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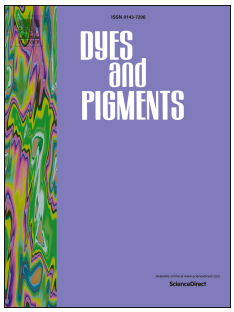


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PII: S0143-7208(19)30896-4

DOI: https://doi.org/10.1016/j.dyepig.2019.107617

Article Number: 107617

Reference: DYPI 107617

To appear in: *Dyes and Pigments*

Received Date: 22 April 2019

Revised Date: 29 May 2019

Accepted Date: 3 June 2019

Please cite this article as: Gebremedhin KH, Li M, Gao F, Gurram B, Fan J, Wang J, Li Y, Peng X, Benzo[a]phenoselenazine-based NIR photosensitizer for tumor-targeting photodynamic therapy via lysosomal-disruption pathway, *Dyes and Pigments* (2019), doi: https://doi.org/10.1016/j.dyepig.2019.107617.

# Benzo[a]phenoselenazine-based NIR Photosensitizer for Tumor-Targeting Photodynamic Therapy via Lysosomal-disruption Pathway

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Abstract:The ultimate goal of cancer therapy is to develop antitumor agents that will destroy selectively tumor cells while sparing the health cell of the patient. Herein, we reported a novel tumor-specific and lysosome dual-targeted NIR photosensitizer, Se-Biotin, by conjugating a biotin ligand into benzo[a]phenoselenazinium derivative dye for selective destruction of tumor cells.Attractively, co-culture model showed that Se-Biotin could selectively target to and retainin biotin receptor-overexpressed tumor cells, which as a result significantly minimized the side effects toward normal cells. As confirmed by the in vitro anticancer mechanism, after cellular internalization, upon irradiation, the effective $^1O_2$ generation ($\Phi_\Delta$ = 0.69)severelydisrupted the lysosomalintegrity and subsequently led to apoptotic cell death. Benefiting from these merits, Se-Biotin successfully achieved a superior anticancer performance with the $IC_{50}$ as low as 85 nM, only under a low light dose irradiation (12 J/cm$^2$, 660 nm). Therefore, these result demonstrated that Se-Biotin will be a promising PDT agent for targeted cancer photodynamic therapy.

Keywords :Benzo[a]phenoselenazinium; Photosensitizer; Tumor-Targeting; Lysosomes; Photodynamic Therapy

## 1. Introduction

Cancer is still the most frequently diagnosed and a major cause of death in the world with high invasion, recurrence and metastasis.[1-3] The world incidence of cancer is increasing at an average annual range of 3 % to 5 %.[4] Therefore, the treatment of malignant tumors has become a major issue of common concern in all countries. Photodynamic therapy (PDT) has been emerged as new cancer therapeutic modality for treating a wide variety of malignant tumors before or after chemotherapy, surgical section and radiotherapy.[5-7] PDT is a two-step approach that uses a combination of light-activated photosensitizer, light and molecular oxygen to destroy tumor cells and tumor vasculature by generating reactive oxygen species (ROS), mainly singlet oxygen ($^1O_2$).[8-10] And also PDT has high site-specificity and the advantages of repeated dose tolerance.[11] Despite the clinical cancer treatment technology, the clinically available conventional PDT agents cannot distinguish malignant cells from the normal cells and also showed low photostability.[12-14]This poor tumor-selectivity of photosensitizer and UV-Vis light excitation becomes a major issue in clinical application, which leads to undesirable cytotoxicity and photodamage to surrounding normal tissue.[15, 16] Therefore, the development of tumor-targeted and specific photosensitizer for malignant cells is essential for improving overall antitumor effect of PDT.

To overcome the above drawback, many researches have focused on developing novel tumor-targeting PDT agents by conjugating a photosensitizer to various tumor-targeting ligands, only overexpressed in tumor cells but not expressed in health cells, which can be used to deliver photosensitizer into tumor tissue. During the past several years, various tumor-specific receptors, such as antibody, [17-19] receptor ligand, and peptides, [20, 21] have been explored and employed to develop novel tumor-selective photosensitizers with improved overall PDT efficacy, which results increasing delivery of PDT agents to the neoplastic tissue and avoiding its undesirable phototoxicity and systemic toxicity.[22-24] However, these tumor-targeted vectors have sill several drawbacks. Considering the high cost, poor penetration into solid tumors and low clearance of antibodies, small-molecule tumor-specific ligands are also investigated and found that Vitamins, including vitamin B12, biotin, riboflavin and folic acid, would serve as excellent biomarkers for tumor-targeted drug delivery.[25-27] The biotin receptor is a growth promoter at the cellular level which is highly overexpressed on the surface of cancer cells or tumor vasculature than that in normal tissue.[28-30] Recently, biotin receptors have been found overexpressed in many cancer cells more than folate and vitamin B-12 receptors.[30]

Moreover, organelle-localized PDT agents can rapidly damage the organelle function under photoactivation, leading to tumor cell deaths.[31] Lysosome is a key degenerative organelle in the cell, and lysosomal enzymes are pivotally involved in apoptosis initiation and execution in cancer cells.[32, 33] Photosensitizers that target lysosome other than mitochondrial and/or nucleus were found in high demand due to their low

dark toxicity and low potential of DNA mutations.[34, 35]Adding on, photooxidative damage of lysosomal membrane leads to release of proteolytic acidic enzymes into cytoplasm and causes a lysosomal membrane destabilization resulting in organelle dysfunction and then leads to cancer cell death, either through apoptosis or necrosis, finally initiated the lysosomal rupture.[36-38] Herein, we report a novel tumor-selective and lysosome-tracking dual-targeted NIR Photosensitizer (**Se-Biotin**, scheme 1) by conjugating a biotin receptor into benzo[a]phenol-selenazinium derivative dye owning to their excellent photophysical property, including a strong absorption in the NIR region (650-700 nm), good water solubility, high singlet oxygen quantum yield and excellent photochemical stability.[39-41]**Se-Biotin**exhibited high $^1O_2$yield ($\Phi_\Delta$ = 0.69 in PBS/ethanol solution, 1:1 v/v), and can cause a tumor-targeted apoptosis of cancer cells via lysosomal-disruption pathway, which is an important PDT agent for improving anticancer effect in clinical application.

## 2. Experimental Section

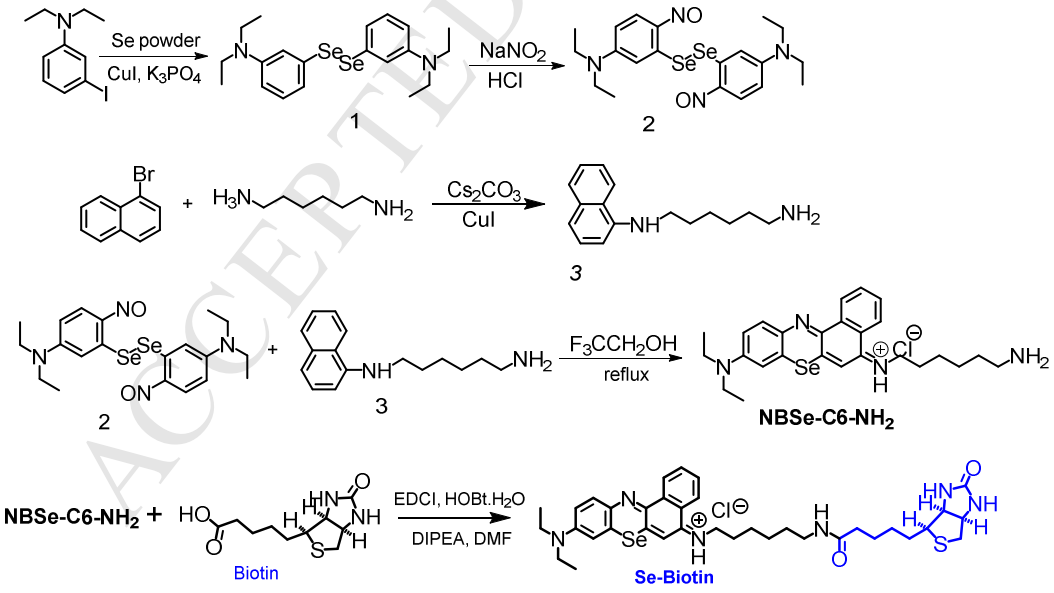
### 2.1. Materials and Reagents

All solvents and reagents were of analytical grade and used directly without further purification. 1,3-diphenylisobenzofuran (DPBF) was obtained from J & K Scientific. The UV-vis absorption spectra were measured on UV-vis spectrophotometer (HP 8453-UV3100, Agilent, USA). The fluorescence spectra were recorded on a FP-6500 fluorescence spectrophotometer (Jasco, Japan). The fluorescence quantum yield of Se-Biotin and NBSe-C6-$NH_2$ were measured directly using quanturus-QY instrument (HAMAMATSU, Japan). All Cellular reagents including 2,7-dichlorofluorescin diacetate (DCFH-DA), MTT, Annexin V-FITC, propidiumiodide (PI), and Calcein-AM were purchased from the Key GEN Bio. TECH Co., Ltd. (Nanjing, China). Cell imaging was carried out with Olympus FV-1000 confocal laser scanning fluorescence microscopy.

### 2.2. Synthesis of Se-Biotin

#### 2.2.1. Synthesis of of Compound 1

A flame-dried test tube containing a magnetic stirring bar was charged with CuI (38 mg, 0.2mmol), $K_3PO_4$ (424.54 mg, 2.0 mmol), Se (158 mg, 2.0 mmol), 1 (275.13 mg, 1.0 mmol), and 2 mL of DMSO under nitrogen atmosphere. The mixture was heated at 100 $^oC$ for 24 h and allowed to cool to room temperature and then treated with water. The resulting mixture was extracted with ethyl acetate (3 x 20 mL). The combined organic layer were dried over anhydrous sodium sulfate and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography with an eluent consisting of petroleum ether and ethyl acetate to give yellow oil in 78 % yield (354.41 mg). $^1$H NMR (500 MHz, DMSO) δ 7.06 (t, *J* = 8.0 Hz, 2H), 6.83 (s, 2H), 6.79 (d, *J* = 7.6 Hz, 2H), 6.54 (dd, *J* = 8.4, 2.3 Hz, 2H), 3.25 (dd, *J* = 14.0, 7.0 Hz, 8H), 1.01 (t, *J* = 7.0 Hz, 12 H). $^{13}$C NMR (126 MHz, DMSO-d6) δ 148.11, 130.31, 116.62, 113.22, 111.03, 43.81, 12.38.HRMS for $C_{20}H_{28}N_2Se_2$, $[M+H]^+$calculated: 457.0656; found: 457.0662.




Scheme 1. Synthetic route of Se-Biotin

#### 2.2.2. Synthesis of compound 2

Compound 2 was synthesized via nitrosylating of diselenide 2 according to Foley, J. W *et.al*.[42]

#### 2.2.3. Synthesis of of Compound 3

20.0 mmol of 1-bromonaphthalene (4.2 g), 40.0 mmol of 16-diaminohexance (4.7 g) and 30 ml 2-methoxyethanol was added into 100 ml dried flask containing a magnetic stirrer. Then, CuI (191 mg, 1.0 mmol) and $Cs_2CO_3$ (4.5 g, 15.55 mmol) was charged and the reaction mixture was heated to

refluxed temperature and continue stirring at 125 ºC for 12 h. after completion of the reaction, the reaction product was cooled to room temperature. The solid was filtered off to yield a pale yellow solution and removed the solvent under reduced pressure.The crude product was purified by column chromatography with $CH_2Cl_2/CH_3OH$ (10:1) and obtains the desired compound 3 as yellow oil (3.5 g, 65 % yield). $^1$H NMR (500 MHz, $CDCl_3$) δ 7.87 – 7.81 (m, 2H), 7.52 – 7.45 (m, 2H), 7.39 (t, *J* = 7.9 Hz, 1H), 7.27 (d, *J* = 8.2 Hz, 1H), 6.65 (d, *J* = 7.5 Hz, 1H), 4.38 (s, 1H), 3.32 (t, *J* = 7.1 Hz, 2H), 2.77 (t, *J* = 6.9 Hz, 2H), 2.03 (dd, *J* = 26.3, 6.8 Hz, 2H), 1.83 (dt, *J* = 14.6, 7.2 Hz, 2 H), 1.54 (dd, *J* = 10.8, 4.1 Hz, 3H), 1.51 – 1.40 (m, 3H). $^{13}$C NMR (126 MHz, $CDCl_3$): δ 143.85, 134.40, 128.50, 126.42, 125.42, 124.35, 123.51, 119.73, 117.12, 104.06, 44.11, 41.10, 31.33, 28.69, 27.12, 26.98. HR MS: m/z calcd for $C_{16}H_{22}N_2$ $[M+1]^+$: 243.1856, found: 242.1825.

### 2.2.4. Synthesis of NBSe-C6-$NH_2$

Compound 2 (1.02g, 1.0 mmol), compound 3 (1.36 g, 6.0 mmol) and 15 ml of 2,2,2-trifluoroethan-1-ol were charged into a flame-dried round bottom flask containing a magnetic stirring bar. After 10 min of stirring, the mixture was heated to reflux temperature. After refluxing for 1.5 h, the solvent was removed under vacuum and the resulting blue waxy solid was dissolved in a mixture of aqueous sodium hydroxide (1 N) solution and $CH_2Cl_2$. The color of the solution turned bright magenta, indicating that deprotonation of dye had occurred. The organic layer was washed twice with brine. Then, the dye was regenerated as a deep blue chloride salt by adding 0.5 mL of concentrated hydrochloric acid to the magenta $CH_2Cl_2$ solution. After removing the solvent and excess hydrochloric acid under vacuum, the desired product was obtained by silica gel column chromatography using $CH_3OH/CH_2Cl_2$ (1:10 to 1:5). Yield 68 % of NBSe-C6-$NH_2$ as blue waxy solid. $^1$H NMR (500 MHz, DMSO-d6) δ 7.83 (dd, *J* = 9.6, 7.6 Hz, 2H), 7.63 – 7.40 (m, 2H), 7.38 (t, *J* = 7.9 Hz, 1H), 7.30 – 7.22 (m, 2H), 6.66 (d, *J* = 7.5 Hz, 1H), 3.74 (s, 1H), 3.34 (m, 2H), 3.32 (m, 2H), 2.46 (m, 2H), 1.89 – 1.74 (m, 12H), 1.64 – 1.55 (m, 6H). $^{13}$C NMR (126 Hz, DMSO-d6) δ 12.63, 27.01, 28.28.17, 28.41, 19.06, 41.72, 45.02, 49.64, 110.38, 11.57, 116.96, 120.01, 120.85, 123.73, 124.59, 129.48, 130.87, 131.11, 131.80, 133.90, 136.59, 146.27, 158.85.HR MS: m/z calcd for $C_{26}H_{33}ClN_4Se_2$ $[M+1]^+$: 516.1559, found: 516.1542

### 2.2.5. Synthesis of Se-Biotin

A mixture of NBSe-C6-$NH_2$ (169 mg, 0.35 mmol), biotin (103 mg, 0.42 mmol), EDCl (135 mg, 0.7 mmol), HOBt.$H_2O$ (61 mg, 0.45 mmol), and DIPEA (0.2 mL, 1 mmol) in DMF was stirred at room temperature under nitrogen for 24 h. The solvent was removed under vacuum, and the crude product was the subject to silica gel column chromatography using $CH_2Cl_2/CH_3OH$ (20:1 v/v) as eluents. The product was isolated as a dark blue solid (78 % yield).$^1$H NMR (500 MHz, DMSO) δ 8.87 (d, *J* = 8.1 Hz, 1H), 8.67 (d, *J* = 8.0 Hz, 1H), 7.96 – 7.70 (m, 2H), 7.52 – 7.36 (m, 2H), 6.44 – 6.26 (m, 2H), 4.33 – 4.18 (m, 2H), 3.71 – 3.54 (m, 6H), 3.24 – 3.09 (m, 4H), 2.92 – 2.76 (m, 4H), 1.97 (dt, *J* = 39.4, 13.1 Hz, 6H), 1.77 (d, *J* = 33.7 Hz, 4H), 1.36 (dd, *J* = 65.3, 31.9 Hz, 8H), 1.27 – 1.08 (m, 4H).$^{13}$C NMR (126 Hz, DMSO-d6) δ 12.76, 25.39, 26.14, 28.10, 28.64, 29.32, 35.13, 38.17, 44.14, 45.31, 55.54, 59.16, 61.12, 103.36, 105.40, 106.86, 116.86, 124.01, 129.24, 131.25, 131.87, 133.56, 136.63, 139.47, 150.53, 153.11, 162.95, 172.95.HR MS: m/z calcd for $C_{36}H_{47}ClN_6O_2SSe$ $[M+1]^+$: 707.2641, found: 707.2618.

### 2.3 Singlet oxygen quantum yield ($\Phi_\Delta$)

The stock solutions of NBSe-C6-$NH_2$ and Se-Biotin were prepared in 1 mM in dimethyl sulphoxide (DMSO), which is then further diluted to the desire working concentrations. The UV-vis absorption spectra and emission spectra were recorded in in different solvents. The photodegradation method of 1,3-diphenlisobenzofuran (DPBF) was employed for the measurement of singlet oxygen ($^1O_2$)quantum yield ($\Phi_\Delta$) of NBSe-C6-$NH_2$ and Se-Biotin in PBS/ethanol (1:1 v/v) solution according to the literature method.[43] Briefly, the absorption spectral intensity of DPBF at 410 nm was adjusted to about 1.0. Then after, a solution of Se-Biotin or NBSe-C6-$NH_2$was added to the cuvette and their absorbance intensity was adjusted to approximately 0.2. Subsequently, each solution was exposed 660 nm red light for 10 second interval. The photodegradation of DPBF spectra at 410 nm was monitored immediately with time. The $^1O_2$ quantum yield of both NBSe-C6-$NH_2$ and Se-Biotin were calculated using Methylene Blue ($\Phi_{\Delta\ MB}$ = 0.52 in PBS/ethanol (1:1 v/v)as the reference photosensitizer using the following equation:

$$\Phi_{\Delta Se\text{-}Biotin} = \Phi\Delta_{MB} . (K_{Se\text{-}Biotin}F_{MB}/ K_{MB}F_{Se\text{-}Biotin})$$

Where, $K_{Se\text{-}Biotin}$ and $K_{MB}$are the decomposition rate constants of DPBF at 410 nm in the presence of test photosensitizer and reference, respectively. The slop of absorbance ratio of DPBF at 410 nm via irradiation time over 10 s intervals is regarded as decomposition rate constant. $F_{Se\text{-}Biotin}$ and $F_{MB}$represent the absorption correction factor of Se-biotin and MB, respectively, which is given by $F = 1\text{-}10^{-OD}$, where OD is the optical density value.

### 2.4. Fluorescence Imaging of Living Cells

Human breast adenocarcinoma (MCF-7) and adherent Monkey Kidney fibroblasts (COS-7) cell lines were cultured in Dulbecco's modified Eagle medium (DMEM) medium supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum. All cells were maintained at 5% $CO_2$ and 95% air at 37 $^o$C for 1-2 days to reach approximately 90% confluence. All drugs and dyes were incubated in the dark. For intracellular fluorescence imaging, cells (~1 x $10^5$) were seeded on a glass-bottom culture dish and cultured for 24 h under normal condition. After removing the old medium, cells were incubated with the Se-Biotin or NBSe-C6-$NH_2$-containg fresh medium (5 μM) for 10 min. Subsequently, cells were rinsed with PBS three times before imaged with an Olympus Laser

Scanning Confocal Microscopy (Japan). To investigate the cellular uptake of Se-Biotin by cancer cells, a co-culture model of MCF-7 cells and COS-7 cells were plated in DMEM culture medium. Furthermore, the cellular uptake of Se-Biotin by MCF-7 cells was also investigated in the presence or absence of free biotin receptor (1 mM).

### 2.5. Colocalization Experiment

Following cell plating in the glass-bottom culture dish, the cells were co-incubated with 2.5μM Se-Biotin and 1μM Lysotracker green (Life Tenchnology), for 30 min in DMEM high-glucose medium. Then cells were washed with phosphate-buffered saline (PBS) two times before imaging. Cell images were collected with 488/520 nm for Lyso-tracker green, and 640 nm/700 nm for Se-Biotin.

### 2.6 Detection of Singlet Oxygen ($^1O_2$) in Vitro

PDT-mediated singlet oxygen generation in MCF-7 cells was evaluated using fluorogenic dye 2,7–dichlorofluorescin-diacetate (DCFH-DA) according to the manufacture's protocol. Briefly, MCF-7 cells were seeded in 35-mm glass-bottom culture dishes and then incubated for 24 h under 5% $CO_2$ and 95% air condition at 37 $^oC$. After removing the old medium, cells are treated with Se-Biotin or NBSe-C6-$NH_2$ for 20 min as follow: Group 1: untreated, Group 2: treated with Se-Biotin (2.5 μM), Group 3: treated with Se-Biotin (2.5 μM) and $NaN_3$ (50 μM) group 4: treated only $NaN_3$ (50 μM). Like Se-Biotin, NBSe-C6-$NH_2$-induced $^1O_2$ generation was also treated under same condition. After treatments, the cells were washed with fresh medium, and then stained with DCFH-DA (2.5 μM) in fresh DMEM medium for 30 min at 37 $^oC$. Then the cells were irradiated with or without 660 nm LED red light for 0 or 1 min. Then, cell imaging was performed after washing with PBS solution (two-times) using Olympus FluoView Laser confocal scanning microscopy under 488 nm excitation wavelength and 500-530 nm emission wavelengths.

### 2.7 Phototoxicity Assay

The Methyl thiazolyltetrazolium (MTT) chemical assay [44] was used to evaluate the phototoxicity of Se-Biotin against the cancer cells and normal cells. MCF-7 and COS-7 cells were seeded at approximately 4 x $10^4$ cells in a 96 well plate per well and incubated for 24 h at 37˚C in a humidified atmosphere of 5% $CO_2$. 2μL stock solution of test photosensitizer (5 mM in DMSO) was diluted into 4mL using DMEM medium to make standard solutions. The standard solution was further diluted with DMEM medium to the desired final concentration varying from 0.02 to 2.5 μM (less than 0.001% DMSO contained in the final solution). The cells were treated with various concentration of Se-Biotin. NBSe-C6-$NH_2$ or commercial photosensitizer Ce6, and incubated for 1 h at 37 $^oC$. Then the cells were immediately irradiated to different light dose (0, 3, 6 and 12 J/$cm^2$) of 660 nm LED red light. After further incubation for 24 h, 20 μL of 5 mg/mL MTT solution was added to each well and the cell were incubated for another 4h. Finally, the medium was removed out carefully, and 200 μL DMSO was added to each well to dissolve blue formazan. Dark control groups were performed in parallel for comparison. The phototoxicity was then determined by measuring absorbance at 570 nm with a microplate reader and the cell viability was obtained by the following equation: Cell viability % = ($OD_{treated}$ _$OD_{blank}$)/$OD_{control}$ - $OD_{blank}$) x 100%, where $OD_{treated}$, $OD_{control}$ and $OD_{blank}$ represents the optical density of sample, cells only and culture medium, respectively. Measurements were performed in triplicate wells and data were computed as the percentage survival of photosensitizer-treated cells compared with that of the untreated control.

### 2.8. Cell Apoptosis Assay

Annexin V-Fluorescein isothiacyanate-Propidium iodide (AV-FITC-PI) apoptosis assay protocol was used to evaluate the cell death induced by Se-Biotin or NBSe-C6-$NH_2$. Briefly, MCF-7 cells were plated with a density 5 x $10^5$ per well in 24-well plate for 24 h at humidified atmosphere (21 % $O_2$, 5% $CO_2$, 37 $^oC$). After replaced the old medium with 1.5 mL fresh medium, 0.5 mL PBS (as control), NBSe-C6-$NH_2$ (2.5 μM) or Se-Biotin (2.5 μM) was added to each well as appropriate. The cells were further incubated for 1 h under humidified atmosphere at 37 $^oC$. The cell plates were irradiated with or without 660 nm red light (6.0 J/$cm^2$) and incubated under the same condition for 12 h. Then, cells were co-stained with AV-FITC-PI Apoptosis assay according to the manufacture protocol before analyzed using flow cytometry.

### 2.9. Dead/Live Staining

Se-Biotin-mediated cell death was also further investigated using Calcein-AM/propidium iodide (PI) co-stained assy. the MCF-7 cells were seeded with a density of 5 x $10^5$ cellinto 35-cell culture dish for 24 h in an atmosphere containing 21 % $O_2$, 5% $CO_2$, 37˚C. After replaced with fresh medium, cells were treated with Se-Biotin (1.0 μM) for 1 h at 37 $^oC$. Next, the cells were irradiated at 660 nm red light for 10 min (10.0 J/$cm^2$) and incubated for 12 h under the same condition. Then after, MCF-7 cells were co-stained with calcein-AM/PI according to the protocol manual for 30 min under standard condition. After washing cells with PBS three times, MCF-7 cells were imaged using Olympus Fluoview confocal laser fluorescence microscopy with Calcein-Am ($\lambda_{ex/em}$:488/515) and PI ($\lambda_{ex/em}$: 559/617).

### 2.10 Lysosomes Disruption Assay

Acridine orange (AO) staining assay was used to investigate the Se-Biotin-mediated cell death mechanism. Since Photooxidative damage of lysosome leads to release of proteolytic acidic enzymes into cytoplasm, leads to cell necrosis or apoptosis.[36-38] For lysosomes disruption assay, the MCF-7 cells were treated with different groups as illustrated in the following; Group 1: treated with 660 nm LED Light (6.0 J/$cm^2$, NIR light only); Group 2: treated with 2.5 μM NBSe-C6-$NH_2$ for 30 min (NBSe-C6-$NH_2$ only); Group 3: treated

with 2.5 µM Se-Biotin for 30 min (Se-biotin only, only); Group 4: treated with 2.5 µM NBSe-C6-$NH_2$ for 30 min and then irradiated with 660 nm LED Light (6 J/$cm^2$, NBSe-C6-$NH_2$ + Light); Group 5: treated with 2.5 µM Se-Biotin for 30 min and then irradiated with 660 nm LED Light (6 J/$cm^2$, Se-Biotin + Light) After respective treatment condition, all group of cells were incubated with AO (2.5 µg/mL) for 20 min and subsequently, cells were imaged with Olympus confocal fluorescence microscopy and images were collected with 488 nm excitation wavelength, and emission wavelength of 510-540 nm for the green channel and 620-670 nm for the red channel.

## 3. Result and Discussion

### 3.1. Photophysical Property of Se-Biotin

All compounds weresynthesized (scheme 1) and confirmed carefully, and the analog, NBSe-C6-$NH_2$ lacking biotin moiety was used as control. The absorption and emission spectra of Se-Biotin and NBSe-C6-$NH_2$ were recorded in different solvents. As shown in Figure 1a and 1c, Se-Biotin and NBSe-C6-$NH_2$ shows a sharp absorption spectrum at 660-670 nm. Upon the excitation of 660 nm, as shown in Figure 1b, Se-Biotin shows a strong fluorescence emission at 690-750 nm similar to NBSe-C6-$NH_2$(Figure 1d). The photophysical and photochemical properties of both Se-Biotin and NBSe-C6-$NH_2$ was recorded in PBS/ethanol (1:1 v/v) solution as summarized in Table 1. This both excitation and emission wavelength of Se-Biotin in the range of NIR region results a minimum background absorbance and autofluorescence in cellular experiments, reduced photodamage to biosamples and increased tissue PDT depth application. This caused negligible skin photosensitivity after PDT treatments.

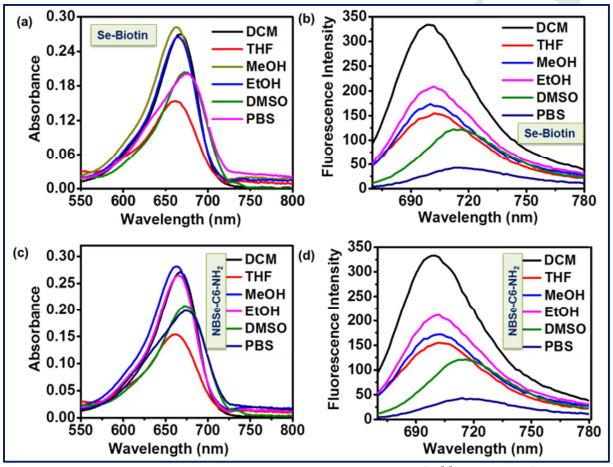



Figure 1.UV-vis spectroscopic spectra in different solvents. (a) absorption and (b) emission spectra of Se-Biotin (5 µM). (c) Absorption and (d) emission spectra of NBSe-C6-$NH_2$ (5 µM). Excitation wavelength is corresponding to maximum absorption wavelength.

The efficiency of singlet oxygen generation of both Se-Biotin and NBSe-C6-$NH_2$ were evaluated using 1,3-diphenliso-benxofuran (DPBF) as photobleaching agent (Figure 2a and Figure 2b). The photooxidation of DPBF by $^1O_2$, Figure 2c, with different irradiation time results in decreased absorbance and therefore provides a means of controlling $^1O_2$ generation.[45]

Table 1. Photophysical property of Se-Biotin and NBSe-C6-$NH_2$

| PDT Agent | $\lambda_{ab}$[a] (nm) | $\lambda_{ab}$[a] (nm) | Ɛ ($M^{-1}cm^{-1}$) | $\phi_f$[b] | $\Phi_\Delta$[c] ($^1O_2$) | $IC_{50}$[d] (nM) |
|---|---|---|---|---|---|---|
| Se-Biotin | 670 | 710 | 5.2 x $10^4$ | 0.026 | 0.69 | 85 |
| NBSe-C6-$NH_2$ | 670 | 710 | 5.3 x $10^4$ | 0.021 | 0.68 | 82 |

[a] Absorption/emission in PBS (1 % DMSO). [b] The absolute fluorescence quantum yield.[c] Relative singlet oxygen ($^1O_2$) quantum yield in PBS/ethanol (1:1 v/v). [d] The half-maximal inhibitory concentration ($IC_{50}$) value. Methylene Blue (MB) was used as standard.

As shown in Figure 2d, the **Se-Biotin**-mediated solution of DPBF shows a rapidly decreased in absorbance upon irradiation of 660 nm over 120 s time interval. Similarly, NBSe-C6-NH2-mediated solution was shown a similar result like it's biotin-conjugated but much higher decay rate than MB. For quantitative evaluation, the$^1O_2$ quantum yield ($\Phi_\Delta$) of bothSe-Biotin and NBSe-C6-$NH_2$ were also calculated using methylene blue (MB), as the reference PDT agent ($\Phi_\Delta$ = 5.2 in ethanol/$H_2O$, 1:1 v/v), to be 0.69 and 0.68, respectively. Notably, such high $^1O_2$ yield was relatively rare over conventional light-sensitized agents, is important to improve PDT efficacy during clinical application.

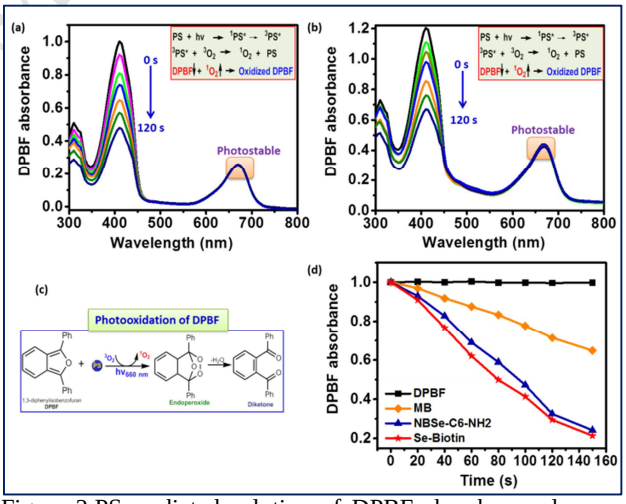



Figure 2.PS-mediated solution of DPBF absorbance decrease over 120 s time intervals under 660 nm red light illuminations (a) Se-Biotin, and (b) NBSe-C6-NH2. (c) Photooxidation of DPBF with $^1O_2$. (d) Comparison of decay rate of DPBF at 410 nm, using Methylene blue as the reference, λex = 660 nm, c = 5 µM in PBS/ethanol (1:1 v/v), 25 °C.

### 3.2 Cellular uptake of Se-Biotin in Live Cell

Since the biotin-ligand has been reported to be highly expressed in several cancer cells, including MCF-7, HepG2, and HeLa.[46, 47] Based on this concept, it can be assumed that Se-Biotin can selectively target cancer cells that overexpressed the biotin receptor, and poorly target the normal cells, such as COS-7 cells. Thus, the cellular uptake of Se-Biotin by MCF-7

cells was assessed in the absence and presence of free biotin receptor. The fluorescence microscopy of MCF-7 cells treated with Se-Biotin displayed an intensive fluorescence (Figure 3a), indicating that binding of the Se-Biotin on the surface of the MCF-7 cells and also localized in endocytic vesicles. In contrast, cells incubated first with 1mM free biotin (Figure 3b), as competitive inhibitor of cellular uptake, and then Se-Biotin displayed only very weak fluorescence indicating that the presence of free biotin receptor can inhibited the uptake of Se-biotin by the cells. Furthermore, the role of cell surface biotin receptors in Se-Biotin was also assessed by incubating with MCF-7 cells (positive biotin receptor) and COS-7 cells (negative biotin receptor) together in co-culture medium. As shown in Figure 3c and 3d, more than 90-fold fluorescent enhancement was obtained in MCF-7 cells than COS-7 cells. This indicates that conjugation of biotin can improve the uptake of Se-Biotin by the biotin-receptor positive cancer cells, and can selectively damage the tumor cells with improved antitumor activity

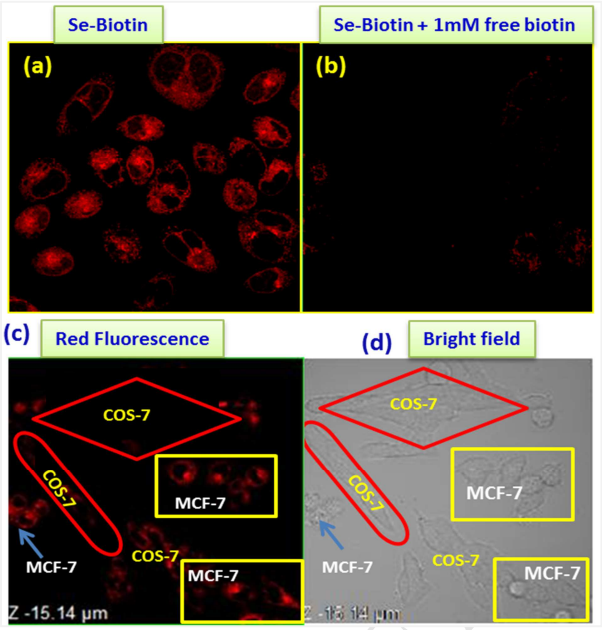



**Figure 3.**Fluorescence image of MCF-7 cells incubated with Se-Biotin (5 µM). Cells were treated in the absence (a) or (b) presence (b) of free biotin receptor (1 mM). (c) Co-culture of MCF-7 cells and COS-7 cells (c) Red Fluorescence, and (d) Bright field. $\lambda_{ex}$ = 640 nm, $\lambda_{em}$ = 650-750 nm.

### 3.3 In Vitro Se-Biotin-mediated $^1O_2$ Generation

To prove the ROS induced by Se-Biotin for antitumor effect of cancer cells, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) was used to detect $^1O_2$ generated during PDT treatment. Owing to its excellent cell membrane permeability, DCFH-DA could easily enter into cells and deacetylated by cellular esterase into a nonfluorescent 2′,7′-dichlorodihydrofluorescein ($DCFH_2$) compound,[48] which is later reacted with $^1O_2$ generated to form green fluorescent 2′,7′-dichlorofluorescein (DCF) upon excitation at 488 nm (Figure 4c). After PDT treatment, the ROS generations in cells were examined using confocal fluorescence microscopy.

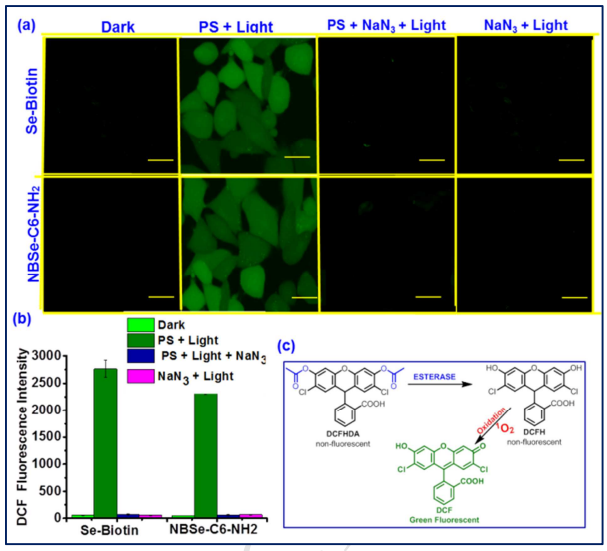



Figure 4. Se-Biotin-mediated Intracellular ROS generation with DCFH-DA staining: (a) Confocal fluorescence images of DCF in MCF-7 cells incubated with Se-biotin (2.0 µM) or NBSe-C6-NH2 (2.0 µM) in different condition after receiving 660 nm laser exposure for 0 or 1 min. (b) Integrated fluorescence intensity of DCF in each group. (c) Schematic illustration of DCFH-DA conversion into Green fluorescent DCF. A green channel emission was collected at 500-530 nm upon excitation at 488 nm. Sodium azide ($NaN_3$, 50 µM) is used as singlet oxygen quencher. Scale: 50 µm.

Upon 660 nm light irradiation, a strong DCF green fluorescence was observed in **Se-Biotin** treated MCF-7 cells (Figure 4a and 4b), demonstrating that abundant $^1O_2$ generation occurred in cells. A very week green fluorescence of DCF was observed in the absence of light illumination or Se-Biotin treated cell only. As a control, sodium azide ($NaN_3$) was used as $^1O_2$ quencher,[49] which was added to the medium and results a negligible green fluorescence of DCF in the cells treated with **Se-Biotin,** indicating that the inhibition of $^1O_2$ generation by $NaN_3$ causes to prevent the cell death. The DCF fluorescence intensity generated in cells were also quantified to analysis the ROS generation. In comparison to the control group, high $^1O_2$ concentrations were produced in cells treated with Se-Biotin like its precursor molecule. These results clearly confirmed that **Se-Biotin** could severely damage the cancer cells selectively whereas leaving adjacent normoxic cells intact.

### 3.4. Phototoxicity of Se-Biotin

A successful antitumor PDT photosensitizer is one that needs to kill tumor cells as effectively as possible while exhibit negligible cytotoxicity to the patient, leading to optimize the therapeutic PDT index and high endocytic internalization into tumor lesion.[50] And it should have also a strong absorption peak in the therapeutic spectra window (650-800 nm), thereby minimizing the skin photosensitivity. The PDT activity of Se-Biotin and NBSe-C6-$NH_2$ was evaluated using MTT Tetrazole assay in both concentration-dependent and light dose-dependent manner. Se-Biotin is almost non-toxic to both MCF-7 cells and COS-7 cell without irradiation under the tested

concentrations (Figure 5a). In contract, the dark toxicity of NBSe-C6-$NH_2$ is little higher than that of Se-Biotin against MCF-7 cells but exhibited high phototoxicity under 660 nm red light irradiation (Figure 5b).The toxicity effect of light treated only was also found to be negligible (data is not shown).

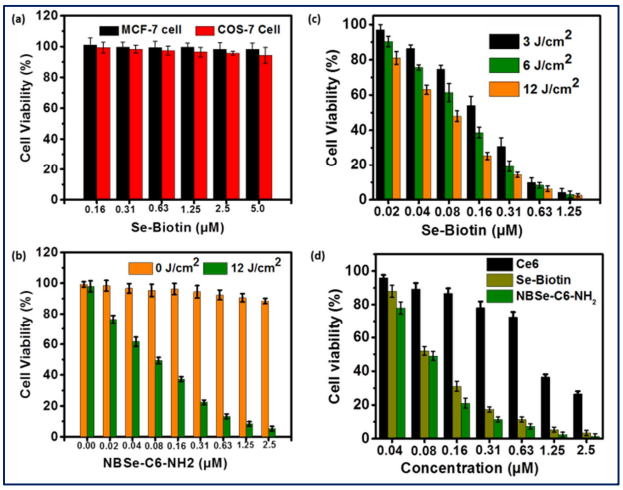



Figure 5. (a) Dark toxicity of Se-Biotin against MCF-7 cells and COS-7 cells. (b) NBSe-C6-$NH_2$-mediated phototoxicity against MCF-7 cells with or without light irradiation (12.0 J/$cm^2$). (c) Se-Biotin-induced toxicity against MCF-7 cells incubated over a varied concentration (0.01 to 2.5 µM) with different power dose (0, 3, 6 and 12.0 J/$cm^2$) at 660 nm red light irradiation. (d) Comparison of cytotoxicity effects on MCF-7 cells treated with NBSe-C6-NH2, Se-Biotin, and Ce6 with a 660 nm red-light illumination (12.0 J/$cm^2$). Data are expressed as mean ± standard error of the mean (SEM, n = 3).

Furthermore, Se-Biotin-induced phototherapy also evaluated over wide range of Se-Biotin concentration (0.02 to 2.5 µM) with light-dose dependent manner (0, 3, 6.0 and 12.0 J/$cm^2$). As illustrated in Figure 5c, Se-Biotin-treated MCF-7 cells showed significant phototoxicity under 660 nm red-light irradiation in all concentration ranges and also show a positive phototoxicity correlation either with Se-Biotin concentration or different light dose. The half-maximal inhibitory concentration ($IC_{50}$) values of NBSe-C6-$NH_2$ and Se-Biotin are summarized in Table 1. Remarkably, the Se-Biotin toxicity efficiency was compared with Ce6 potent clinical photosensitizer (Figure 5d). The $IC_{50}$ of Se-Biotin (0.085 µM) is much lower than that of Ce6 (1.42 µM), which is in nanomolar range. Therefore, Se-Biotin can act as a powerful photosensitizer owing to its minimal background toxicity and severe phototoxicity under NIR light illumination, which is of a great important for the in vivo tumor therapy.

### 3.5. Se-Biotin-induced Cell Apoptosis

To explore the Se-Biotin-induced cancer cell death following PDT treatment, we further investigated the cell death mechanism using live/dead cell staining assay. Recently, cell apoptosis, such as Annexin V-FITC/ PI assay and calcein-AM/PI assay, have been engaged in PDT studies to evaluate the cell death after exposure to PDT light.[51]

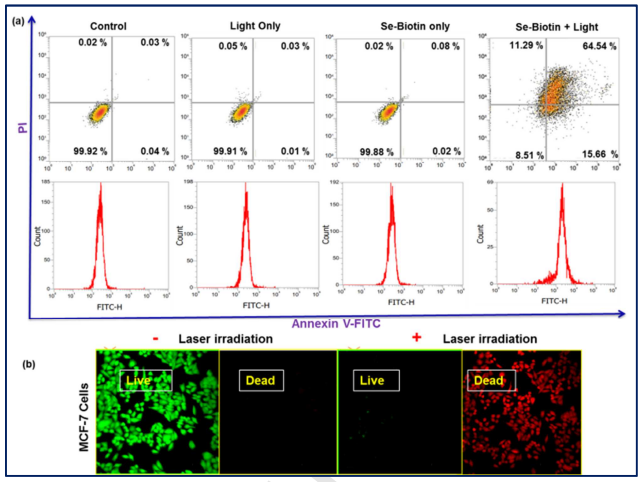



Figure 6. (a) AV-FITC /PI stained MCF-7 cells treated with or without Se-Biotin (2.5 µM) in the absence or presence of 660 nm red light irradiation (6 J/$cm^2$). (b) Calcien-Am/PI stained MCF-7 cells treated with or without Se-Biotin (2.5 µM) with or without illuminated to 660 nm red light (6 J/$cm^2$). Confocal fluorescence imaging was carried out with calcein-AM (Ex/Em: 488/515 nm) and PI (Ex/Em: 559/617 nm). Scale: 50 µm.

To quantify the Se-Biotin-induced cell death, the PDT treatment effect of MCF-7 cells was determined using Annexin V-FITC/ PI cell apoptosis kit. After cells exposed to 660 nm red light (6 J/$cm^2$), cells were double stained with AV–FITC/PI for 5 min before analyzing with flow cytometery. As illustrated in Figure 6a, the Se-biotin treated cell alone and in other control groups showed a negligible cell apoptotic (<1 %), whereas, a significant enhanced apoptotic cells (80.20 %) were found in cells treated with 660 nm NIR light irradiation, which is consistent with the cell viability experimental result. In addition, low necrotic cells (11.29 %) were found against MCF-7 cells treated with NIR light. As shown in Figure 6b, cells treated with Se-Biotin displayed green fluorescence without light irradiation. When Se-biotin-treated cells were exposed to 660 nm red light (6 J/$cm^2$), significant dead cells with red fluorescence was found. These results illustrates that Se-Biotin with NIR light irradiation showed excellent abilities to kill tumor cells. Therefore, all results of the in vitro experiments confirmed that the tumor targeting Se-Biotin could effectively enhance the antitumor PDT effect in cells.

### 3.6. Se-Biotin-Mediated PDT Antitumor Effect

To understand the detailed mechanism of antitumor effect of Se-Biotin, we then further studied the intracellular colocalization using commercial available Lyso-Green Tracker. As manifested in Figure 7a, the red fluorescence of Se-Biotin overlapped well with the green fluorescence of LysoTracke Green (correlation coefficient of 0.84), indicating that Se-Biotin intracellular localized in lysosome.In contrast, very poor correlation coefficients (~0.22 and~0.080) were obtained between our NIR PDT agent (Se-Biotin) and green MitoTracker FM or Hoechst 33342, respectively, probably due to its hydrophobic and weak basic nature of

dialkylaminobenzophenoselenazine scaffolds and also a similar results obtained to the previous related works.[52-54] Recently, many researches, including ours, have reported that acridineorgange (AO) dye was used as the PDT-induced lysososmal membrane destruction indicator.[55, 56] therefore, we used AO as lysosome integrity indicator to assess the Se-Biotin-mediated lysosome dysfunction. For control samples, including untreated, Se-Biotin and light-treated cells, an obviously red fluorescence (yellow arrow) of AO observed, suggesting the lysosomal functions were still intact. Upon 660 nm light illumination, the AO red fluorescence was disappeared in the cells treated with Se-Biotin, indicating that the integrity of lysosomes was several disrupted (Figure 7b). These result demonstrated that Se-biotin-mediated phototherapy could damage the lysosome via $^1O_2$ generation under 660 nm light irradiation, leading to apoptotic cell death via lysosome-disruption pathway.

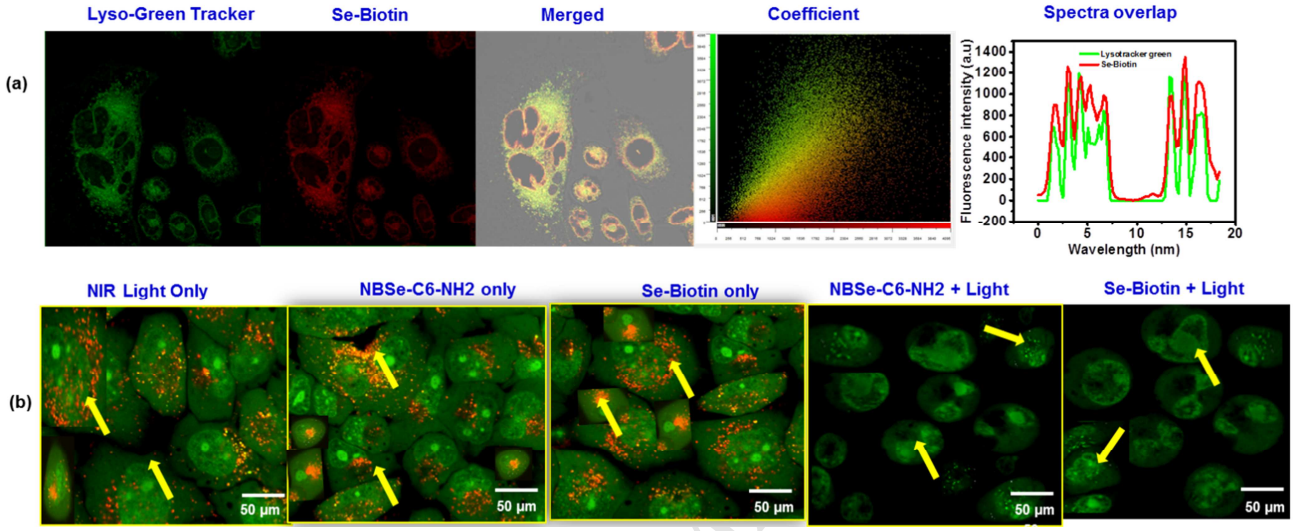



Figure 7. (a) Co-localization of Se-Biotin (2.5 μM) with Lyso-Green tracker (1.0 μM). Green Channel ($\lambda_{ex}$ = 488 nm, $\lambda_{em}$ = 510-550 nm), and Red Channel ($\lambda_{ex}$ = 640 nm, $\lambda_{em}$ = 650-700 nm). (b) AO staining and imaging of MCF-7cells treated with Se-Biotin or NBSe-C6-$NH_2$ with or without PDT light at 660 nm light (6 J/$cm^2$). Cells were stained with AO (2.5 μM). All images were acquired from CLSM, excitation wavelength was fixed at 488 nm, and the emission region scanned between 515-545 nm (green channel) and 610-640 nm (red channel).

## Conclusion

In summary, we have successfully synthesized and developed a new dual NIR photosensitizer, **Se-Biotin**, by conjugating benzo[a]phenoselenazinium derivative dye with biotin as the tumor-targeting ligand via 1,6-diaminohexane linker. The introduction of the biotin-targeted drug moiety in the NBSe-C6-$NH_2$ photosensitizer can completely discriminate between tumor cells (MCF-7 cells) and health cells (COS-7 cells) in difficult co-culture conditions, indicating its excellent tumor-targeting ability. The lysosome-targeting ability of Se-Biotin can further lead to the lysosome of the cancer cells, directly induce lysosome-disruption pathway, and then cause cell apoptosis and necrosis. The $IC_{50}$ value of Se-Biotin is about 85 nM toward biotin overexpressed tumor cells with ultra-low photodynamic action (12 J/$cm^2$) in the NIR region. In addition, the dark toxicity of Se-biotin toward tumor cells was much lower than its non-targeted precursor, NBSe-C6-$NH_2$, suggesting that Se-Biotin may be a safer PDT agent. These result demonstrated that Se-Biotin will be a promising PDT agent for development of novel tumor and lysosome dual-targeted photosensitizer for cancer treatments in the future.

## Supporting Information

The Supporting Information is available free of charge.

## AUTHOR INFORMATION

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## ACKNOWLEDGMENT

This work was financially supported by the National Science Foundation of China (21421005, U1608222, 21422601, 21576037 and 21406028).

## Highlight

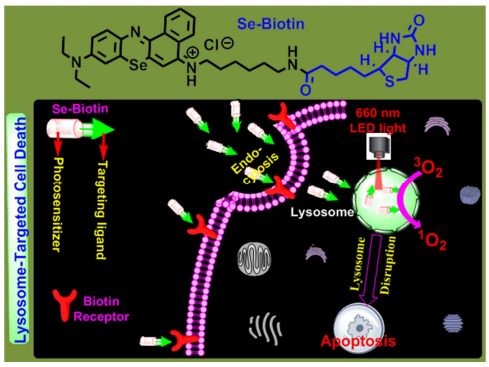
- A benzo[a]phenoselenazine-based dual-targeted NIR photosensitizer,Se-Biotin, is synthesized.

- Se-Biotin exhibits excellent discrimination between MCF-7 cells and Cos-7 cells, and also improved antitumor effect compared to classical photosensitizer Ce6.

- Se-Biotin-mediated $^1O_2$ generation can disrupt lysosomes of cancer cells.

- Se-Biotin-mediated PDT can induce cell apoptosis and death via lysosomal-disruption pathway.

## Graphic Abstract

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