

# A Selenamorpholine and Pyrimidine-based Redox-responsive Fluorescent Probe and Its Response Mechanism

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## Research Article

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# Abstract

A new type of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) fluorescent probe **Pyrimidine-Se** was synthesized from selenomorpholine and pyrimidinyl and the large Stokes shift ( $\Delta\lambda > 140$  nm) was exhibited. The fluorescence intensity of **Pyrimidine-Se** is very sensitive to pH, and its  $pK_a$  value is 9.06. While the probe is reacted with  $\text{H}_2\text{O}_2$ , the selenomorpholine changes from Se (II) to Se (IV), which enhances the electron-withdrawing ability of the **Pyrimidine-Se** electron-withdrawing group. Based on this, the probe **Pyrimidine-Se** was used to detect  $\text{H}_2\text{O}_2$  by the fluorescence spectrum. The detection limit of the probe Pyrimidine-Se was 1.3  $\mu\text{M}$ . At the same time, we also found that **Pyrimidine-Se** displayed the reversibility back and forth between  $\text{H}_2\text{O}_2$  and GSH. The reaction mechanism with  $\text{H}_2\text{O}_2$  was verified by mass spectrometry and simulation on the Gaussian 09 program.

## Introduction

Hydrogen peroxide is a light blue liquid compound combined with a covalent bond. It can be miscible with water in any proportion and easily penetrate the cell membrane. It is generally considered that high concentration of  $\text{H}_2\text{O}_2$  has cytotoxicity to a variety of animals, plants, and bacteria [1–2], and it is also an important product of cell life activities. It is known that the  $\text{H}_2\text{O}_2$  in the cell is mainly produced by the NADPH oxidase complex, and  $\text{H}_2\text{O}_2$  appears as a by-product in a variety of enzymatic reactions. For example, the conversion of glucose to glucose lactone by the oxidation of glucose oxidase can produce  $\text{H}_2\text{O}_2$  in the cell [3–6]. The normal concentration of peroxide concentration plays an important role in regulating cell proliferation, differentiation, aging, and signal transduction [7]. The abnormal cell content of  $\text{H}_2\text{O}_2$  may cause cancer, Alzheimer's disease, etc [8–14]. The redox state of cells is dynamically regulated by reactive oxygen species and biological thiols, which can maintain the normal life activities of cells [15–18]. Therefore, a means to dynamically monitor the oxidation-reduction state is needed.

At present, the main methods that have been developed to detect hydrogen peroxide are: titration [19], spectrophotometry [20], bioluminescence [21], mass spectrometry [22], chromatography [23], electrochemistry [24], resonance spectroscopy [25–26], and fluorescence analysis [27–32]. Compared with other detection methods, fluorescence analysis has become an important tool for biological imaging detection due to its excellent selectivity, high sensitivity, high spatial and temporal resolution, non-destructive detection and low cost of use. A variety of  $\text{H}_2\text{O}_2$  fluorescent probes have been reported before, and most of these probes are intensity-based turn-on fluorescent sensors. Changes in probe position, probe concentration, probe environment, and excitation intensity may affect the measurement of fluorescence intensity as well as emission collection efficiency and excitation intensity, which may further affect the accuracy and reliability of fluorescence intensity measurement. Moreover, there are still very few  $\text{H}_2\text{O}_2$  fluorescent probes capable of cycling redox reactions [33–34]. For this reason, we designed and synthesized a new type of  $\text{H}_2\text{O}_2$  fluorescent probe Pyrimidine-Se, which is composed of selenomorpholine group [35–36] and pyrimidine group connected with N, N-dimethylaminophenyl group

(scheme 1). The selenomorpholine group is the recognition group of  $\text{H}_2\text{O}_2$ , and GSH can reduce and oxidize the selenomorpholine group.

**Pyrimidine-Se** responds quickly to  $\text{H}_2\text{O}_2$ , and the reaction with excess  $\text{H}_2\text{O}_2$  is almost instantaneous. The fluorescence intensity of **Pyrimidine-Se** has a good linear relationship with 0–20 times the concentration of  $\text{H}_2\text{O}_2$ , and the linear correlation coefficient ( $R^2$ ) is 0.996. **Pyrimidine-Se** has good specificity and some ions can detect a variety of reactive oxygen species. The concentration of reactive oxygen species (ROS), amino acids, ions, etc. ( $100\ \mu\text{M}$ ) is much greater than  $\text{H}_2\text{O}_2$  ( $20\ \mu\text{M}$ ), and the fluorescence intensity of other detected substances is not significant. The change. At the same time, we also tested the probe's responsiveness to  $\text{H}_2\text{O}_2$  and GSH, and the results showed that the cyclic response to both can last 4 times. We simulated the reduced, oxidized, and acidified probes on the Gaussian 09 software through the B3LYP/6–31\* OPT FREQ level. We also used nuclear magnetic titration and mass spectrometry to explain the mechanism of the probe detecting  $\text{H}_2\text{O}_2$ : **Pyrimidine-Se** After responding to  $\text{H}_2\text{O}_2$ , the valence state of Se changes from Se(II) to Se(IV), and the electron transfer intensity inside the probe increases, which leads to an increase in the fluorescence intensity of **Pyrimidine-Se**.

## Materials And Equipment

The chemicals used are all purchased from suppliers such as Anaiji, Macleans, Aladdin, and Daimo, and no further purification is required before use. In the synthesis process, the high-efficiency thin-layer plate was used for TLC analysis (thin-layer chromatography (TLC) analysis), and the product was purified by silica gel column chromatography using Qingdao Ocean Silica Gel (200-300 mesh).  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra were recorded on Bruker DRX-600. The HR-ESI-MS was detected by Bruker Solarix XR FTMS from the Analysis and Testing Center of Peking University. All fluorescence data detection uses Toshiba F-2700 fluorescence spectrophotometer, and absorption spectrum detection uses SP-1920 UV-Vis spectrophotometer of Shanghai Spectrometer Co. Ltd. The pH measurement used METTLER TOLEDO's FE28 type pH meter. According to Xu's [35] work, selenomorpholine was synthesized. Reactive oxygen solution (ROS) was prepared according to Jiao Shan's [37] work. The water used in the configuration solution is ultrapure water (micropores,  $\geq 18\text{M}\Omega$ ).

### Synthesis

The first step of **Pyrimidine-Se** synthesis is to synthesize the fluorophore host compound **1** by the Knauvengel reaction of *p*-dimethylaminobenzaldehyde and 2-chloro-4, 6-dimethylpyrimidine.  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR, and high-resolution mass spectrometry were used to characterize the structure of compound **1** and **Pyrimidine-Se**. (Fig.S2-Fig.S7) Fortunately, we obtained its crystals (Fig. **1**) (CCDC number: 2097485) by slowly evaporating a solution of **Pyrimidine-Se** (methanol: acetonitrile (V/V)=4:1) at room temperature.

### Synthesis of Selenomorpholine

Under the protection of Ar, selenium powder (7.9 g, 0.10 mmol) was stirred in anhydrous ethanol (100 mL) in a 250 mL three-necked flask at -10 °C. Sodium borohydride (4.3 g, 0.11 mmol) was gradually added to the solution in portions until colorless. Then sodium hydroxide (4.4 g, 0.11 mmol) was added to the solution in portions and stirred for 30 minutes. An ethanol solution of bis(2-chloroethyl)amine hydrochloride (17.8 g, 0.10 mmol) was gradually added and refluxed for 6 h. The mixture was cooled to room temperature, filtered to remove the insoluble matter, and then the solvent was removed by rotary evaporation. Distillation under reduced pressure (56 °C/1.32 kP) gave a colorless oil with a yield of 30%.

### Synthesis of Compound 1

In a 150 mL thick-walled pressure bottle, 2-chloro-4, 6-dimethylpyrimidine (142.6 mg, 1 mmol), *p*-dimethylaminobenzaldehyde (895.1 mg, 6 mmol), 1 mL of concentrated hydrochloric acid were dissolved in 20 mL absolute ethanol. The reaction was refluxed for 24 h. After TLC (Thin layer chromatography), the solvent was removed by rotary evaporation and column chromatography (PE: DCM(V/V)=10:1) to obtain a green solid (110mg, 0.4mmol) with a yield of 40%. <sup>1</sup>H NMR (600 MHz, DMSO) δ 7.80 (d, *J* = 15.9 Hz, 1H), 7.55 (d, *J* = 8.6 Hz, 2H), 7.38 (s, 1H), 6.92 (d, *J* = 15.9 Hz, 1H), 6.74 (d, *J* = 8.6 Hz, 2H), 2.98 (s, 6H), 2.42 (s, 3H). <sup>13</sup>C NMR (151 MHz, DMSO) δ(ppm): 170.3, 165.8, 159.8, 151.4, 139.0, 129.6, 122.5, 118.8, 116.0, 112.0, 39.8, 23.4. HR-ESI-MS: *m/z* for C<sub>15</sub>H<sub>16</sub>ClN<sub>3</sub><sup>+</sup> Found: [M+H<sup>+</sup>] = 274.11053, calcd: [M+H<sup>+</sup>] = 274.11055

### Synthesis of Pyrimidine-Se

In a 15 mL reaction tube, under the protection of argon, selenomorpholine (150 mg, 1 mmol), compound 1 (68.5 mg, 0.25 mmol), and TEA (30.4 mg, 0.3 mmol) were dissolved in 5mL anhydrous DMF at 90°C (Oil temperature) Reaction for 12 h. After TLC, the solvent was evaporated and then purified by column chromatography (PE: EA=10:1, V/V) to obtain a yellow solid (46.3 mg, 0.12 mmol), yield: 48%.

<sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>) δ 7.67 (d, *J* = 15.8 Hz, 1H), 7.47 (d, *J* = 8.5 Hz, 2H), 6.74-6.67 (m, 3H), 6.40 (s, 1H), 4.37-4.29 (m, 4H), 3.01 (s, 6H), 2.76 - 2.69 (m, 4H), 2.32 (s, 3H). <sup>13</sup>C NMR (151 MHz, CDCl<sub>3</sub>) δ(ppm): 167.9, 163.6, 160.8, 151.0, 136.0, 129.0, 124.0, 121.9, 112.1, 107.9, 40.3, 38.1, 36.3, 24.3. HR-ESI-MS: *m/z* for C<sub>19</sub>H<sub>25</sub>N<sub>4</sub>Se<sup>+</sup> Found: [M+H<sup>+</sup>] = 389.12346, calcd: [M+H<sup>+</sup>] = 389.12390

## Results And Discussion

The UV-Vis spectrophotometer was used to determine the maximum absorption wavelength of the probe **Pyrimidine-Se** (10 μM) in different solvent systems (Fig. 2), and the probe **Pyrimidine-Se** was measured in the range of 400 nm-500 nm with a fluorescence spectrophotometer for six different The solvent is screened. The maximum absorption wavelength ( $\lambda_{ex}$ ) of the probe **Pyrimidine-Se** in the six solvents is 382 nm. The fluorescence intensity of **Pyrimidine-Se** varies greatly in different solvent systems (Fig. 3). The highest brightness is in DMSO, the highest The weak one is in ethanol (the photomultiplier tube voltage is 400V).

We evaluated the optical response of the probe **Pyrimidine-Se** to pH (Fig. 4 and Fig. 5). When the pH value is from 3 to 5, the fluorescence intensity of **Pyrimidine-Se** at 525 nm changes greatly, almost an increase of 18 times. Using the Henderson-Hasselbalch equation to analyze the data, the pKa of **Pyrimidine-Se** was calculated to be 9.06. The absorbance of the probe **Pyrimidine-Se** at 395 nm decreased rapidly, and the absorbance at 455 nm increased rapidly (Fig. 6). Due to the basicity of the pyrimidine on **Pyrimidine-Se** and the influence of the nitrogen atom on the selenomorpholine group, the fluorescent probe is greatly affected in a highly acidic environment. By calculating the B3LYP/6-31\* OPT FREQ level on the Gaussian 09 program (Fig. 16), the selenomorpholine of **Pyrimidine-SeH<sup>+</sup>** is in phase with the selenomorpholine groups of **Pyrimidine-Se** and **Pyrimidine-SeO**. The deflection of nearly 90 ° occurs, and the energy difference between the excited state (LUMO) and the ground state (HOMO) becomes smaller. The charge of **Pyrimidine-SeH<sup>+</sup>** is mainly concentrated near the benzene ring, which is very different from the fluorescence quenching in an acidic environment. Big relationship.

After the probe **Pyrimidine-Se** reacts with H<sub>2</sub>O<sub>2</sub>, its maximum absorption wavelength is red-shifted from 382 nm to 388 nm (Fig. 7), and the fluorescence emission wavelength is also red-shifted (Fig. 8).

The response time of **Pyrimidine-Se** (10 μM) to H<sub>2</sub>O<sub>2</sub> (10 μM, 20 μM, 100 μM) was tested, and the results showed that the probe has a high sensitivity to H<sub>2</sub>O<sub>2</sub> (Fig. 9). **Pyrimidine-Se** and excess H<sub>2</sub>O<sub>2</sub> can quickly complete the reaction. In a solution of (pH=7.4, 10 mmol, 30% DMSO), the wavelength of the excitation wave is 388 nm, and the probe shows weak fluorescence at 528 nm. After being oxidized by H<sub>2</sub>O<sub>2</sub>, the fluorescence of the probe at 528nm increases.

As shown in Fig. 11, the fluorescence intensity of the probe **Pyrimidine-Se** increases with the increase of H<sub>2</sub>O<sub>2</sub> concentration and reaches the peak at the concentration of 700 μM. What attracts us most is that the fluorescence intensity shows a good linear relationship in the range of H<sub>2</sub>O<sub>2</sub> concentration between 0-200 μM. By setting the H<sub>2</sub>O<sub>2</sub> concentration (μM) as the abscissa, the fluorescence intensity of **Pyrimidine-Se** at 525 nm is set as the ordinate. , The linear curve between the probe **Pyrimidine-Se** at 525 nm and the concentration of H<sub>2</sub>O<sub>2</sub> is fitted. As shown in the figure, the two have a good linear relationship when the H<sub>2</sub>O<sub>2</sub> concentration is between 0-40 μM. The fitting equation is  $y=0.57661x+93.9575$ , and the detection limit is 1.3 μM.

The reaction of **Pyrimidine-Se** (10 μM) to reactive oxygen species and other analytes was studied in PBS Buffer (30% DMSO, 10 mM, pH=7.4). According to reports, selenomorpholinyl has competitive adaptability to the detection of H<sub>2</sub>O<sub>2</sub> and hypochlorite. As shown in the Fig. 15, **Pyrimidine-Se** has high specificity for H<sub>2</sub>O<sub>2</sub> detection, and the fluorescence of H<sub>2</sub>O<sub>2</sub> detection ( $\lambda_{em}=525$  nm) is much stronger than other he fluorescence intensity of other analytes increased slightly, but it was negligible. As shown in the figure, when other analytes and H<sub>2</sub>O<sub>2</sub> are both present, the detection of H<sub>2</sub>O<sub>2</sub> by **Pyrimidine-Se** will not be interfered.

## Fluorescence reaction of Pyrimidine-Se on redox cycle

Biological thiols such as glutathione, cysteine, and homocysteine can reduce the active groups oxidized in cells. Therefore, biological thiols play an important role in maintaining the balance of intracellular reactive oxygen species concentration. Selenium is an important trace element in the human body. Its unique chemical properties have excellent performance in eliminating reactive oxygen species and free radicals. **Pyrimidine-Se** responds to the redox cycle of  $H_2O_2$  and glutathione. It can be seen from Fig. 16 that the fluorescence of **Pyrimidine-Se** probe increased significantly after adding 5 eq  $H_2O_2$ . After adding 3 eq of GSH for two hours, it can be seen that the fluorescence of the probe decreases close to the initial state, and then add 5 eq of  $H_2O_2$  to the solution to wait for fluorescence enhancement. The redox process can be cycled at least 4 times. It is proved that the probe **Pyrimidine-Se** can realize the continuous cyclic response between  $H_2O_2$  and GSH, indicating that **Pyrimidine-Se** has the potential for real-time imaging of redox cycle.

### Pyrimidine-SeH<sup>+</sup>

Fig.17 predicted the detection mechanism of **Pyrimidine-Se** on  $H_2O_2$ . The oxidation reaction of **Pyrimidine-Se** with  $H_2O_2$  was verified by MS spectroscopy. After adding  $H_2O_2$ , the valence state of Se in **Pyrimidine-Se** changed from Se(II) to Se(IV), and then **Pyrimidine-SeO** was formed. It can be seen in the MS spectrum (Fig. S1) that  $[M^+]=386.8$  and  $[M^+]=402.7$ , indicating **Pyrimidine-Se** produces **Pyrimidine-SeO**. The oxidized selenomorpholine enhances the charge attraction ability of the pyrimidine group, enhances the ICT process of the probe molecule and its fluorescence intensity.

Using density functional theory (DFT) to optimize the structure of **Pyrimidine-Se**, **Pyrimidine-SeO** and **Pyrimidine-SeH<sup>+</sup>** at the B3LYP/6-31\* OPT FREQ level on the Gaussian 09 program. The structure, electron density, and molecular electrostatic potential are analyzed accordingly. As shown in the Fig. 17, the selenomorpholines of **Pyrimidine-Se**, **Pyrimidine-SeO**, and **Pyrimidine-SeH<sup>+</sup>** present boat-like configurations. The electrons in the ground state (HOMO) of **Pyrimidine-Se** and **Pyrimidine-SeO** are mainly concentrated in the N, N-dimethylaminophenyl group. In the excited state (LUMO) state of the two, the charges are attracted by the strongly attracting pyrimidine groups and then migrate. When Se(II) is oxidized to Se(IV), the charge is enriched in Se=O, which enhances the intensity of charge transfer (ICT) in the molecule, and thus the fluorescence increases (Fig. 18). The selenomorpholine of **Pyrimidine-SeH<sup>+</sup>** undergoes a nearly 90 ° twist after accepting hydrogen ions. From its ESP diagram, it can be seen that its charge is mainly concentrated in the pyrimidine group and mainly exhibits an excited state (LUMO). The ground state (HOMO) and excited state (LUMO) energies of **Pyrimidine-SeH<sup>+</sup>** are much smaller than those of **Pyrimidine-Se** and **Pyrimidine-SeO**, so the fluorescence of **Pyrimidine-SeH<sup>+</sup>** is quenched in an acidic environment.

## Conclusion

A novel hydrogen peroxide fluorescent probe **Pyrimidine-Se** with large Stokes shift was synthesized. It was synthesized by a simple combination of selenium morpholine and pyrimidine fluorescent groups.

Through the change of Se (Ⅱ) to Se (Ⅳ) after reaction with H<sub>2</sub>O<sub>2</sub>, the probe affects the electron absorption ability of pyrimidine in pyrimidine fluorescent group, so as to realize the change of fluorescence intensity. The probe can quickly and specifically recognize H<sub>2</sub>O<sub>2</sub>, which is less affected by other reactive oxygen species (ROS) and ions. The fluorescence intensity of the probe **MNG** has a good linear relationship in the range of H<sub>2</sub>O<sub>2</sub> concentration 0-200 μM. In addition, the **Pyrimidine-Se** redox cycle can last at least four times. Therefore, it has the potential of real-time imaging of the redox process.

## Declarations

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**Conflict of Interest** There are no conflicts to declare.

**Ethics approval/declarations** Not applicable

**Consent to participate** I would like to declare on behalf of my co-authors that the work described was original research that has not been published previously, and not under consideration for publication elsewhere, in whole or in part. All the authors listed have approved the manuscript that is enclosed.

**Consent for publication** We would like to submit the enclosed manuscript entitled "A selenamorpholine and pyrimidine-based redox-responsive fluorescent probe and its response mechanism", which we wish to be considered for publication in "Journal of Fluorescence".

**Availability of data and material/ Data availability** Not applicable

**Code availability** Not applicable

**Authors' contributions** Tao Ma: Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Writing-review & editing, Project administration. Yiyun Li: Visualization. Changchun Yuan, Wenbing Ma, Zhichun Li, Ling Ma: Conceptualization, Methodology, Resources, Writing-review & editing, Supervision.

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## Scheme 1

Please see the Supplementary Files for the Scheme 1.

## Figures

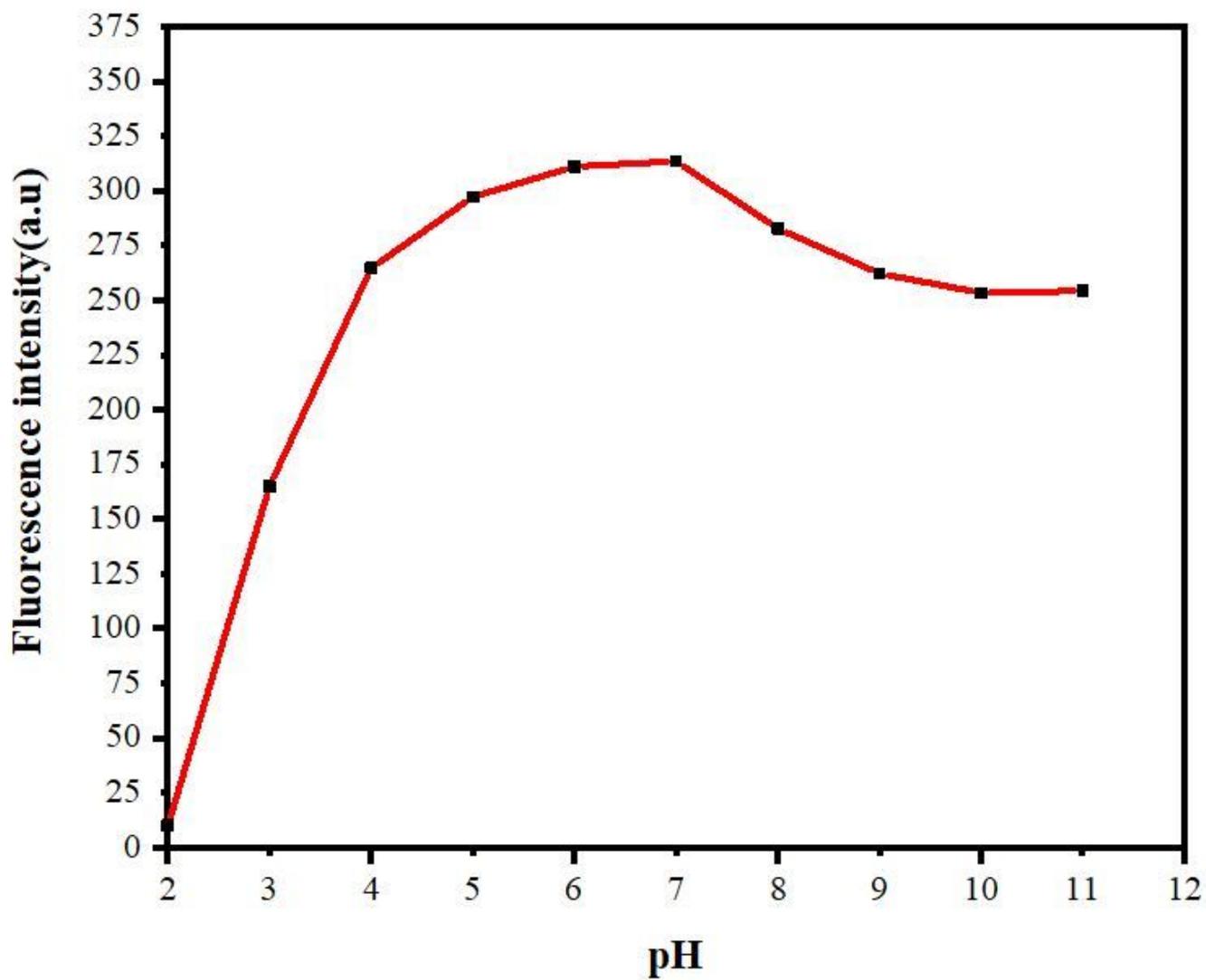
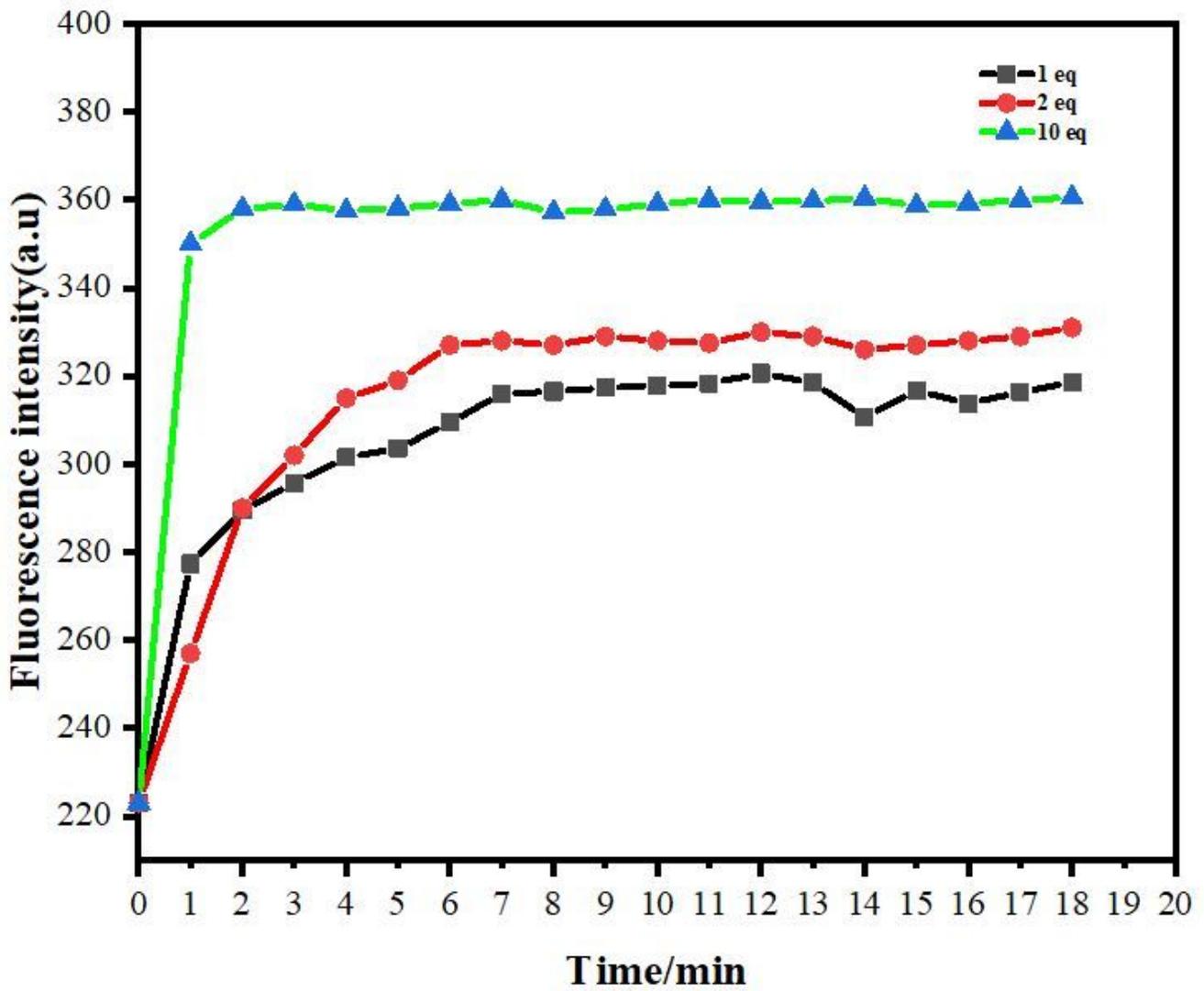


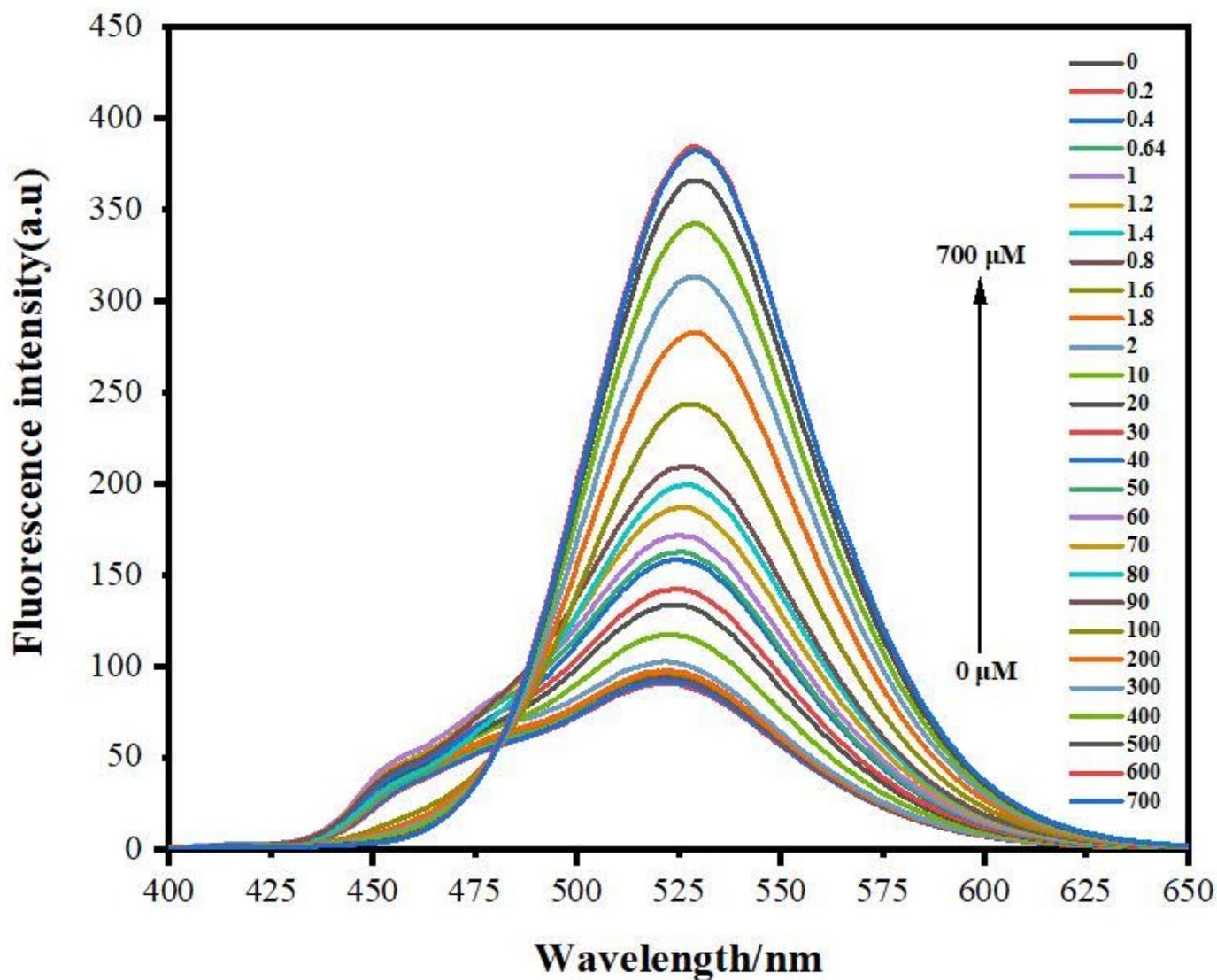
Figure 5

Fluorescence intensity of Pyrimidine-Se (10  $\mu$ M) in PBS Buffer(10 mmol) of different pH containing 30% DMSO



**Figure 9**

The response rate of Pyrimidine-Se (10  $\mu$ M) to 10  $\mu$ M, 20  $\mu$ M, 100  $\mu$ M hydrogen peroxide in PBS Buffer (10 mmol, pH=7.4, 30% DMSO)



**Figure 10**

Fluorescence intensity of Pyrimidine-Se (10 μM) in PBS Buffer (pH=7.4, 10 mmol, 30% DMSO) in response to hydrogen peroxide (0-700 μM)

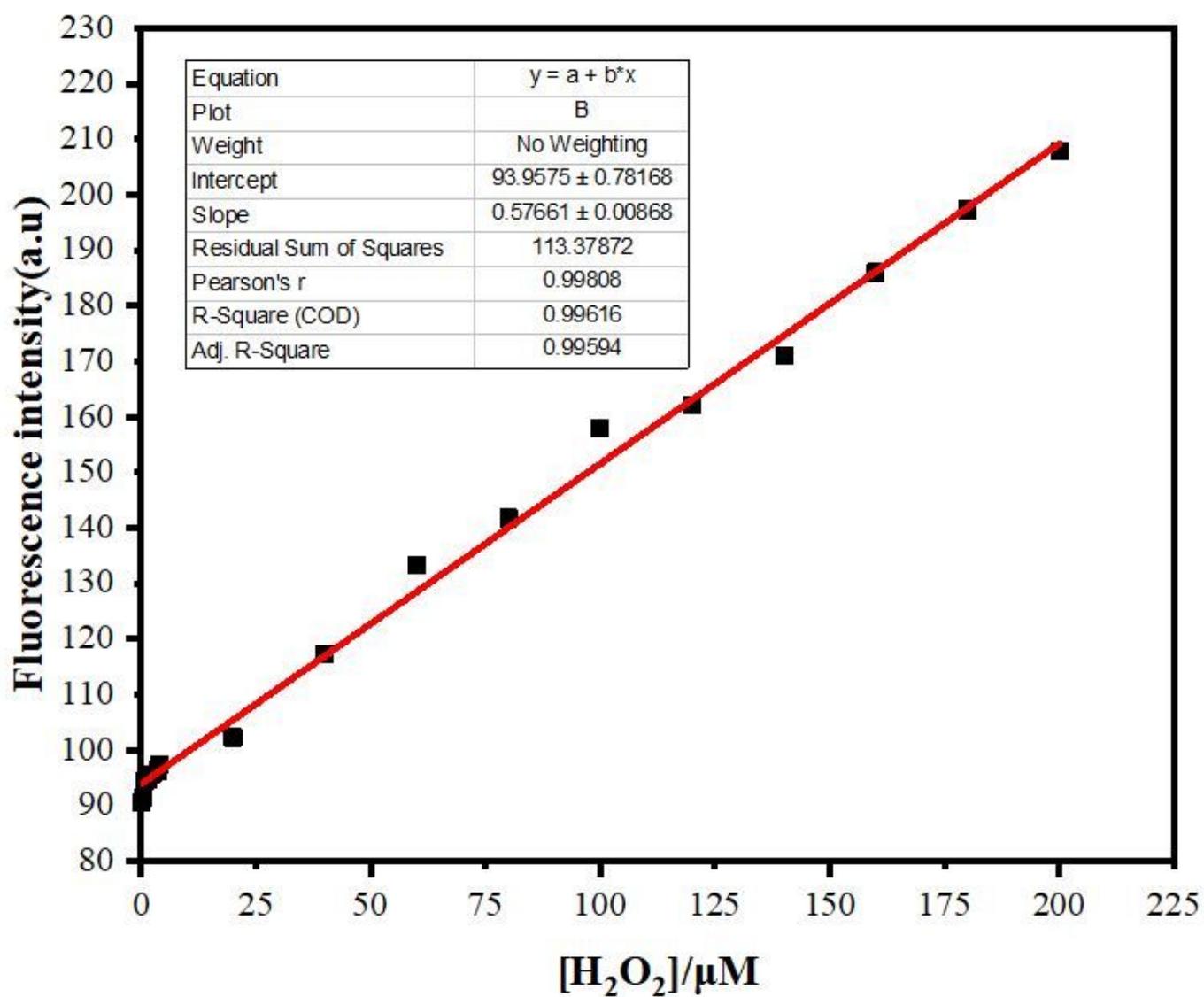
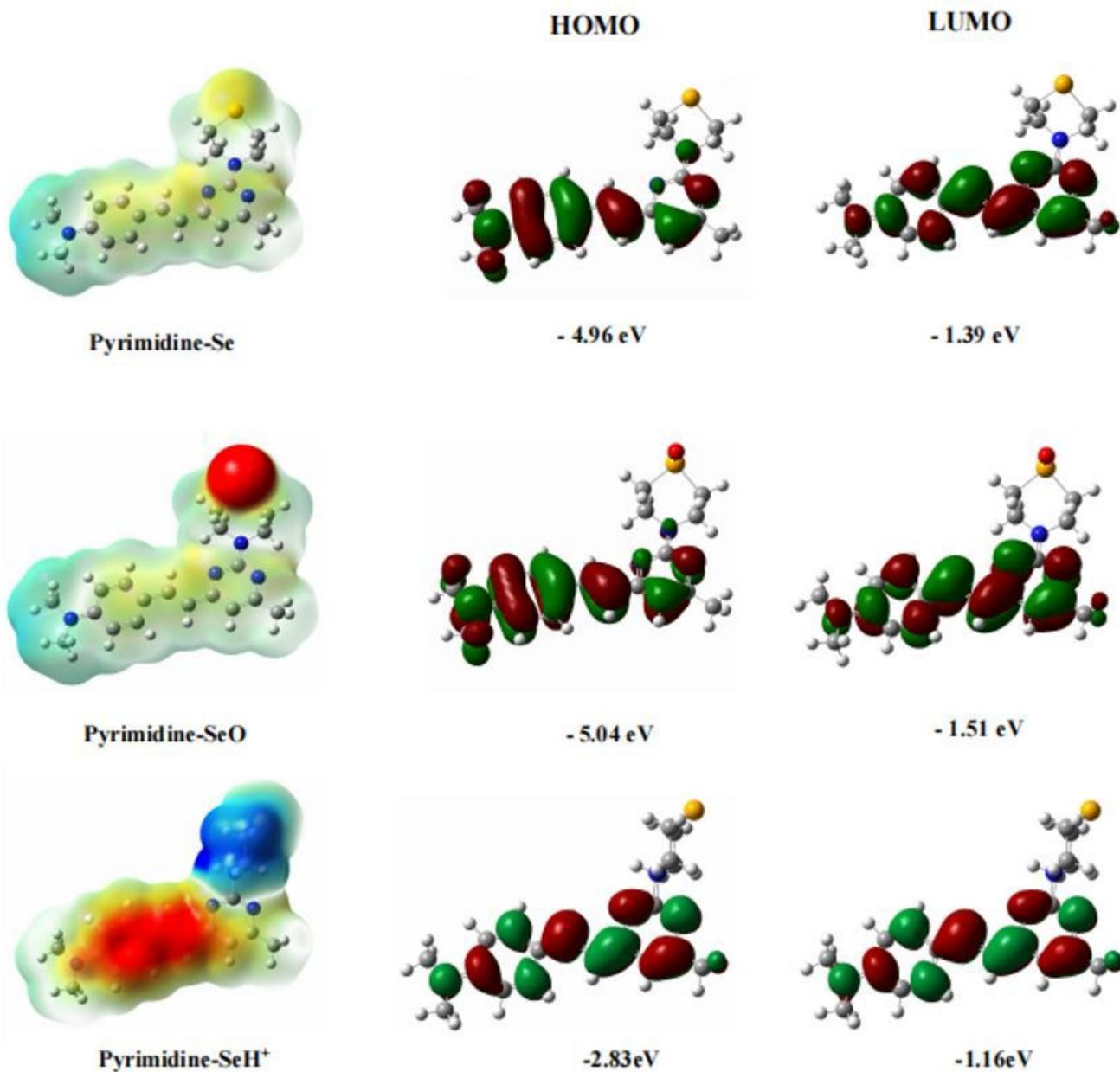


Figure 12

H<sub>2</sub>O<sub>2</sub> concentration is in the 0-200 μM range, and the probe fluorescence intensity is linearly related to the H<sub>2</sub>O<sub>2</sub> concentration.





**Figure 18**

Density functional theory (B3LYP/6-31\* OPT FREQ) calculated molecular orbitals and molecular electrostatic potential diagrams of Pyrimidine-Se, Pyrimidine-SeO and, Pyrimidine-SeH<sup>+</sup>