c, d) The quantitative analyses of the corresponding results.

Figure 6. (a, b) CLSM images of HepG2 cells incubated with rhodamine 123 and Mito-NIR-H$_2$Se-MSN. (c, d) The quantitative analyses of the corresponding results from (a) and (b).
MSN were coincubated for 6 h with HepG2, HeLa, and MCF-7 cells, respectively, and before CLSM imaging, relevant organelles were labeled with commercially available dyes. In Figure 4, a well-overlapped image was obtained for each nanoprobes and organelle dye in HepG2 cells (Pearson’s correlation coefficient, \( \rho_a = 0.652 \), \( \rho_b = 0.572 \), \( C = 0.786 \)). And the similar phenomenon was observed in HeLa and MCF-7 cells (Figures S11 and S12). The organelle localizations of the three nanoprobes in HepG2 cells were also observed by TEM (Figure S13). These results proved that the three nanoprobes are effective tools for targeting organelles.

In our previous work, higher H\(_2\)Se content was observed in HepG2 cells when Na\(_2\)SeO\(_3\) was induced under hypoxic conditions, exhibiting a positive correlation with the cell death level.\(^9\) However, because the distribution of H\(_2\)Se in the subcellular organelles is still unclear, it becomes a huge obstacle to fully elucidate the biological functions of H\(_2\)Se in Na\(_2\)SeO\(_3\) anticancer signaling pathways. Therefore, the detection of H\(_2\)Se in subcellular organelles was examined in HepG2, HeLa, and MCF-7 cells. The Na\(_2\)SeO\(_3\) was incubated with the cells for different incubation time or concentrations (Figure 5), and followed by coincubation with Cyto-NIR-H\(_2\)Se-MSN, Lyso-NIR-H\(_2\)Se-MSN, and Mito-NIR-H\(_2\)Se-MSN for 6 h. In Figure 5, relatively weak fluorescence intensities were observed in the three groups of the stained HepG2 cells, which have not been treated with Na\(_2\)SeO\(_3\). Nevertheless, the intracellular fluorescence from Mito-NIR-H\(_2\)Se-MSN-stained cells performed much stronger intensity when the cells were induced by Na\(_2\)SeO\(_3\) for a longer time or a higher concentration, demonstrating that higher amount of H\(_2\)Se only accumulated in mitochondria, rather than cytoplasm or lysosome. Similarly, with the increasing incubation concentration of Na\(_2\)SeO\(_3\), the fluorescence was obviously enhanced only in mitochondria. The accumulation of H\(_2\)Se in mitochondria was also evaluated in HeLa and MCF-7 cells (Figures S14 and S15).

As mitochondria plays a crucial role in regulating cell death, selenite-induced H\(_2\)Se accumulation in mitochondria was investigated to check damaged mitochondria’s function. The change of mitochondrial membrane potential (MMP) can be monitored using rhodamine 123 staining, which was an early event in apoptosis process triggered by mitochondria. Figure 6 shows that treatments of HepG2 cells with selenite displayed higher amount of H\(_2\)Se accumulation in mitochondria and induced a significant loss of MMP. The same tendency was observed in HeLa and MCF-7 cells (Figures S16 and S17). These suggest that in Na\(_2\)SeO\(_3\)-stimulated cancer cells, selenite-induced H\(_2\)Se accumulation triggers functional and structural disruption in mitochondria that leads to cell’s apoptosis and death.

**CONCLUSIONS**

In summary, three MSN-based nanoprobes were developed for understanding the distribution of subcellular H\(_2\)Se among corresponding organelles. These nanoprobes own excellent sensitivity to H\(_2\)Se and high selectivity over other interferes. In vitro experiments indicated the nanoprobes’ decent biocompatibility and targeting for cytoplasm, lysosomes, and mitochondria in living cells. Furthermore, in Na\(_2\)SeO\(_3\)-stimulated cancer cells, H\(_2\)Se only can be detected in mitochondria was verified for the first time. In addition, clear evidence of H\(_2\)Se accumulation induced by selenite-triggered functional and structural disruption in mitochondria was obtained, which leads to cell apoptosis. These findings offer novel tools to understand the underlying mechanisms of cell death in cancer cells induced by Na\(_2\)SeO\(_3\) under hypoxic conditions and offer new perspectives for future clinical cancer therapies.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.8b02206.

Characterization of the three nanoprobes, the kinetics study, the selectivity of the three nanoprobes for H\(_2\)Se, the MTT assay, endocytic inhibition studies, TEM images (PDF).

**AUTHOR INFORMATION**

Corresponding Authors

*E-mail: xukehua@sdnu.edu.cn (K.X.).
*E-mail: tangbi@sdnu.edu.cn (B.T.).

ORCID®

Bo Tang: 0000-0002-8712-7025

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Author Contributions

B.H. and R.C. contributed equally to this work.

Notes

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

This work was supported by National Natural Science Foundation of China (21535004, 91753111, 21390411, 21775091, 21575081, 21507075, and 21705098).

**REFERENCES**


