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Electronic Supplementary Information

Intermolecular Interaction of Chloroquine with Peptide-Bonded-(N)-Ethyl Selenol-(C)-Ethanethiol

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Contents

1. General information

1.1 Materials

Ethanol (EtOH, 99%) was purchased from Shanghai LingFeng Chemical Reagent CO., LTD. Sodium bicarbonate ($NaHCO_3$, 99%), 1,3- diphenylisobenzofuran (DPBF, ≥98%), anhydrous magnesium sulfate ($MgSO_4$, 99%), dimethyl sulfoxide (DMSO, 99.9%), chloroquine diphosphate salt (CQ, >98%), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, 99%) and tetrazolium bromide (MTT, ≥99%) were purchased from Adamas. 2',7'dichlorofluorescin diacetate (DCFH-DA, ≥97%) and 1,2-bis(triethoxysilyl)ethane (BTEE, 96%) were obtained from Sigma-Aldrich. Dichloromethane (DCM, 99.5%), sodium borohydride ($NaBH_4$, 99%), methylene blue (MB, 99%), ethyl acetate (EtOAc, 99%), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI, 98%), Triethylamine (TEA, 99%) and selenocystamine dihydrochloride (97%) were acquired from Aladdin. 3,3'-Disulfanediyldipropanoic acid (>99%), 1-hydroxybenzotriazole monohydrate (HOBt, >97%) and 1-chloro-2, 4-dinitrochlorobenzene (DNCB, >99%) were provided by TCI. Hexadecyltrimethylammonium bromide (CTAB, 99%), hydrochloric acid (HCl, 99%), Sodium hydroxide (NaOH, 99%), sodium Chloride (NaCl, 99%), L-histidine (His,99.8%) and ammonia ($NH_3 \cdot H_2O$， 99%) were purchased from Sinopharm Chemical Reagent Co., Ltd.

1.2 Characterization

Fourier transform infrared (FT-IR) spectra were acquired using an IR Prestige-21 (Shimadzu) spectrophotometer. Electrospray ionization mass spectrometry (ESI-MS) was measured on an Agilent 1100 Series LC/MSD (ESI). High Performance Liquid Chromatography (HPLC) was conducted on an Acchrom S6000 system using a Unitary C18 column （4.6×250 mm, 5 μm）; acetonitrile (degassed)/water (degassed) = 15:85 (v/v), 25 ℃, 0.5 mL/min, λ = 230 nm (DAD detector). X-ray photoelectron spectroscopy spectra were obtained by the ESCALAB Xi^+ (Thermo Fisher, USA) X-ray

photoelectron spectrometer. UV-visible spectra were acquired by UV2300 spectrophotometer (Shimadzu, Japan).

2. Thermodynamic and kinetic calculations

Thermodynamic and kinetic calculations were employed to investigate the interactions among CQ and selenol/thiol. For comparison, the interactions of imidazole (IM) with selenol and thiol were also carried out. The selenol and thiol were modeled by ethyl selenol (CH_3CH_2SeH) and ethanethiol (CH_3CH_2SH), CQ were separated into triethylamine (TEA) and chloroquinoline (CQN). The energy barriers ($\Delta G^{\neq}$) and energy changes (ΔG) for the formation of triethylammonium selenolate (TEA-Se), triethylammonium thiolate (TEA-S), chloroquinolinium selenolate (CQN-Se), chloroquinolinium thiolate (CQN-Se), imidazolium selenolate (IM-Se) and imidazolium thiolate (IM-S) ion pairs, as well as the interaction energies (E_{int}) for TEA-Se, CQN-Se, IM-Se were calculated by quantum-chemical calculations at the density functional theory (DFT) level with Gaussian 16W software. The values of $\Delta G^{\neq}$ were obtained by subtracting the sum energy of neutral acid and neutral base from the energy of transition state (TS), and ΔG values were calculated by subtracting the sum energy of neutral acid and base from the energy of the product complex. E_{int} values were computed by subtracting the sum energy of isolated cation and anion in the geometry of the complex from the energy of the ionic complex. The optimized geometries of molecules, ions, and ion pairs were calculated by the dispersion-corrected B3LYP functional (B3LYP+GD3 method) in conjunction with the 6-31+G (d, p) basis set. The Polarizable Continuum Model (PCM) of water, specifically the integral equation formalism variant (IEFPCM) was employed to account for solvation effects.[1] $\Delta G^{\neq}$, ΔG for different chemical reactions and E_{int} of TEA-Se, CQN-Se and IM-Se were computed using the dispersion-corrected M062X functional (M062X+GD3 method) in combination with the ma-def2-TZVP basis set. The Solvation model of water based on density (SMD) was utilized to incorporate solvation effects.

3. Supplementary chemically synthesized methods

3.1 Preparation of peptide-bonded-(N)-ethyl selenol-(C)-ethanethiol (Pep-Se-S)

We successfully designed and synthesized a TrxR-simulative small molecule, peptide-bonded-(N)-ethyl selenol-(C)-ethanethiol (Pep-Se-S), which exhibits reduction activity toward DTNB comparable to that of TrxR. To prepare Pep-Se-S, 3,3'-disulfanediyldipropanoic acid (0.3 mmol), EDCI (0.65 mmol), HOBt (0.65 mmol) and selenocystamine dihydrochloride (0.6 mmol) were added to DCM (24 mL). Subsequently, 0.36 mL of triethylamine (TEA) and 6 mL of ethanol were gradually added to the mixture with stirring at 0 ℃ for 30 min in darkness under a N_2 atmosphere. Once the selenocystamine had completely dissolved, the solution was gradually warmed to room temperature and stirred overnight. After that, solvent was removed using a rotary evaporator and the yellow residue was sequentially washed with 5% HCl (15 mL, three times), 5% $NaHCO_3$ (20 mL), deionized water (20 mL), and saturated salt water (20 mL). The residue, now designated as Pep-Se-S Precursor (Pep-Se-S-P), was subsequently collected by centrifugation. For further reaction, Pep-Se-S-P was dispersed to 8 mL of ethanol and stirred for 30 min under a N_2 atmosphere at 0 ℃ in darkness. Next, $NaBH_4$ ethanol dispersion (1.8 mmol, 2 mL) was slowly added to the above mixture and the reaction was proceeded for additional 2 h. Afterwards, 3 mL of degassed 1 M HCl was added to the above solution. The combined organic phase was then extracted thrice using a degassed mixed solution of DCM and EtOAc (7:3, v/v, 20 mL each time) and subsequently dehydrated with anhydrous $MgSO_4$. The anhydrous $MgSO_4$ was removed via vacuum filtration under a N_2 atmosphere. Finally, Pep-Se-S was obtained as a white waxy solid after solvent removal using a rotary evaporator and stored under an argon atmosphere at 4 ℃.

3.2 Preparation of peptide-bonded-(N)-ethyl selenol-(C)-ethanethiol and CQ complex (Pep-Se-S-CQ)

CQ (in free base form, 0.1 mM) was dissolved in 8.3 mL of a degassed solution containing DCM and ethanol (2:1, v/v), followed by the addition of an equivalent amount of Pep-Se-S, all under a N_2 atmosphere. The solution was shaken at room temperature for 6 h and then left to stand at 4 ℃ overnight in a N_2 atmosphere. Subsequently, the solvent was evaporated to 1.66 mL under a N_2 atmosphere and the solid product was isolated by centrifugation. The as-prepared Pep-Se-S-CQ was stored under an argon atmosphere at 4 ℃ The UV-vis spectra of CQ, Pep-Se-S and Pep-Se-S-CQ were obtained by dissolving them in a mixed solution of degassed DCM and ethanol (2:1, v/v).

3.3 TrxR activity assay

The thioredoxin reductase (TrxR) activity assay utilized the Thioredoxin Reductase Activity Assay Kit (Solarbio, China). Initially, 1 mL of reagent 1 from the kit was add to 5×10^6 4T1 cells in proportion to prepare a cell suspension. Cellular TrxR was extracted from 4T1 cells using ultrasonic treatment in an ice bath. The ultrasonic power is set to 300 W, the duration was 1 s with a 9 s interval, resulting in a total treatment time of 9 min. Subsequently, the sonicated cell fluid was centrifuged at 4 ℃ and 12000 rpm for 10 min, yielding the centrifuged supernatant. The protein concentration in the centrifuged supernatant was quantified using the Bradford protein concentration assay kit. To assess the inhibitory effect of CQ on TrxR, 200 μg of total protein was supplemented with reagent 1 to make the total volume of 100 μL. The mixture was initially reduced by 2 mM NADPH for 5 min at room temperature (in triplicate). Afterward, 2 μL of EtOH, 2 μL of CQ (15 mM, dissolved in EtOH), and 2 μL of DNCB (15 mM, dissolved in EtOH) was added separately to each of the three above solutions, which were then co-cultured at 37 ℃ for 2 hours. After the co-incubation, 2 μL of 2-vinylpyridine diluted 10 times with EtOH was added to each sample, followed by

continued incubation for 30 minutes. The TrxR activity was evaluated using the TrxR Activity Assay Kit (Solarbio, China), with the following procedure: To the test solution, separately add 350 μL of reagent 1, 50 μL of reagent 2, and 50 μL of reagent 3. Mix thoroughly and immediately measure the absorbance at 412 nm at 10 s. After incubating in a 37 ℃ water bath for 5 min, measure the absorbance again at 412 nm at 310 s. Calculate the difference in absorbance between the two time points to determine the reduction activity of each group.

3.4 Pep-Se-S activity assay

To prepare Pep-Se-S and Pep-Se-S-CQ, all solvents were degassed and purged with nitrogen (N_2) immediately before use to maintain an inert atmosphere. In this nitrogen environment, 4 mL of water was added to a mixture of Pep-Se-S-P (0.03 mmol) and $NaBH_4$ (0.26 mmol) with stirring at 0 ℃. The reaction was proceeded for 1 h in darkness. Subsequently, the pH of the solution was adjusted to 6.0 using 1 M HCl. A 0.2 mL aliquot of this solution was then diluted with 1.8 mL of water (duplicate). After this, the two aforementioned identical solutions were treated as follows: one with the addition of CQ (in free base form, 0.018 mmol) and the other with the addition of blank solvent (EtOH, same volume to the CQ ethanol solution). The mixtures were then left to react overnight with shaking, again in the dark and under a N_2 atmosphere at room temperature.

To investigate the TrxR-like reduction activity of Pep-Se-S and the influence of CQ on Pep-Se-S activity, 90 μL of solution 1 from the TrxR activity assay kit was first reduced by 2 mM NADPH for 5 min at room temperature (duplicate), followed by an incubation period of 2 h at 37 ℃. Then, 2 μL of 2-vinylpyridine diluted 10 times with EtOH was introduced into the above solutions and incubated for 0.5 h at 37 ℃. Finally, the two identical solutions were each supplemented with 10 μL of 200 μM Pep-Se-S or 10 μL of 200 μM Pep-Se-S-CQ, respectively. The reducing activity of the samples were tested using the TrxR activity assay kit according to the same procedure mentioned in 3.3.

3.5 Calculation of CQ protonation and selenol and thiol ionization at various pH

The formula for calculating the dissociation degree of monoprotic weak acids selenol (pKa 5.8) and thiol (pKa 8.3) is as follows:

$$\frac{[A^-]}{[HA]} = 10^{pH-pKa} \quad (3\text{-}1)$$

$[A^-]$ represents the concentration of acid anions; [HA] represents the concentration of the weak acids, and pKa is the acidity constant of the weak acids. Assuming the total concentration sum of acid anion and weak acid as 1, we can calculate the percentage content of selenol anion and thiol anion at different pH values.

For the diprotic weak base CQ, whose triethylamine group (pKa 10.2) and chloroquinoline group (pKa 8.1) can undergo protonation. The formula for calculating the monoprotonation degree of CQ is as follows:

$$\frac{[BH^+]}{[B]} = 10^{pKa1-pH} \quad (3\text{-}2)$$

The formula for calculating the diprotonation degree of CQ is as follows:

$$\frac{[BH_2^{2+}]}{[B]} = 10^{pKa1+pKa2-2pH} \quad (3\text{-}3)$$

$[BH_2^{2+}]$ represents the concentration of diprotic CQ; $[BH^+]$ represents the concentration of the monoprotic CQ; pKa1 and pKa2 are the acidity constants of triethylamine group and chloroquinoline group, respectively. Assuming the total concentration of diprotic CQ, monoprotic CQ and CQ as 1, we can calculate the percentage content of diprotic CQ and monoprotic CQ at different pH values.

3.6 Statistical Analysis

Statistical analyses were performed using either one-way ANOVA followed by Dunnett post-hoc test for multiple comparisons by GraphPad Prism 8.0. All data are presented as mean ± SD, derived from at three independent experiments. A p-value of less than 0.05 was considered statistically significant.

4. Supplementary figures

4.1 The molecular structure of chloroquine in water

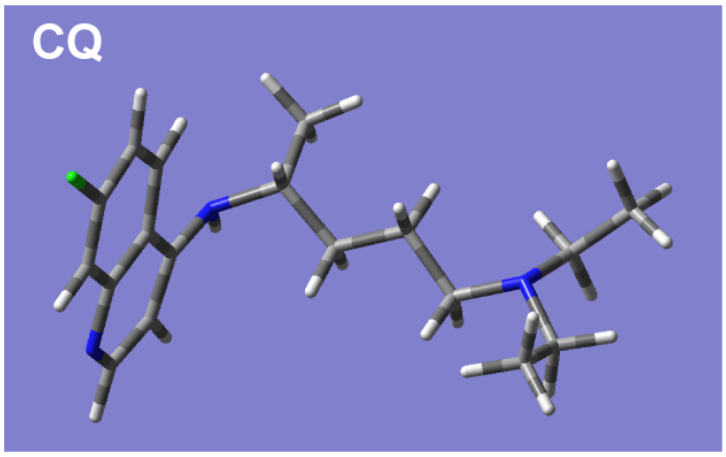

Figure S1. Optimized structure of chloroquine at the B3LYP-D/6-31+G (d, p) level of theory.

4.2 The molecular structure of Pep-Se-S in water

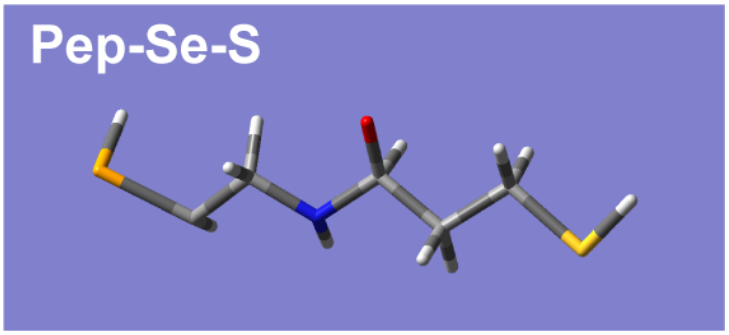

Figure S2. Optimized structure of Pep-Se-S at the B3LYP-D/6-31+G (d, p) level of theory.

4.3 The molecular structure of Pep-Se-S-CQ in water

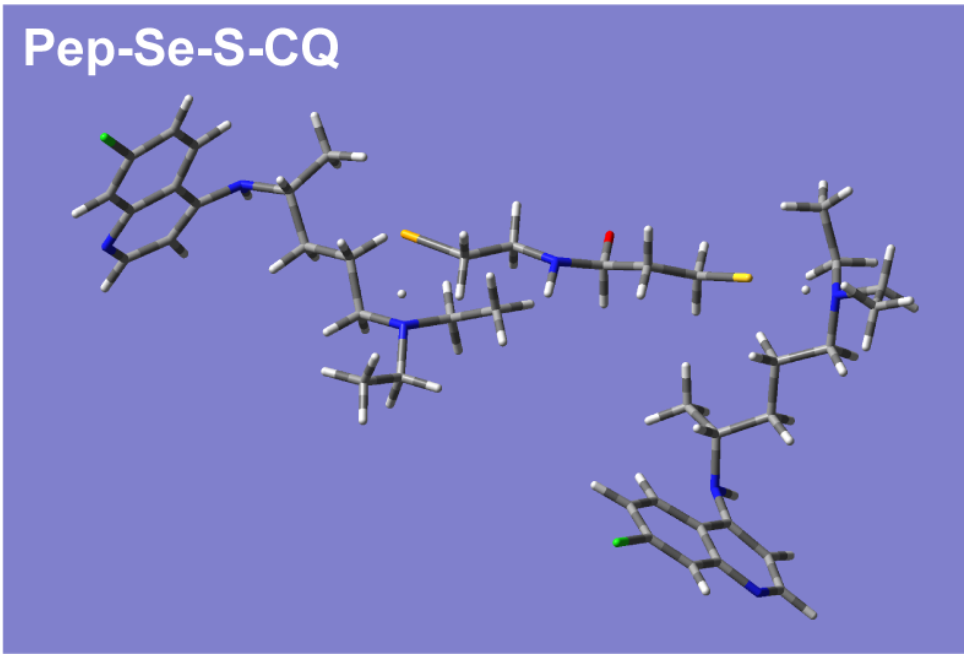

Figure S3. Optimized structure of Pep-Se-S-CQ ion pairs at the B3LYP-D/6-31+G (d, p) level of theory.

4.4 The spatial structures of reactants in water

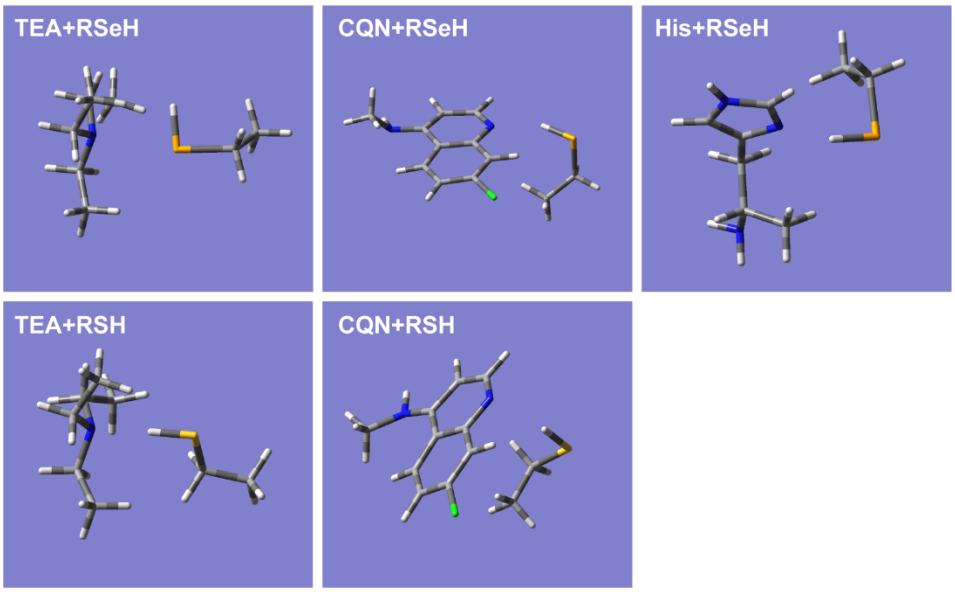

Figure S4. Optimized structures of various pairs of molecular reactants at the B3LYP-D/6-31+G (d, p) level of theory.

4.5 The spatial structures of transition states in water

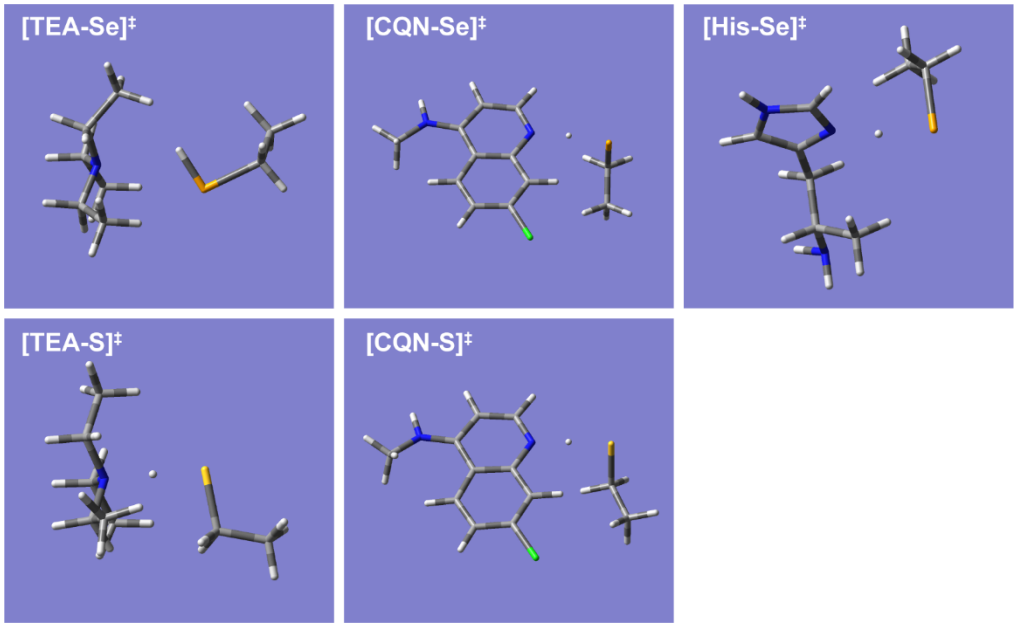

Figure S5. Optimized transition state structures of various ion pairs at the B3LYP-D/6-31+G (d, p) level of theory.

4.6 The spatial structures of ion pairs in water

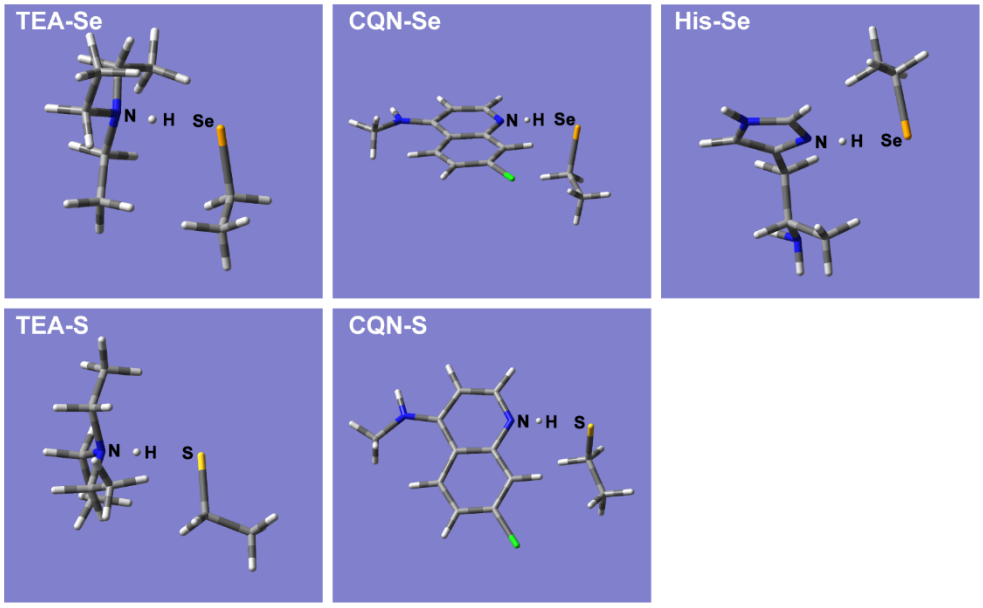

Figure S6. Optimized structures of various ion pairs at the B3LYP-D/6-31+G (d, p) level of theory.

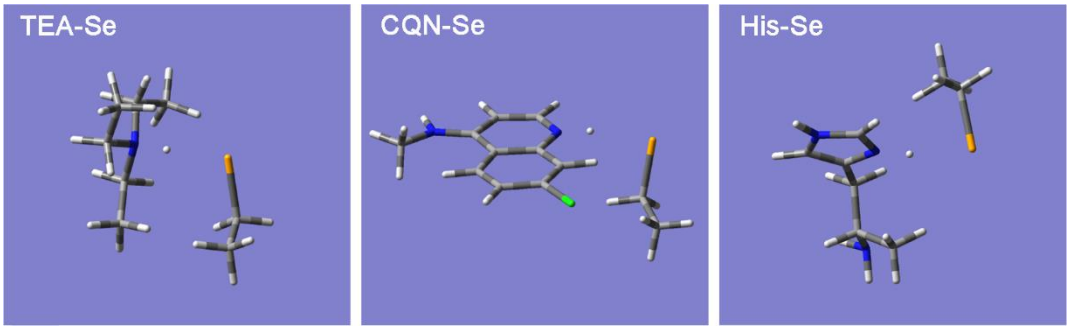

Figure S7. The Optimized cation and anion geometries at the B3LYP-D/6-31+G (d, p) level of theory.

5. References

1. I. V. Fedorova, M. A. Krestyaninov and L. P. Safonova, *J Phys Chem A*, 2017, **121**, 7675-7683.