

## **Oxovanadium-salen and -salan complexes as effective labels for electrochemical immunosensing: A case study for estradiol detection.**

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### **Supporting Information**

Details of synthesis procedures and characterizations of complexes by elemental analyses  
(ESI, RMN, RPE and mass spectroscopy)

## MATERIALS AND METHODS

### • Reagents and Materials.

Estradiol (E2), N-Boc-1,6-hexanediamine hydrochloride, ethyl bromoacetate, sodium ethoxide, potassium bisulfate, acetyl chloride, Tin(IV) chloride, vanadyl acetylacetonate, 3-tert butyl-4-hydroxyanisole, paraformaldehyde, tributylamine, (benzotriazol-1-yloxy)tris(dimethylamino) phosphonium hexafluorophosphate (BOP) were purchased from Sigma-Aldrich (France). Z-Dab(Z)-OH was purchased from Bachem (Switzerland). All the reactions were monitored using TLC analysis, DC-Fertigfolien Alugram Xtra SIL G/UV254 (Macherey-Nagel Germany). The chemicals and organic solvents required for syntheses were purchased from Aldrich (France). All the  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded using a JEOL 400 MHz spectrometer. Mass spectral analysis was performed using electrospray ionization mass spectrometry (ESI-MS) (Thermo Scientific, France). Liquid chromatography analyses were carried out using a Thermo Fisher Scientific LC/MS device, Accela HPLC coupled to a LCQ Fleet equipped with an electrospray ionisation source and a 3D ion-trap analyser.

### • Chemical syntheses and characterizations.

#### Synthesis of linker spacer arm.

(Benzotriazol-1-yloxy)tris(dimethylamino) phosphonium hexafluorophosphate (BOP) (663 mg, 1.5 mmol) was added to a stirred solution of Z-Dab(Z)-OH (387 mg, 1 mmol) and triethylamine (278  $\mu\text{L}$ , 2 mmol) in 5 mL of DCM and DMF mixture (4:1 v/v) at room temperature. After 10 min, N-Boc-1,6-hexanediamine hydrochloride (280 mg, 1.1 mmol) was added, followed by triethylamine (278  $\mu\text{L}$ , 2 mmol). After a 3 h stirring at room temperature, the mixture was supplemented with ethyl acetate (20 mL) and then washed successively with HCl (1M, 3 x 5 mL), H<sub>2</sub>O (1 x 5 mL), NaHCO<sub>3</sub> (1M, 3 x 5 mL) and H<sub>2</sub>O (2 x 5 mL). The organic phase was dried over anhydrous sulphate and evaporated under vacuum. The crude

compound was purified by column chromatography with ethyl acetate / dichloromethane (6:4) as eluent, leading to 484 mg (0.83 mmol, 83%) of compound 1. Deprotection was performed by dropwise addition of 6 mL of 4 M solution of hydrochloride in ethyl acetate to a solution of compound 1 (484 mg, 0.83 mmol) in ethyl acetate (10 mL). The reaction mixture was stirred at room temperature for 60 min. The resulting mixture was evaporated under vacuum and the residue was dissolved in 20 mL of ethyl acetate. The solution was again evaporated and the residue was triturated with anhydrous ether, leading to 422 mg (97%) of spacer arm 2 (Figure 2).

#### Addition of Estradiol acidic derivative.

Estradiol acidic derivative was synthesized as described previously.<sup>1</sup> 494 mg (1.11 mmol) of BOP was added to a mixture of DCM and DMF (4:1 v/v) containing 258 mg (0.78 mmol) of Estradiol acidic derivative **3** and triethylamine (240  $\mu$ L, 1.72 mmol). After stirring for 10 min, 420 mg (0.8 mmol) of spacer arm **2** were added and followed by triethylamine (240  $\mu$ L, 1.72 mmol). The resulting mixture was stirred for 4 h at room temperature. The reaction mixture was supplemented with ethyl acetate (20 mL) and then washed successively with HCl (1M, 3 x 5 mL), H<sub>2</sub>O (1 x 5 mL), NaHCO<sub>3</sub> (1M, 3 x 5 mL) and H<sub>2</sub>O (2 x 5 mL). TLC analysis (ethyl acetate/ dichloromethane, 6:4) indicated full consumption of the starting material. The mixture was then dried over anhydrous sulphate and evaporated to dryness, affording 545 mg (87%) of compound **4** (Figure 2).

#### Deprotection of N-benzyloxycarbonyl group

54.5 mg of Pd/C (10% Pd basis) were added to a stirred solution of compound 5 in 20 mL of methanol under hydrogen atmosphere. The resulting mixture was stirred overnight at room temperature, complete disappearance of compound **4** was demonstrated by TLC (ethyl acetate /dichloromethane, 7:3). The heterogeneous mixture was filtered on celite and the filtrate was

concentrated under reduced pressure. The residue was washed in 15 mL of diethyl ether and evaporated under vacuum, yielding 350 mg of compound **5** (96%) (Figure 2).

#### Preparation of the ligands

H<sub>2</sub>L<sup>E2</sup> Salen (**6**): To a stirred solution of compound **5** (238 mg, 0.45 mmol) in anhydrous chloroform (8 mL), 234 mg (1.12 mmol) of 3-*tert*-butyl-4-methoxy-2-hydroxybenzaldehyde and 12 mg of paratoluene sulfonic acid (5%) were added. The resulting solution was heated at reflux overnight. The reaction mixture was concentrated under reduced pressure and purified by column chromatography using ethyl acetate / dichloromethane (7:3) as eluent, leading to 262 mg (64%) of compound **6** as yellow powder (Figure 2). 3-*tert*-butyl-4-methoxy-2-hydroxybenzaldehyde was synthesized as described previously.<sup>2</sup>

H<sub>2</sub>L<sup>E2</sup> Salan: 21 mg (0.0235 mmol) of L<sup>E2</sup> Salen was dissolved in methanol (5 mL) and a slight excess of NaBH<sub>4</sub> was slowly added. After 2 hours the solution became pale yellow, indicating its complete reduction. The reaction mixture was diluted with water (5 mL), acidified to pH 2 using 4M HCl, and then re-adjusted to pH 8 with Na<sub>2</sub>CO<sub>3</sub>. The mixture was extracted with ethyl acetate (2x20 mL). The organic phase was washed with brine, dried over anhydrous sulphate and evaporated to dryness, affording 17 mg (81%) of H<sub>2</sub>L<sup>E2</sup> Salan.

#### Preparation of the oxovanadium complexes

A general procedure was applied for the synthesis of oxovanadium complexes. To a stirred solution of ligand (1 equiv) in methanol (5 mL), vanadyl acetylacetonate (1 equiv) dissolved in methanol (5 mL) was added dropwise. The resulting solution was heated at reflux for 1 hour. The resulting mixture was washed with diethyl ether and dried under vacuum. VOL<sup>E2</sup> Salen **7** and VOL<sup>E2</sup> Salan **8** were obtained respectively as dark blue and turquoise blue solids (Figure 2).

- **Electrochemical immunosensor protocol.**

Electrochemical measurements were performed using an Autolab PGSTAT100

electrochemical station (Eco Chemie B.V., The Netherlands). Screen-printed electrode (SPE) systems, with carbon paste as working and counter electrodes and Ag/AgCl as a reference electrode, were fabricated using a DEK 248 screen-printing system. E2 was firstly dissolved in ethanol (1 g/L) and then diluted in phosphate buffer saline (PBS 1X). Buffer components and Tween 20 were purchased from Sigma (France). Monoclonal antibody (anti-E2, developed in mouse) against E2 was obtained from Thermo Scientific.

Pretreatment of each electrode was carried out by scanning the potential from -0.6 V to 0.6 V in 0.5M H<sub>2</sub>SO<sub>4</sub>. Adsorption of anti-E2 was then performed for 20 min using 50 µL of PBS containing anti-E2 at dilution 1/500. The competition step was performed during 20 min using 25 µL of E2 standard solutions at different concentrations and 25 µL of VOL<sup>E2</sup> solution at 50 µM in PBS. Electrochemical detection was carried in three steps. A preliminary stabilization step was carried out by applying a potential of 0 V vs. Ag/AgCl for 20 s. Then, a 20 s treatment was performed at a potential of 0.02 V vs. Ag/AgCl, ensuring the oxidation of the metallic complex on the working electrode. Finally, chronoamperometric measurements were performed by automatically switching the applied potential to -0.2 V vs. Ag/AgCl, allowing the reduction of bound metallic complexes. The height of the resulting reduction peak was recorded and plotted against target concentration to give a calibration curve. Washing steps were performed between each step using 100 µL of PBS (1X). Assays were performed in duplicate.

## ***Characterization of Chemical syntheses***

### ***Compound 1***

**<sup>1</sup>H RMN 400 MHz (DMSO-d<sub>6</sub>) δ/ppm:** 7.82 (t, J = 5.2 Hz, 1H), 7.44 (d, J = 8.2 Hz, 1H), 7.34 (m, 10H), 7.2 (t, J = Hz, 1H), 6.76 (d, J = 5.5Hz, 1H), 5.01 (m, 4H), 3.95 (q, J = 8.2Hz, 1H), 3.60 (t, J = 6.7 Hz, 1H), 3.02 (m, 4H), 2.87 (m, 2H), 1.75 (m, 2H), 1.63 (m, 1H), 1.36-1.15 (m, 15H).

**<sup>13</sup>C NMR (DMSO-d<sub>6</sub>) δ/ppm:** 171.89, 156.62, 156.44, 156.19, 137.63, 137.48, 128.90, 128.35, 128.29, 128.20, 77.94, 65.97, 65.81, 53.14, 38.99, 37.91, 32.60, 29.95, 29.47, 28.78, 26.49.

**MS (m/z)** 607.20 [M + Na]<sup>+</sup>.

### ***Spacer arm 2***

**<sup>1</sup>H RMN 400 MHz (DMSO-d<sub>6</sub>) δ/ppm:** 7.90 (t, J = 5.5 Hz, 1H), 7.84 (s, 3H), 7.46 (d, J = 8.2 Hz, 1H), 7.34 (m, 10H), 7.24 (s, 1H), 5.00 (m, 4H), 3.95 (q, J = 8.2Hz, 1H), 3.03 (m, 4H), 2.73 (m, 2H), 1.76 (m, 1H), 1.65 (m, 1H), 1.52-1.15 (m, 7H),

**<sup>13</sup>C NMR (DMSO-d<sub>6</sub>) δ/ppm:** 171.91, 156.61, 156.44, 137.69, 137.54, 128.92, 128.36, 128.32, 128.23, 65.96, 65.80, 53.17, 38.91, 37.95, 32.62, 29.35, 27.43, 26.31, 26.02.

### ***Compound 4***

**<sup>1</sup>H RMN 400 MHz (DMSO-d<sub>6</sub>) δ/ppm:** 7.99 (t, J = 7.0 Hz, 1H), 7.82 (t, J = 6.4, 1H), 7.43 (d, J = 8.0 Hz, 1H), 7.33 (m, 10H), 7.17 (m, 1H), 7.15 (d, J = 8.5 Hz, 1H), 6.67 (dd, J<sub>1</sub> = 8.5 Hz, J<sub>2</sub> = 2.6 Hz, 1H), 6.62 (d, <sup>4</sup>J = 2.6 Hz, 1H), 5.00 (m, 4H), 4.49(d, J = --Hz, 1H), 4.37 (s, 2H), 3.96 (q, J = 6.1Hz, 1H), 3.50 (q, J = 8.0Hz, 1H), 3.10-3.00 (m, 7H), 2.74 (m, 2H), 2.24 (m, 1H), 2.08 (m, 1H), 1.77 (m, 4H), 1.61 (m, 2H), 1.45-1.10 (m, 15H), 0.66 (s, 3H).

**<sup>13</sup>C NMR (DMSO-d<sub>6</sub>) δ/ppm:** 171.78, 168.17, 156.56, 156.42, 156.08, 137.98, 137.70, 137.55, 133.49, 128.89, 128.33, 128.24, 126.74, 115.02, 112.72, 80.59, 79.86, 79.53, 79.20, 67.58, 66.18, 65.95, 65.79, 60.32, 53.15, 50.05, 44.08, 43.35, 39.09, 38.94, 38.71, 38.61, 37.96, 37.12, 32.69, 30.45, 29.81, 29.60, 29.53, 27.38, 26.54, 23.34, 21.33, 14.65, 11.81.

**MS (m/z)** 819.14 [M + Na]<sup>+</sup>.

### **Compound 5**

**<sup>1</sup>H RMN 400 MHz (DMSO-d<sub>6</sub>) δ/ppm:** 8.02 (t, J = 5.8 Hz, 1H), 7.83 (t, J = 5.6, 1H), 7.18 (d, J = 8.8 Hz, 1H), 6.70 (dd, J<sub>1</sub> = 8.8 Hz, J<sub>2</sub> = 2.3 Hz, 1H), 6.62 (d, <sup>4</sup>J = 2.3 Hz, 1H), 4.38 (s, 2H), 3.51 (t, J = 8.3 Hz, 1H), 3.16-2.96 (m, 4H), 2.74 (m, 2H), 2.61 (s, 1H), 2.24 (m, 1H), 2.09 (m, 1H), 1.85 (m, 3H), 1.59 (m, 2H), 1.45-1.09 (m, 19H), 0.66 (s, 3H).

**<sup>13</sup>C NMR (DMSO-d<sub>6</sub>) δ/ppm:** 168.18, 156.08, 137.98, 133.49, 126.74, 115.02, 112.71, 80.58, 67.58, 53.52, 50.05, 49.15, 44.09, 43.35, 39.10, 38.70, 37.12, 30.44, 29.82, 29.70, 29.60, 26.57, 23.34, 11.82.

**MS (m/z)** 541 [M + Na]<sup>+</sup>.

### ***Salen and salan ligands:***

#### ***H<sub>2</sub>L<sup>E2</sup> Salen 6:***

**<sup>1</sup>H RMN 400 MHz (Methanol-D<sub>4</sub>) δ/ppm:** 8.43 (s, 1H), 8.33 (s, 1H), 7.16 (d, J = 8.5 Hz, 1H), 6.92 (d, <sup>4</sup>J = 2.8 Hz, 1H), 6.88 (d, <sup>4</sup>J = 2.8 Hz, 1H), 6.71-6.62 (m, 5H), 4.37 (s, 2H), 4.05 (m, 1H), 3.70-3.60 (m, 10H), 3.17 (q, J = 6.1 Hz, 4H), 2.77 (m, 2H), 2.40-2.16 (m, 3H), 2.10 (m, 1H), 2.01-1.86 (m, 2H), 1.84 (m, 1H), 1.65 (m, 1H), 1.45-1.10 (m, 40H), 0.73 (s, 3H).

**<sup>13</sup>C NMR (Methanol-D<sub>4</sub>) δ/ppm:** 172.30, 169.98, 168.27, 166.93, 155.57, 154.40, 154.19, 151.71, 151.48, 138.48, 138.25, 137.91, 133.71, 126.17, 118.53, 118.23, 118.10, 117.73, 114.35, 111.94, 111.64, 81.12, 71.41, 66.88, 55.69, 54.84, 49.93, 48.71, 39.01, 38.91, 38.44, 36.65, 34.53, 34.48, 34.40, 29.45, 29.36, 28.93, 28.75, 28.49, 28.27, 27.08, 26.21, 25.99, 25.91, 22.71, 10.40.

**MS (m/z)** 909.38 [M + H]<sup>+</sup> and 931.46 [M + Na]<sup>+</sup>.

#### ***H<sub>2</sub>L<sup>E2</sup> Salan 7:***

**<sup>1</sup>H RMN 400 MHz (Methanol-D<sub>4</sub>) δ/ppm:** 7.17 (d, J = 8.5 Hz, 1H), 6.70-6.68 (m, 5H), 6.63 (m, 1H), 6.45 (d, J = 2.8 Hz, 1H), 6.38 (6.38, d = 2.8, 1H), 4.40 (s, 2H), 3.95 (d, J = 13 Hz, 1H), 3.84 (s, 2H), 3.73-3.54 (m, 10H), 3.37-3.09 (m, 11H), 2.81-2.63 (m, 4H), 2.31-2.26 (m, 1H), 2.18-1.92 (m, 4H), 1.87-1.66 (m, 4H), 1.53-1.12 (m, 28H), 0.74 (m, 3H).

**<sup>13</sup>C NMR (Methanol-D<sub>4</sub>) δ/ppm:** 173.98, 170.04, 169.98, 169.35, 155.60, 152.55, 152.26, 152.08, 150.19, 150.04, 138.42, 138.15, 137.91, 137.57, 133.71, 126.16, 123.42, 123.17, 118.52, 114.35, 112.24, 111.95, 111.34, 111.14, 81.12, 70.93, 66.91, 58.25, 54.87, 54.77, 51.44, 50.27, 49.94, 48.38, 44.71, 44.21, 44.00, 43.01, 39.02, 38.72, 38.45, 36.65,

34.50, 34.37, 32.39, 29.45, 29.37, 29.00, 28.91, 28.76, 28.73, 28.61, 28.46, 27.07, 26.21, 26.10, 25.90, 22.70, 10.37.

**MS (m/z)** 913.38 [M + H]<sup>+</sup>.

***Oxovanadium complexes:***

**VOL<sup>E2</sup> Salen 8:** A dark blue solid was obtained. **MS (m/z)** 973.05[M-H]<sup>-</sup>.

**VOL<sup>E2</sup> Salan 9:** A turquoise blue was obtained. **MS (m/z)** 977.07 [M-H]<sup>-</sup>.

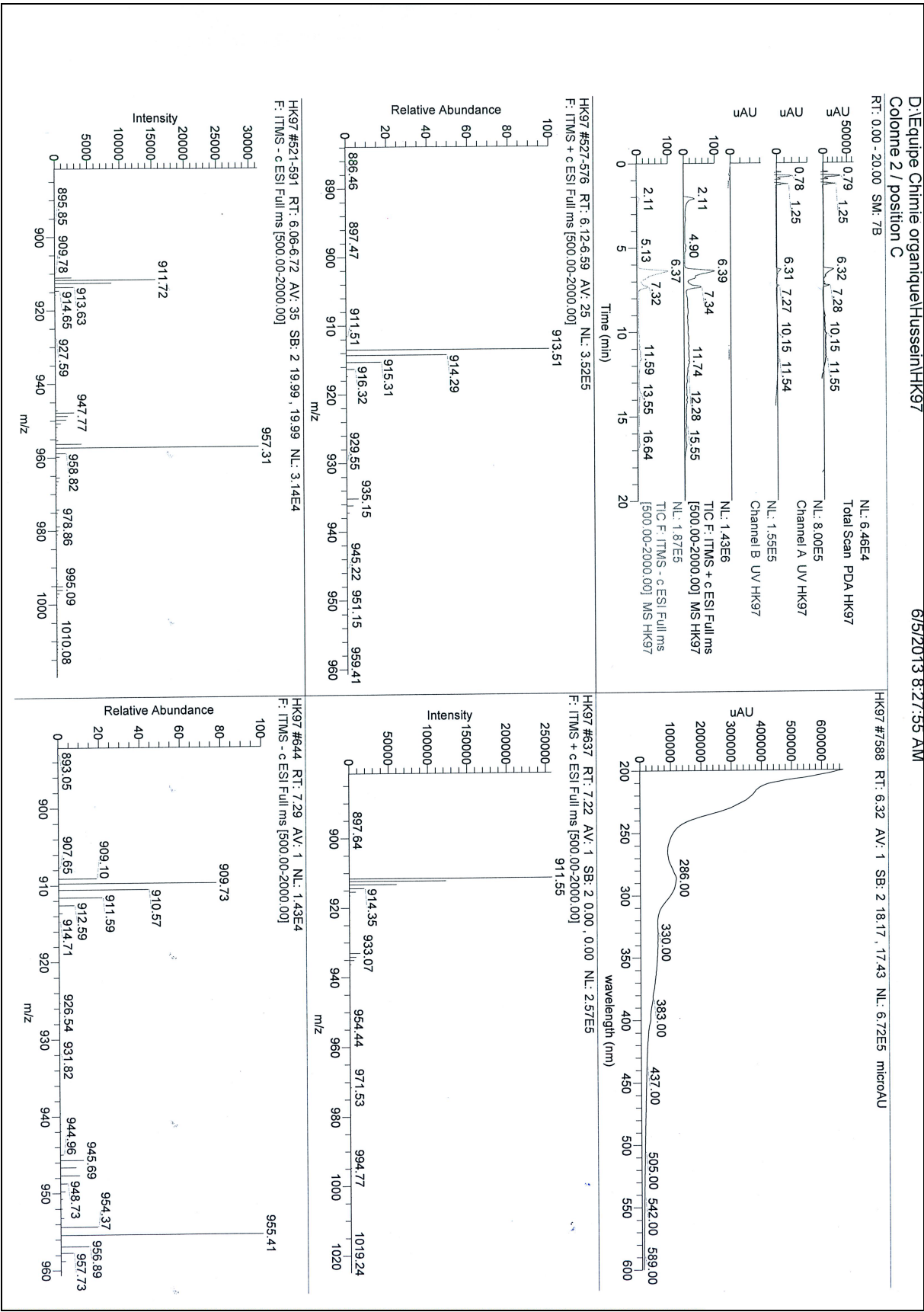


Figure S1: LC/MS chromatogram of VOL<sup>E2</sup> salan.

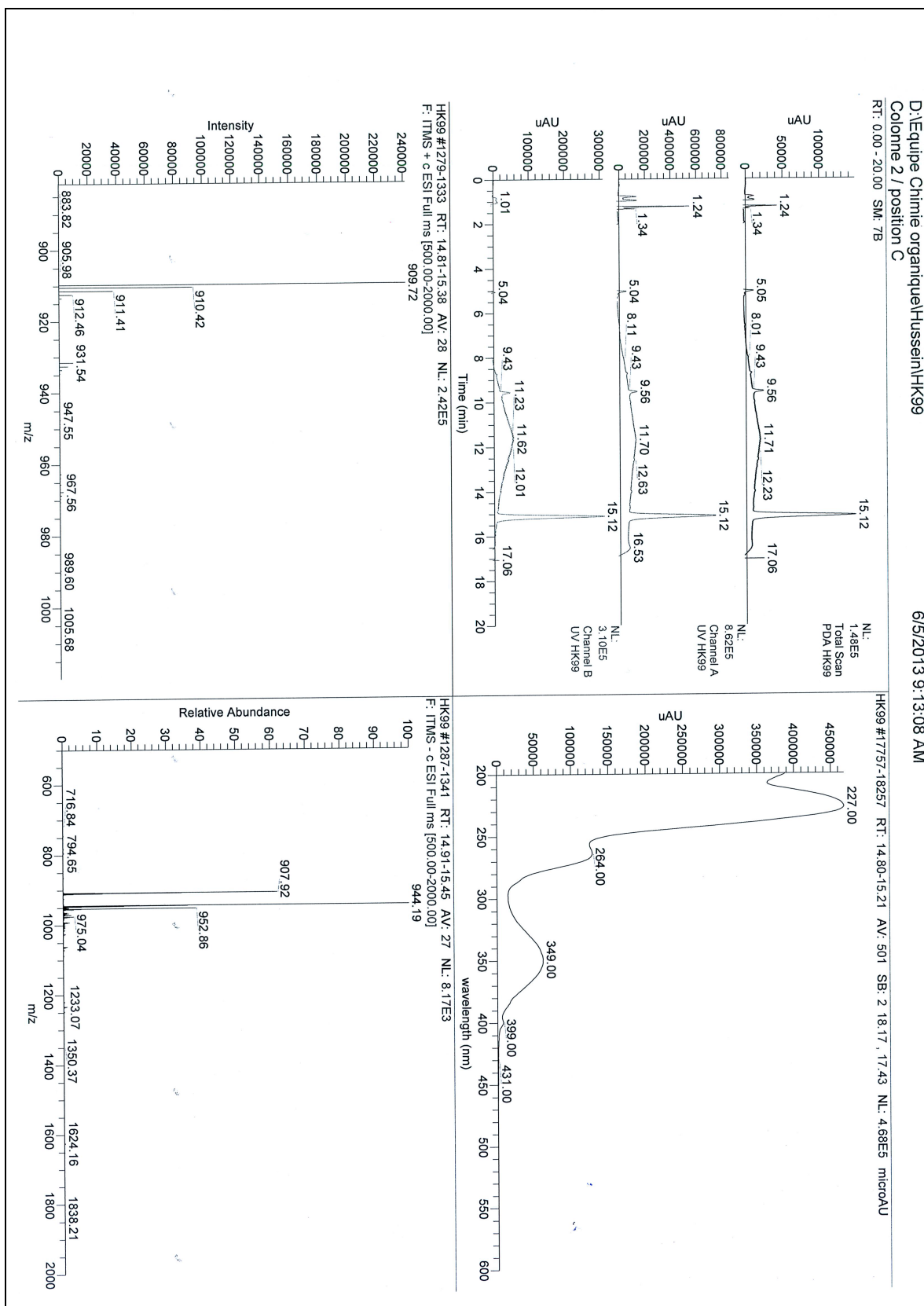


Figure S2: LC/MS chromatogram of freshly prepared VOL<sup>E2</sup> salen.

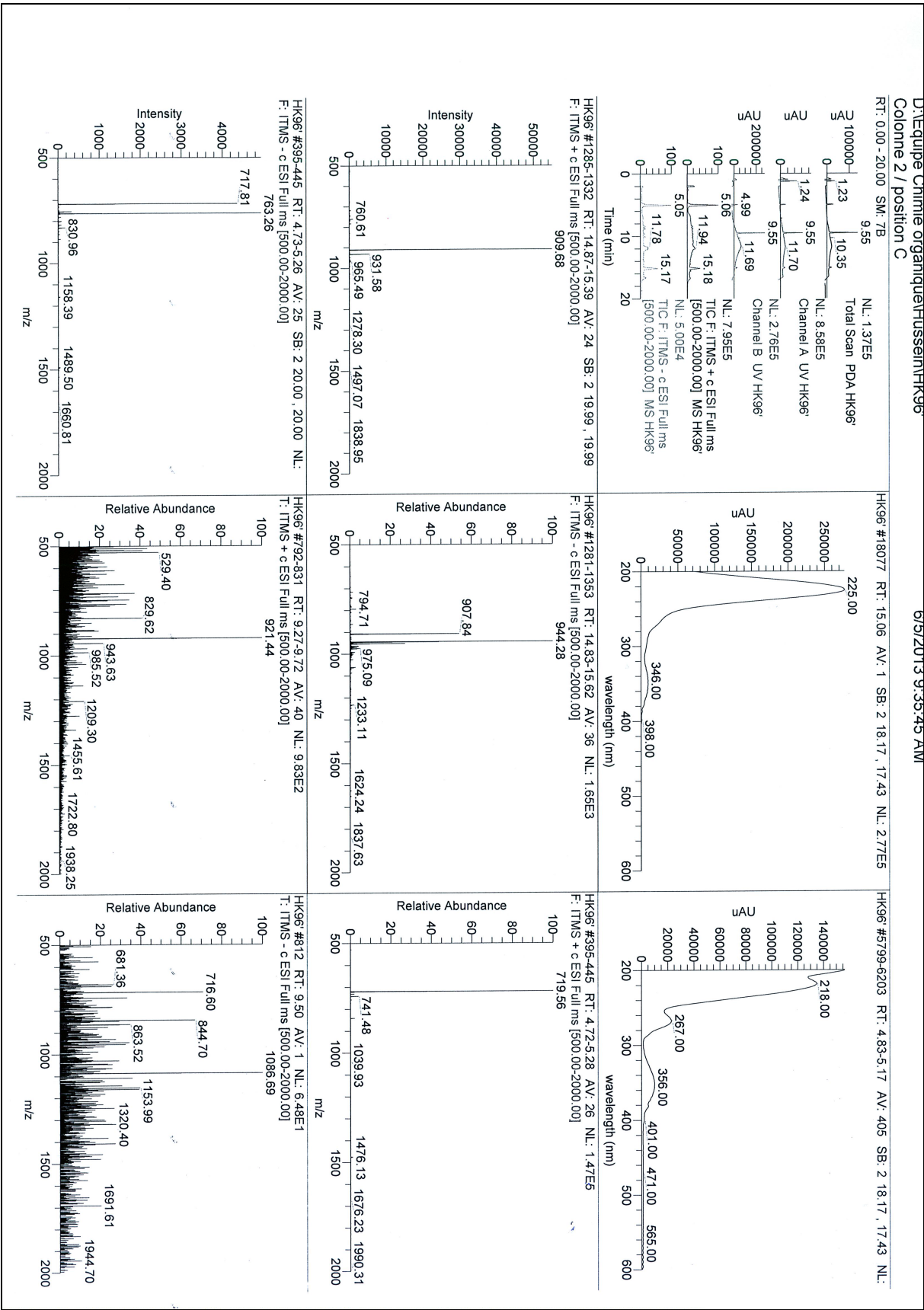


Figure S3: LC/MS chromatogram of VOL<sup>E2</sup> salen after one week storage.

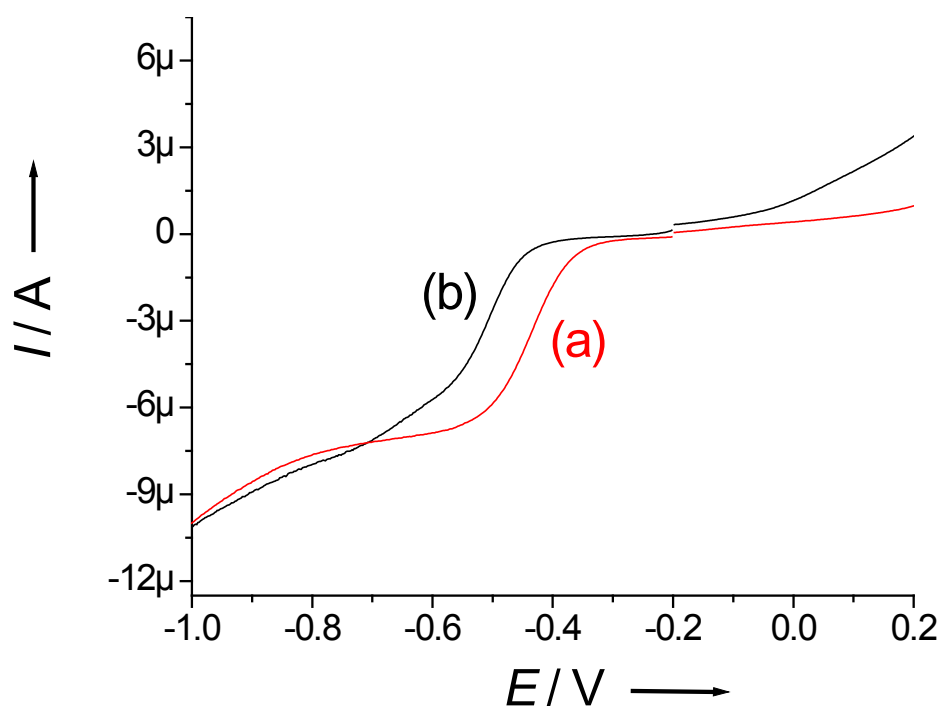


Figure S4: Rotating Disc Electrode voltammetry curves of (a)  $\text{VOL}^{\text{E2}}$  salen and (b)  $\text{VOL}^{\text{E2}}$  salan in 0.5 mM  $\text{CH}_2\text{Cl}_2$  solution (+ 0.1 M TBAP); carbon disc,  $\nu = 500$  rpm,  $T = 298$  K. The potentials are given relative to the  $\text{Fc}^+/\text{Fc}$  reference.

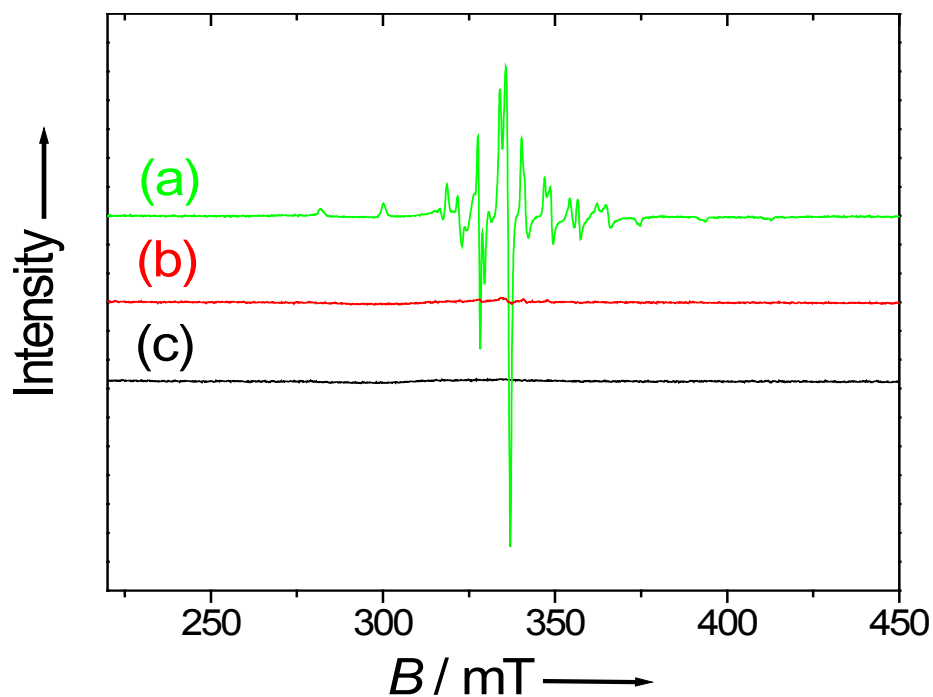


Figure S5: EPR spectra of: (a)  $\text{VO}(\text{acac})_3$  in 0.5 mM MeOH frozen solution; (b) (a)  $\text{VOL}^{\text{E2}}$  salen and (c)  $\text{VOL}^{\text{E2}}$  salen in 0.5 mM  $\text{CH}_2\text{Cl}_2$  (+ 0.1 M TBAP) frozen solutions. Microwave freq.: 9.44 GHz, power : 5 mW; Mod. Freq. 100 KHz; Mod. Amp. : 0.3 mT;  $T = 100 \text{ K}$ .

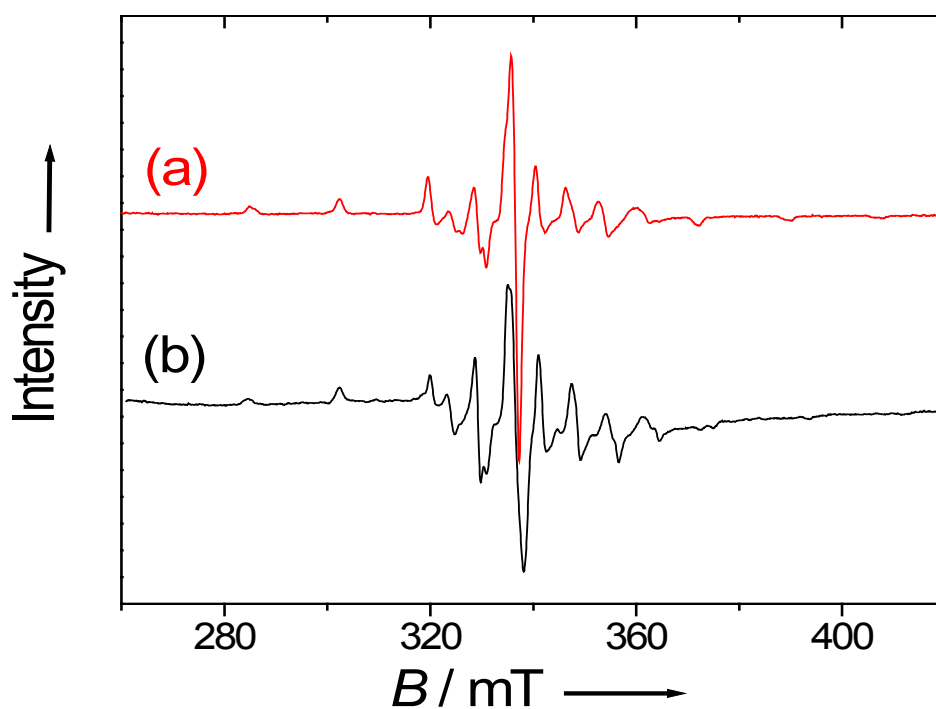


Figure S6: EPR spectra of electrochemically reduced complexes ( $E_{\text{applied}} = -0.6 \text{ V}$  under Argon atmosphere at 273 K): (a)  $\text{VOL}^{\text{E2}}$  salen and (b)  $\text{VOL}^{\text{E2}}$  salen in 0.5 mM  $\text{CH}_2\text{Cl}_2$  (+ 0.1 M TBAP) frozen solutions.

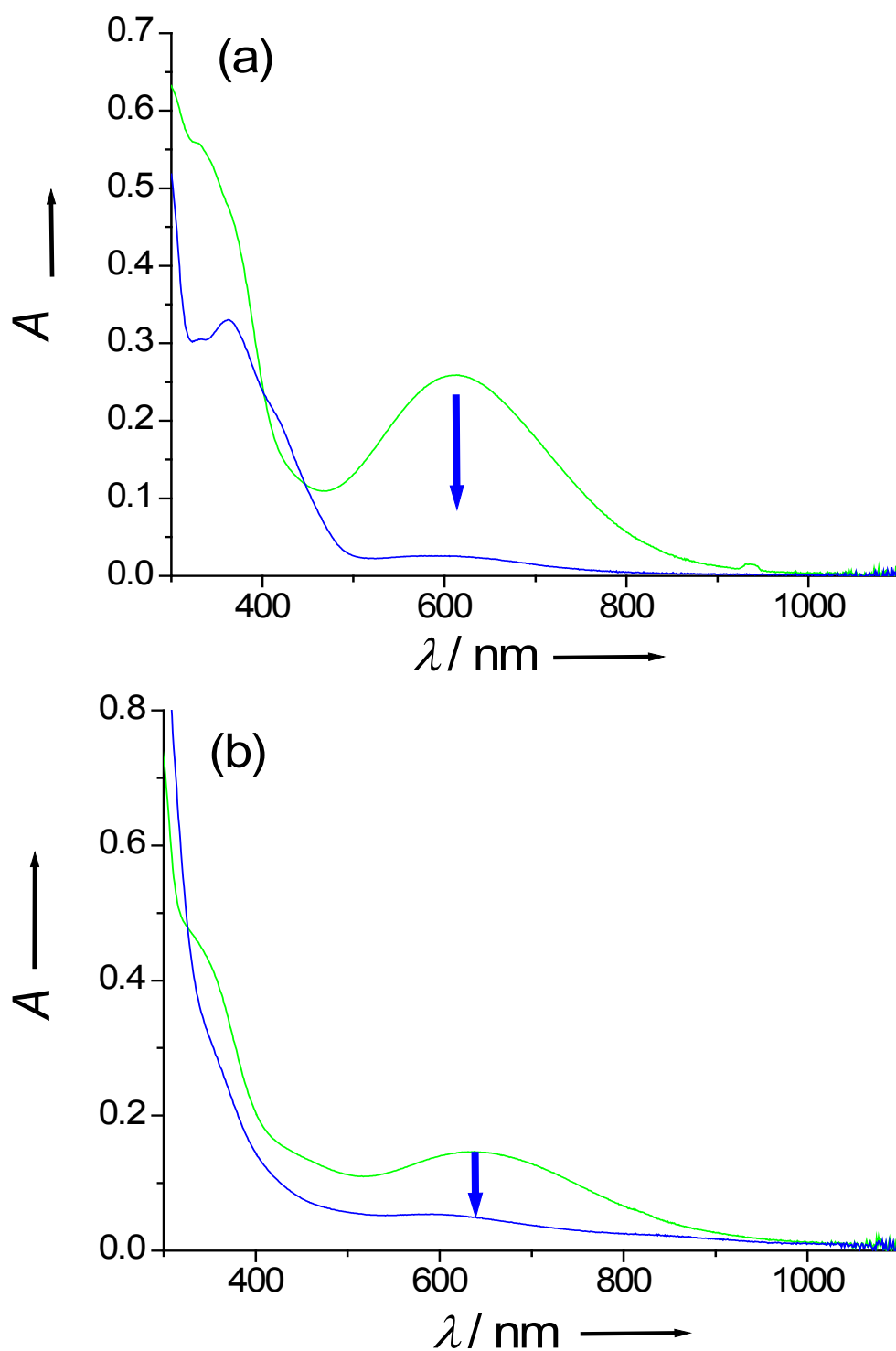


Figure S7: UV-Vis spectra of (a)  $\text{VOL}^{\text{E2}}$  salen and (b)  $\text{VOL}^{\text{E2}}$  salan in  $\text{CH}_2\text{Cl}_2$  solution (+ 0.1 M TBAP): green before electrochemical reduction, blue after electrochemical reduction complexes ( $E_{\text{applied}} = -0.6 \text{ V}$  under Argon atmosphere at 273 K). The blue arrow indicates the spectral changes upon reduction.  $c = 0.5 \text{ mM}$ ,  $T = 298 \text{ K}$ ,  $l = 0.100 \text{ cm}$ .

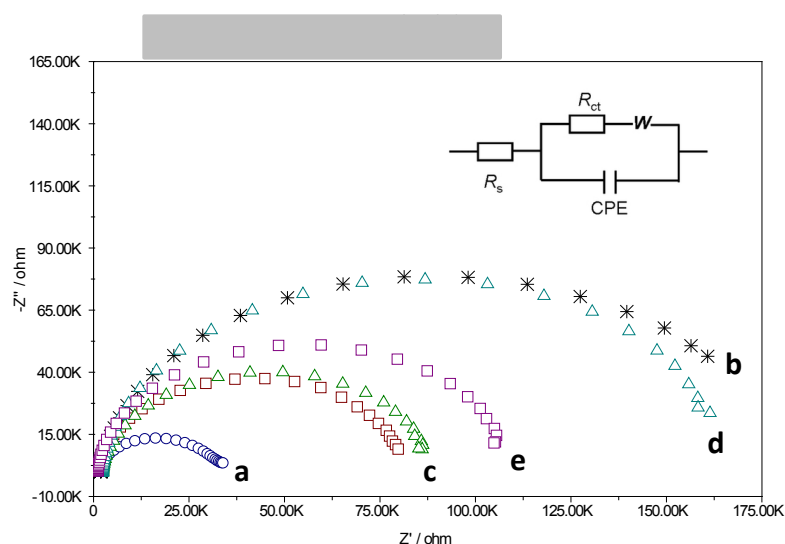


Figure S8: Nyquist plots of (a) bare SPE, (b) after anti-E2 IgG adsorption, (c) after binding of 0.025 mM VOL<sup>E2</sup> salen ( $\Delta$ ) and salan ( $\square$ ) complexes freshly prepared, (d) and (e) after binding of 0.025 mM VOL<sup>E2</sup> salen ( $\Delta$ ) and salan ( $\square$ ) complexes after one week storage.

## References:

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2. F. Michel, F. Thomas, S. Hamman, E. Saint-Aman, C. Bucher, and J.-L. Pierre, *Chem. – Eur. J.*, 2004, **10**, 4115–4125.