# ChemComm

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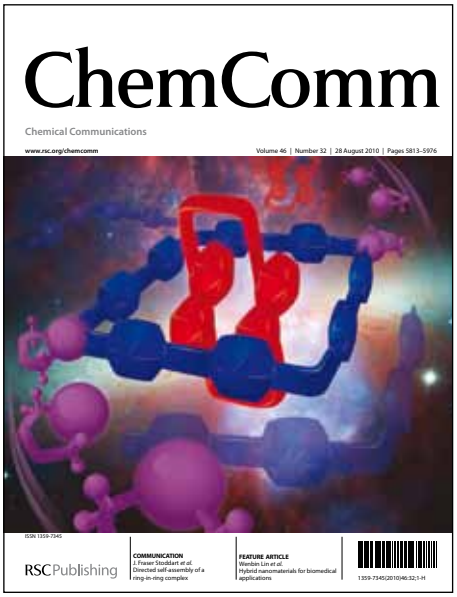


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# Oxovanadium-salen and -salan complexes as effective labels for electrochemical immunosensing: A case study for estradiol detection.

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**Oxovanadium complexes are presented as new labels for the development of electrochemical immunosensors. The concept was successfully applied to the accurate detection of estradiol, an emerging environmental pollutant, at concentrations ranging from 4 ng.L$^{-1}$ to 5 µg.L$^{-1}$.**

Due to their low cost and ease of miniaturisation, immunosensors hold great promise for many applications including environmental analysis. Immunosensors are directly inspired from immunoassays, they are based on the highly selective interaction of antibodies to antigens, the binding event being transformed into a measurable signal by the transducer.[1] Transduction can be performed using several methods involving optical,[2] piezoelectrical,[3,4] atomic force microscopy,[5-7] scanning electrochemical microscopy,[8-10] and other electrochemical techniques.[11,12] Although direct detection can be performed by monitoring changes in capacitance and/or resistance of the electrode, a widely used format in immunosensing involves immunospecies labelled with enzymes, producing an electroactive product from an added substrate.[12]

Despite their high specificity, these devices suffer from several limitations inherent to enzyme-labelled systems like temperature sensitivity, non-specific binding and potential inactivation by natural inhibitors. An attractive yet uncommon alternative to enzyme-based systems is the labelling of analytes with redox non-innocent complexes, and further detection by electrochemical techniques.

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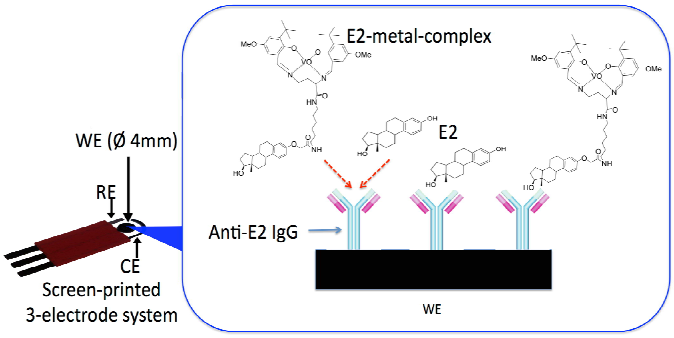
We have described in a previous work the development of novel electrochemical immunosensors for sensitive detection of 17-β estradiol (E2) and ethinylestradiol (EE2) in surface waters.[13] These emerging pollutants are of particular interest and have been recently proposed to be included in the list of priority pollutants established by the European Commission (COM(2011)0876). The described sensor was based on competition between free hormone and immobilised synthetic derivatives for the binding sites of the primary antibody, with subsequent revelation using alkaline phosphatase-labelled secondary antibody. Under the optimized working conditions, the electrochemical immunosensors showed a highly sensitive response to E2 and EE2, with respective detection limits of 1 and 10 ng.L$^{-1}$. In the present work, we demonstrate for the first time that oxovanadium-salen and -salan complexes could favourably replace enzymes as labels in an immunosensor assay for E2. The assay is based on the competition between free estradiol and a metal complex-estradiol conjugate (tracer) for their binding to immobilised antibodies (Scheme 1).




Scheme 1. Principle of the electrochemical immunosensor based on competition between E2 and E2-linked oxovanadium complex (RE = Reference electrode, WE = Working electrode, CE = Counter electrode).

The tracers were synthesized by coupling E2 to metal complexes using an appropriate spacer arm grafted onto the carboxylic function of E2-3-acetic acid (3, Fig. 1). The

synthesis of spacer-hormone conjugate 6 was accomplished in four steps with an overall yield of 67%. The reduction of compound 6 ($H_2L^{E2}$ salen) with NaBH4 yielded quantitatively the corresponding salan ligand N,N'-bis(3-tert-butyl-5-metoxy-salicylaminato)-1,3-propane diamine ($H_2L^{E2}$ salan).

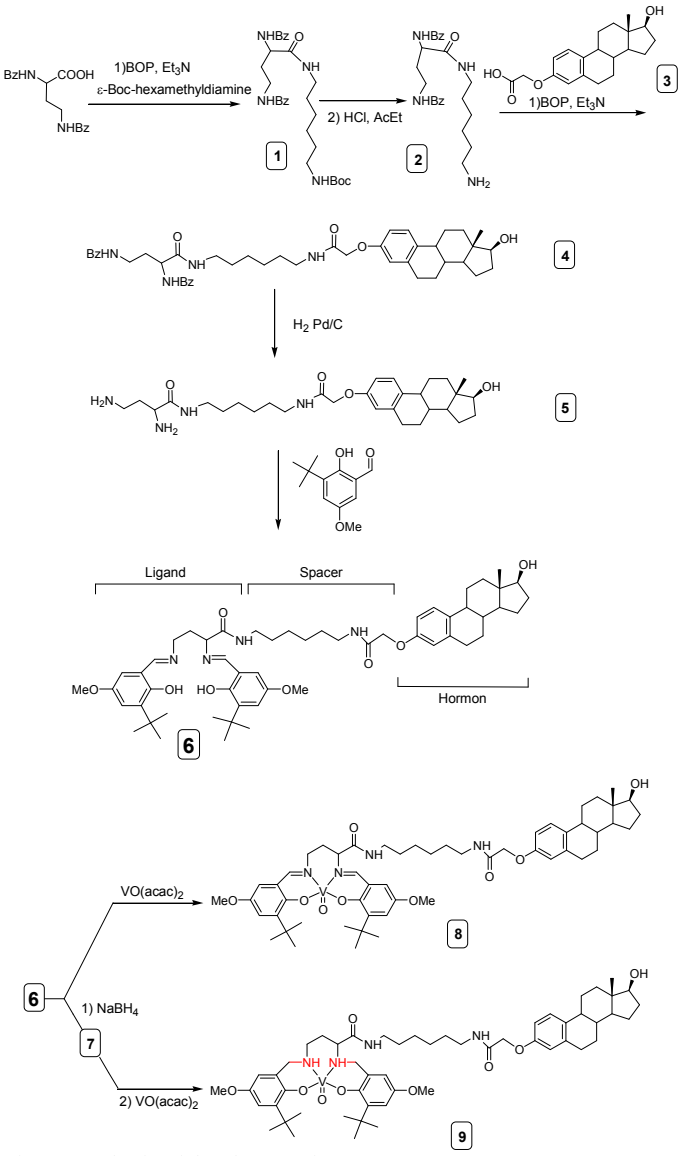



Fig. 1. Synthesis of the electroactive tracers.

Cyclic voltammetry experiments showed that complexes $VOL^{E2}$ salan and $VOL^{E2}$ salen display a reversible reduction wave at E1/2 = -0.51 V and -0.40 V vs. $Fc^+/Fc$, respectively, which is assigned to the V(V)/V(IV) redox couple (Fig. 2 and ESI,† Fig. S4). Electron paramagnetic resonance (EPR) spectroscopy of both $VOL^{E2}$ salan and $VOL^{E2}$ salen showed silent spectra (ESI,† Fig. S5), suggesting that the complexes are present in their oxidized, oxovanadium (V), forms. Consistent with this assumption, electrochemical reduction of $VOL^{E2}$ salan and $VOL^{E2}$ salen at -0.6 V generates paramagnetic (S = ½) species whose EPR signature is characteristic of mononuclear oxovanadium V(IV) complexes (ESI,† Fig. S6). Further support for the (+V) oxidation state of the metal ion in $VOL^{E2}$ salan and $VOL^{E2}$ salen comes from visible spectroscopy, which features an intense transition above 600 nm for both compounds. Based on its high intensity ($\varepsilon > 2000\ M^{-1}cm^{-1}$) it cannot correspond to ligand field excitations of a V(IV) ion, but instead arises from a phenolato → V(V) charge transfer transition (ESI,† Fig. S7).[14] Thus complexes $VOL^{E2}$ salan and $VOL^{E2}$ salen have undergone aerobic oxidation in organic solvents, affording V(V)–bis(phenolato) compounds.[14]

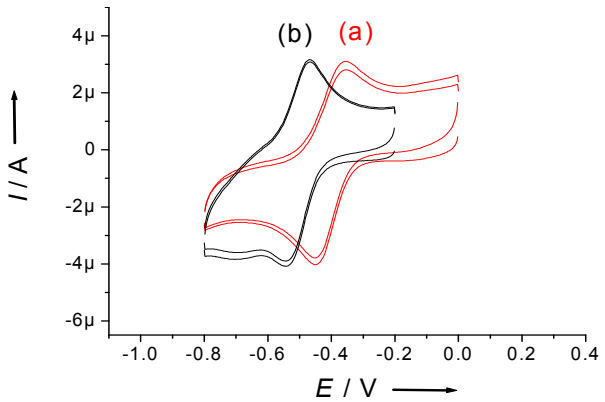



Fig. 2: Cyclic voltammetry curves of (a) $VOL^{E2}$ salen and (b) $VOL^{E2}$ salan in 0.5 mM $CH_2Cl_2$ solution (+ 0.1 M TBAP); carbon disc, T = 298 K. The potentials are given relatively to the $Fc^+/Fc$ reference.

For designing the immunosensors, a layer of anti-estradiol antibodies (anti-E2 IgG) was first adsorbed on the working electrode surface. Because the number of anti-E2 IgG binding sites must be constant, controls were systematically performed by using electrochemical impedance spectroscopy (EIS). Nyquist plots of Faradaic impedance spectra after IgG deposition, tracer binding and competition with free estradiol are depicted in Fig. 3.

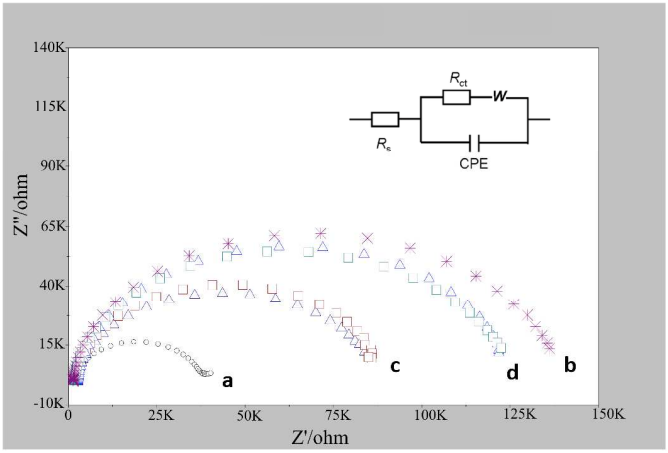



Fig. 3. Nyquist plots of (a) bare SPE, (b) after anti-E2 IgG adsorption, (c) after binding of 0.025 mM $VOL^{E2}$ salen (△) or salan (□) complexes, (d) after competition between 0.025 mM $VOL^{E2}$ (△ salen, □ salan) and 0.18 µM (50 $\mu g.L^{-1}$) of free E2.

An increase of charge transfer resistance ($R_{ct}$) from 35.26 kΩ to 140 kΩ was observed after anti-E2 IgG immobilisation, indicating that the formation of the biorecognition layer retards the charge-transfer process. The deposition of anti-E2 IgG was highly reproducible, as shown by the similar $R_{ct}$ measured for different preparations. In addition, the response did not evolve during at least 4 hours, indicating that the layer is rather stable (Fig. 3, b and d). Binding of tracer $VOL^{E2}$ to anti-E2 IgG

induced a significant decrease of $R_{ct}$, from 140 to 86.5 kΩ. This decrease is related to the metal complex that facilitates the charge-transfer process. In the presence of a high concentration of E2 (50 µg.L$^{-1}$), the charge transfer resistance increases back to a maximum value 122 kΩ. Under these conditions the tracer VOL$^{E2}$ is therefore replaced by free E2, devoid of charge-transfer capacity, on anti-E2 IgG binding sites. Similar EIS experiments conducted after one week storage (ESI,† Fig. S8) at 4°C of the tracers show a large increase of $R_{ct}$ in the case of VOL$^{E2}$ salen, which is assigned to major degradation of the metal complex, consistent with hydrolysis of salen fraction observed in LC-MS (ESI,† Fig. S3).

Due to its high sensitivity and selectivity, chronoamperometry is the most appropriate detection method for immunosensors measurements. As the metal complexes are present in their oxidized form, the binding of tracers on immobilised antibodies was examined at -0.2 V vs Ag/AgCl, by recording the steady-state cathodic current after direct reduction of oxovanadium (V) to oxovanadium (IV). The chronoamperometric response of the biosensor coated with anti-E2 IgG in PBS (a) and in presence of 0.025 mM of either VOL$^{E2}$ salen (b) or VOL$^{E2}$ salan (c) was measured (Fig. 3). Binding of VOL$^{E2}$ salen induced a signal change from -0.1 to -0.18 µA, while under the same conditions VOL$^{E2}$ salan induced a remarkable drop of the reduction peak down to -1 µA. This behaviour cannot be explained by a difference in binding mode of the complexes on anti-E2 IgG sites. We rather believe that the electronic delocalisation is higher in salen complexes, due to conjugation between the imine and phenolate moieties. Delocalisation is disrupted in salan complexes due to replacement of the imine by an amine bond, thus enhancing the electroactivity of salan over salen complexes. It is noteworthy that after aging of the solution, the small shift observed in impedance (ESI,† Fig. S9) does not affect its electrochemical activity in chronoamperometric tests (Fig. 4).

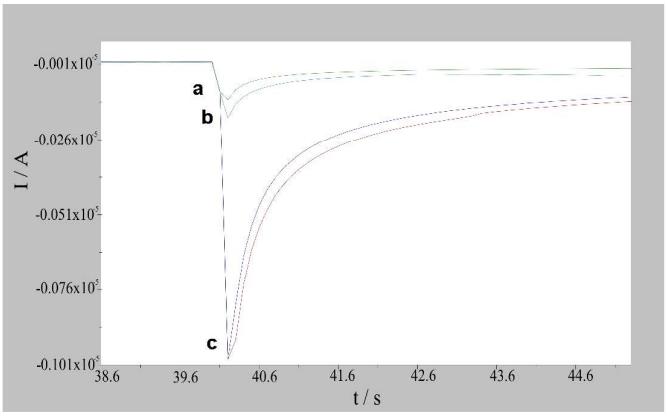



Fig. 4. Chronoamperometric response of anti-E2 IgG immunosensor in presence of PBS buffer (a), after VOL$^{E2}$ salen binding (b), or after VOL$^{E2}$ salan (freshly prepared or after one week storage) binding (c).

These promising results led us to perform an immunoassay based on the competition between free E2 and the tracers for binding to antibody adsorbed on the working electrode. In such assay, the intensity of the response is inversely proportional to the concentration of free E2. Calibration curves were established for E2 by using either VOL$^{E2}$ salen or VOL$^{E2}$ salan as tracer (Fig. 5).

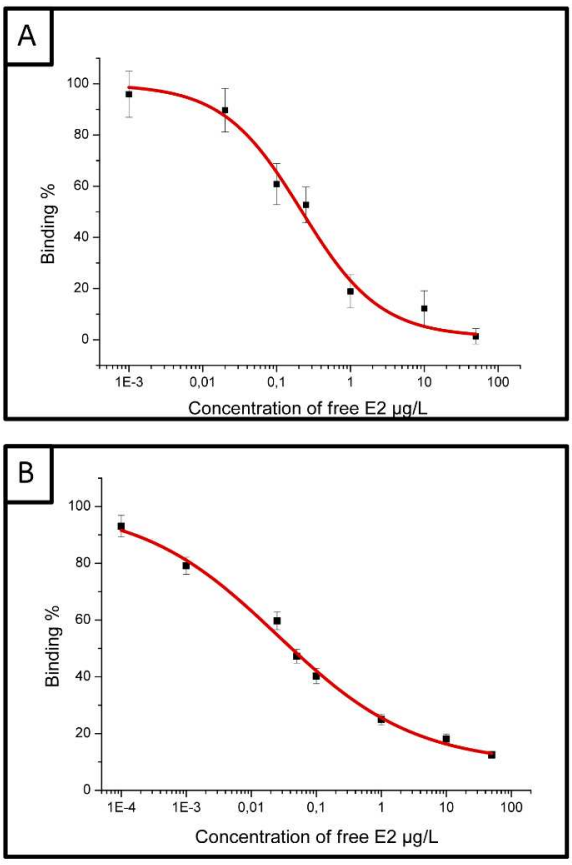



Fig. 5. Calibration curves of E2 obtained using VOL$^{E2}$ salen (A) and VOL$^{E2}$ salan (B) tracers.

Due to the low experimental error associated to electrochemical detection (<5%), the limit of detection (LOD) was arbitrary calculated for an estrogen concentration that induces a signal decrease of 15%. In both cases, the LOD corresponded to the limit of quantification (LOQ). LOD values of 4 and 45 ng.L$^{-1}$ were obtained for VOL$^{E2}$ salan and VOL$^{E2}$ salen, respectively, confirming the higher sensitivity of the immunosensor based on salan complex. In addition, a wider dynamic range was achieved using salan derivative as tracer, allowing quantification of E2 at concentrations ranging from 4 ng.L$^{-1}$ to 5 µg.L$^{-1}$ (RSD=2.14%) (Fig. 5B). Comparatively the salen derivative VOL$^{E2}$ salen was less efficient, as judged by the narrower concentration range of E2, comprised between 45 ng.L$^{-1}$ and 2 µg.L$^{-1}$ (RSD=6.5%) (Fig. 5A).

A summary comparison of VOL$^{E2}$-based immunosensors with the previously described immunoenzymatic sensor[13] is presented in Table 1. The LOD obtained using VOL$^{E2}$ salan as tracer is remarkably low and fairly compares with values observed for immunosensor based on enzyme labelling. An additional advantage of using this tracer is the shorter analysis time related to the simplification of assay, which avoids revelation step using enzyme substrate.

Table 1: Comparison of performances of immunosensors based on $VOL^{E2}$ tracers and enzyme-labelled immunosensor.

| Method | | LOD ($ng.L^{-1}$) | Quantification Range ($ng.L^{-1}$) | Time of analysis (h) |
|---|---|---|---|---|
| Enzyme-labelled immunosensor | | 1 | 1 – 1000 | 2 |
| Immunosensor based on electroactive tracer | $VOL^{E2}$ salen | 45 | 45 – 2000 | 1 |
| | $VOL^{E2}$ salan | 4 | 4 – 5000 | |

In conclusion, this study clearly establishes that oxovanadium complexes are very powerful labels for immunosensing. These metal complexes open new perspectives for the design of sensitive and stable immunosensors. We also demonstrate that oxovanadium salan complexes are more efficient than the more classical salen derivatives. Among their numerous advantages we noted a higher stability in solution, better reproducibility, enhanced electroactivity and consequently higher sensitivity.

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