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Poly(benzoxazine)s Modified with Osmium Complexes as a Class of Redox Polymers for Wiring of Enzymes to Electrode Surfaces

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Benzoxazine-based redox polymers bearing Os complexes are synthesized and used as an immobilization matrix for glucose oxidase (GOx) as a model system for a reagentless biosensor. The polymers are formed by electrochemically induced anodic polymerization of the corresponding benzoxazine monomers modified with Os complexes. The precursors are synthesized in a Mannich-type reaction between bisphenol A, formaldehyde, and the corresponding Os complexes or ligands, which contain free amino groups. The Os complexes are redox active within the polymer films, and thus, can be used as redox relays for the electron transfer between the electrode surface and the prosthetic group within the enzyme. Entrapment of GOx within the poly(benzoxazine) film is achieved successfully, as shown by the biocatalytic activity of the poly(benzoxazine)/GOx films upon the addition of glucose.

Introduction

Poly(benzoxazine) materials are based on derivatives of 3,4-dihydro-2 H-1,3-benzoxazine monomers, which are readily derived from the reaction between a phenol derivative, a primary amine, and formaldehyde through a Mannich-type approach.[1–3] Poly(benzoxazine)s are used widely as protective layers because of their thermal stability and mechanical properties.[2,4–8]

The chemical polymerization of benzoxazine monomers can be initiated thermally or in combination with an acid catalyst.[1–3,9] Moreover, the electrochemically induced anodic polymerization of benzoxazine monomers can be achieved in basic acetonitrile or methanol.[4,5,10,11] It is assumed that the electrochemical polymerization is induced by oxidation of the deprotonated oxazine ring. The formed radicals further react with the aromatic systems of the phenol moiety within the benzoxazine monomers, and propagation then leads to the formation of a dense network of a poly(benzoxazine) resin.[5,10]

Recently, our research group described the electrochemically induced deposition of poly(benzoxazine) films onto electrode surfaces from aqueous suspensions through the anodic oxidation of water-soluble benzoxazine derivatives at high potentials.[12] The polymer formed a stable film on the electrode surface and was further used as an immobilization matrix for glucose oxidase (GOx) by codeposition of the polymer film and the enzyme. This poly(benzoxazine)/GOx layer was applied successfully in an amperometric biosensor for the determination of glucose through the oxidation of H_2O_2 at the underlying glassy carbon electrode, which was decorated with Pt nanoparticles.[12] However, this approach requires high operating potentials of the glucose sensor and the use of Pt nanoparticles to lower the applied potential for the oxidation of H_2O_2.

Os complexes containing 2,2′-bipyridine (bpy) ligands are well-investigated redox mediators for the electron transfer between electrode surfaces and prosthetic groups within enzymes.[13–18] The use of hydrogels modified with Os complexes allows the wiring of the enzyme to the electrode surface within a stable immobilization matrix. The redox relays within the active layer ensure a fast and efficient electron transfer between the enzyme and the electrode. Such polymers modified with Os complexes have been used successfully in so-called reagentless amperometric biosensors[13–16] and in biophotoelectrochemical applications.[17,18]

Herein, we describe the synthesis and electrochemically induced polymerization of novel benzoxazine monomers modified with Os complexes, and their application as an immobilization matrix for GOx from *Aspergillus niger* used as a model enzyme, to further investigate the potential of poly(benzoxazine)s modified with osmium complexes as new materials for the development of enzymatic biosensors.

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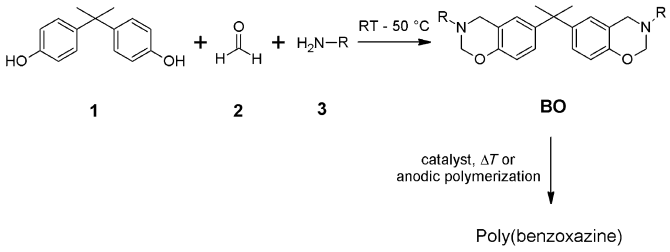
Results and Discussion

Synthesis of benzoxazine monomers modified with Os complexes

The formation of benzoxazine monomers (**BO**) is based on the reaction of a phenolic compound (bisphenol A, **1**) with an aldehyde (formaldehyde, **2**) and a primary amine (**3**) under mild conditions (Scheme 1). The polymerization of the resulting monomers is induced by heating in the presence or absence of an acid catalyst or by anodic oxidation (Scheme 1).



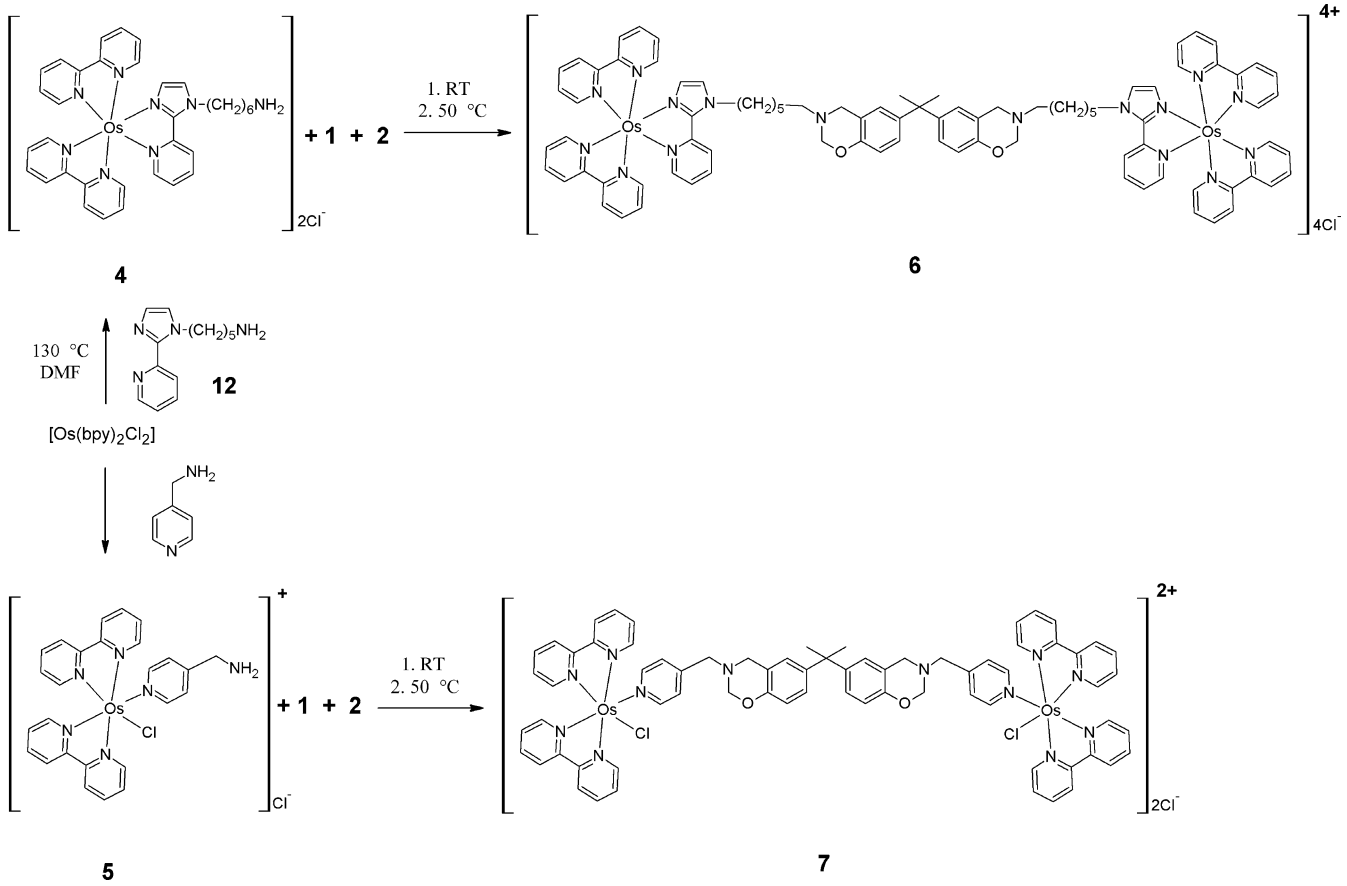
Scheme 1. Synthesis of poly(benzoxazine)s through polymerization of a benzoxazine monomer (**BO**) formed in the reaction of bisphenol A (**1**), formaldehyde (**2**), and an amine derivative (**3**).

For the introduction of Os complexes at the benzoxazine moiety, two different synthetic pathways were investigated: a) the direct reaction of Os complexes **4** and **5** bearing an amine functionality in the ligand sphere with bisphenol A (**1**) and formaldehyde (**2**) to form the benzoxazine monomers modified with Os complexes **6** (top) and **7** (bottom), respectively (Scheme 2); and b) the formation of a benzoxazine monomer containing a mono- (**8**) or a bidentate (**9**) ligand (Scheme 3, top) for later coordination at the Os center by substitution of singly or doubly labile chloro ligands in $[Os(bpy)_2Cl_2]$ (bpy = 2,2′-bipyridine) to yield **10** and **11** (Scheme 3, bottom). The monomers **6** and **11** reveal the same molecular structure but were synthesized through the two different synthetic pathways (a and b). In the following, the syntheses of the benzoxazine monomers modified with Os complexes and the **BO**-based ligands are discussed in detail.

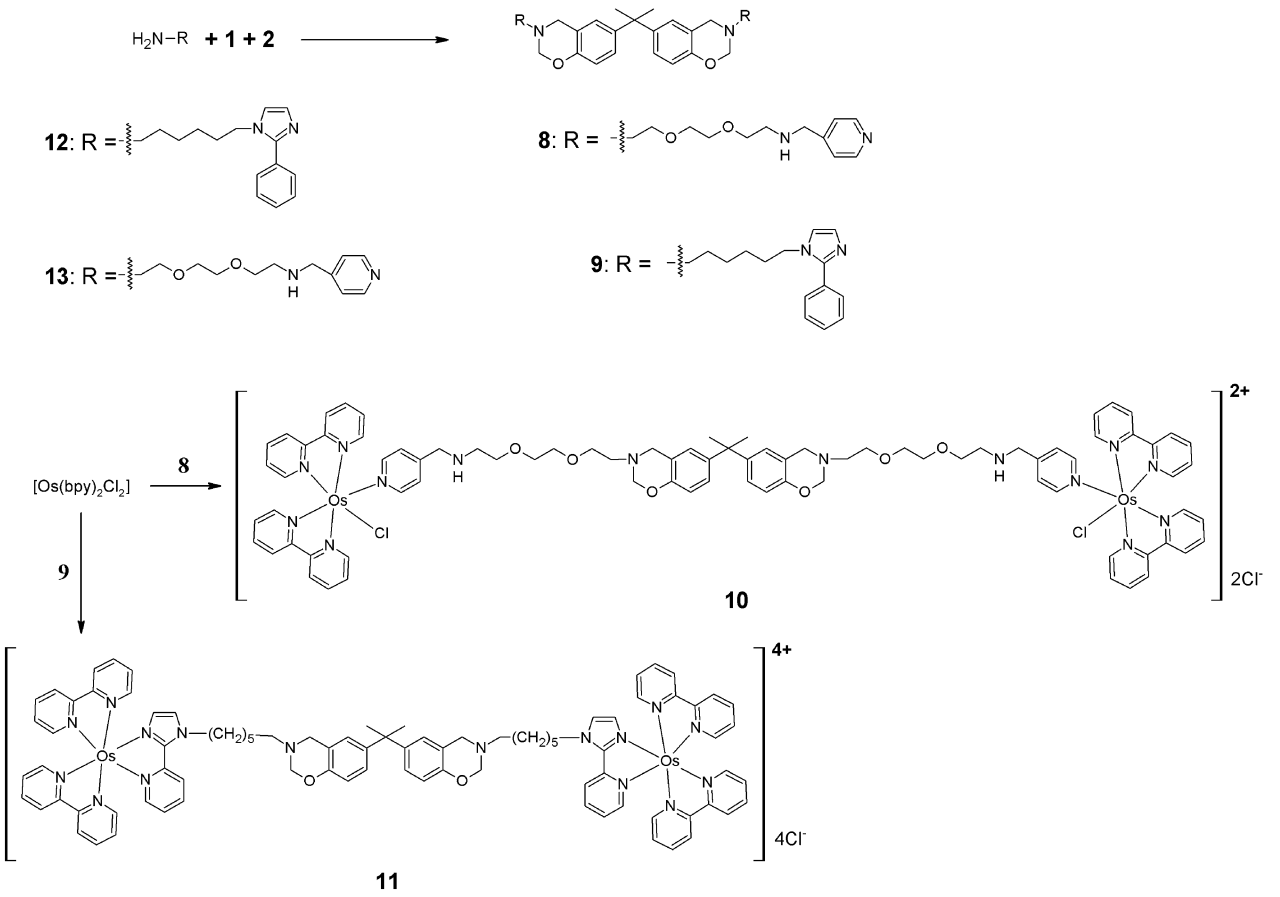
Synthesis of benzoxazine monomers modified with Os complexes 6 and 7 through synthetic pathway (a)

Synthesis of 6

Compound **4** bearing a free amino group was synthesized through the substitution of both chloro ligands in $[Os(bpy)_2Cl_2]$



Scheme 2. Synthesis of the Os-modified benzoxazine monomers **6** and **7** through reaction of Os complexes **4** and **5** with bisphenol A (**1**) and formaldehyde (**2**). The Os complexes **4** and **5** were obtained by ligand exchange reactions of $[Os(bpy)_2Cl_2]$ with the bidentate ligand **12** or with 4-(aminomethyl)pyridine, respectively.



Scheme 3. Synthesis of the Os-modified monomers **10** and **11** through a ligand exchange reaction of $[Os(bpy)_2Cl_2]$ with **8** or **9** (bottom), respectively. Ligands **8** and **9** were synthesized by the reaction of bisphenol A (**1**) and formaldehyde (**2**) with amine **12** or **13**, respectively (top).

in dimethylformamide (DMF) at 130 °C by the bidentate ligand **12** (Scheme 2, top) which was synthesized following the procedure described in Ref. [16] starting from 6-amino-1-hexanol and 2-pyridinecarboxaldehyde (see Supporting Information for experimental details). The reaction of **4** with **1** and **2** at 50 °C leads to **6** in moderate yield (Scheme 2, top).

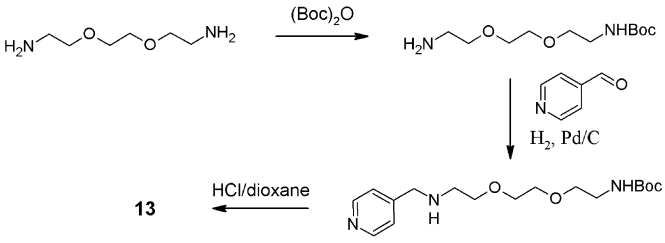
Synthesis of 7

Monomer **7** was obtained through the straightforward reaction of **5** with **1** and **2**. The Os-complex precursor **5** was synthesized by exchanging one of the chloro ligands in $[Os(bpy)_2Cl_2]$ with 4-(aminomethyl)pyridine in ethylene glycol reflux, as described in reference [19].

Synthesis of benzoxazine monomers modified with Os complexes 10 and 11 through synthetic pathway (b)

Synthesis of 10

The precursor **8** bearing two monodentate pyridine ligands was prepared through the Mannich reaction of amine **13** with **1** and **2** at room temperature (Scheme 3, top). Compound **13** was obtained in three steps starting from 2,2′-(ethylenedioxy)-bis(ethylamine) (Scheme 4). The selective protection of one of the amino groups in the starting compound with di-*tert*-butyl dicarbonate allows the modification of only one amino group with a pyridine moiety through reductive amination with 4-pyridinecarboxaldehyde. Removal of the protecting group under anhydrous conditions with HCl in dioxane gives the free amine **13** in good yield (Scheme 4). Substitution of a single chloro ligand in $[Os(bpy)_2Cl_2]$ by **8** finally leads to the monomer modified with Os complexes **10** (Scheme 3, bottom).



Scheme 4. Synthesis of the amine precursor **13** bearing a monodentate pyridine ligand through selective protection of only a single amino group in 2,2′-(ethylenedioxy)bis(ethylamine) with a Boc protecting group followed by reductive amination using 4-pyridinecarboxaldehyde and deprotection of the amino group; $(Boc)_2O$ = di-*tert*-butyl dicarbonate, Boc = –COO*t*Bu.

Synthesis of 11

Monomer **11** was synthesized through the reaction of the bidentate ligand **12** with bisphenol A and formaldehyde. Characteristic signals[6,7,20] of the CH_2 groups in the newly formed oxazine ring at $\delta = 3.89$ (–*N*–CH_2–aryl) and 4.79 ppm (–O–CH_2–*N*–) in the ^{1}H NMR spectrum indicate the successful formation of the monomer precursor **9**. The ligand exchange reaction with $[Os(bpy)_2Cl_2]$ yields the monomer modified with Os complexes **11** (Scheme 3, bottom).

Optical properties of the monomers modified with Os complexes

The variation in the coordination spheres of the Os center in the monomers **6**, **7**, **10**, and **11** leads to variations in the UV/Vis absorption behaviors. Figure 1 shows the absorption spectra of the corresponding monomers in water in the spectral range 340–800 nm. Os complexes bearing one chloro ligand show rather broad absorption bands located at 379 and ≈480 nm (**7**, red line) and at 386 and 495 nm (**10**, black line), respectively. Complexes **6** (green line) and **11** (blue line) show distinct absorption bands located at 371, 398, 441, and 485 nm, as well as a broad and weak signature in the range 600–700 nm.

Electrochemical characterization of monomers 6, 7, 10, and 11

The electrochemical properties of the monomers modified with Os complexes were investigated by means of differential pulse voltammetry (DPV) in 1.5 M aqueous KCl solutions at a glassy carbon (GC) electrode. The positive charge at the Os center ensures sufficient solubility of the synthesized monomers in the aqueous electrolyte.

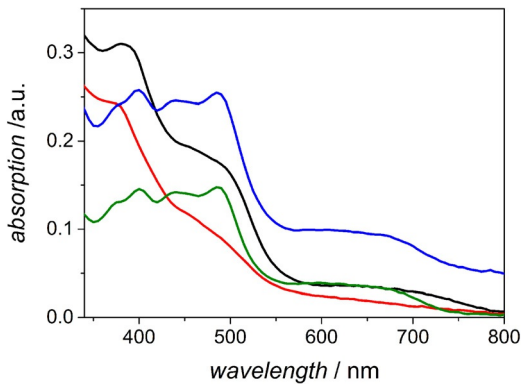


Figure 1. UV/Vis spectra of compounds **6** (green), **7** (red), **10** (black), and **11** (blue) in water in the range 340–800 nm.

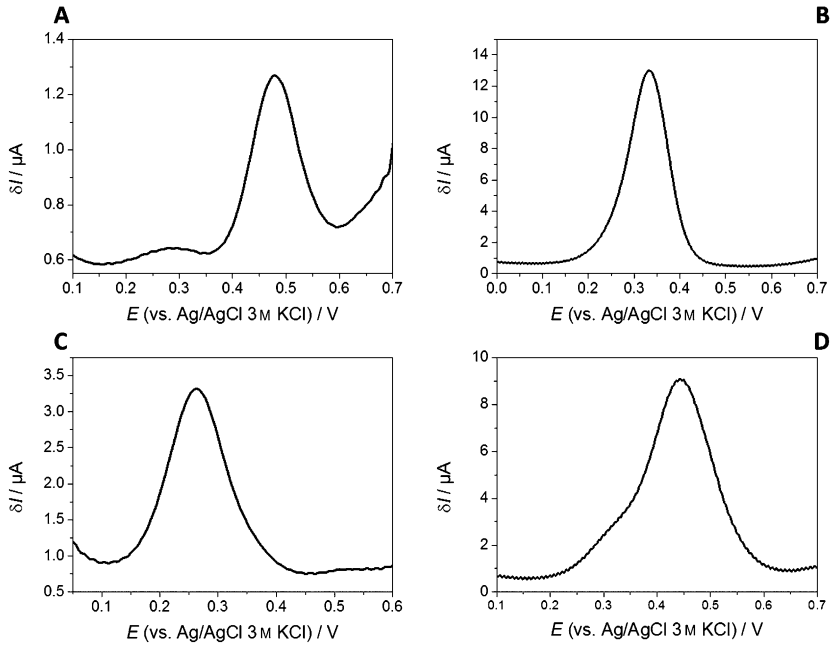


Figure 2. Differential pulse voltammograms of the Os-modified benzoxazine monomers **6** (A), **7** (B), **10** (C), and **11** (D) in 1.5 M KCl/water at a glassy carbon electrode (pulse amplitude = 25 mV, pulse duration = 0.05 s, interval time = 0.5 s, step potential = 5 mV).

Figure 2 depicts the differential pulse voltammograms of **6** (A), **7** (B), **10** (C), and **11** (D) measured in potential windows between +0.0 and +0.7 V (vs. Ag/AgCl/3 M KCl/water) with a pulse amplitude of 25 mV. In this potential window, no oxidation of the benzoxazine moiety is expected.[12] All Os complexes within the **BO**-based monomers **6**, **7**, **10**, and **11** show pronounced oxidation peaks. The potentials of maximum currents (E_{max}, Table 1) are at +0.26 V (**10**), +0.33 V (**7**), +0.44 V (**11**), and 0.48 V (**6**). The lowest values are obtained for monomers **7** (+0.33 V) and **10** (+0.26 V), which is attributed to the strong electron-donating character of the chloro ligand. As expected, the E_{max} values of compounds **6** (+0.48 V) and **11**

Table 1. Characteristic potential values for the Os-modified benzoxazine monomers **6**, **7**, **10**, and **11** as well as for the polymers **P6**, **P7**, **P10**, and **P11** derived from differential pulse voltammetry in water containing KCl as the supporting electrolyte (1.5 or 3.0 M) at GC electrodes; polymers were measured in monomer-free electrolyte after electrochemically induced polymer deposition.

Compound	E_{max} (vs. Ag/AgCl 3 M KCl) [V]
6/P6	+0.48/≈ +0.6[a]
7/P7	+0.33/+0.26[b]
10/P10	+0.26/+0.30[b]
11/P11	+0.44/+0.56[b,c]

[a] Oxidation potential determined from cyclic voltammetry; chemically irreversible electron transfer reaction. [b] Determined from differential pulse voltammograms of films deposited with the potential *pulse profile A* (20 repetitions). [c] A second oxidation process at −0.03 V is visible, which is attributed to remaining $[Os(bpy)_2Cl_2]$.

(+0.44 V), which differ only in their synthetic pathways, are almost identical. In the corresponding *I–E* curves of **6** (Figure 2A) and **11** (Figure 2D), weak signals at around +0.3 V are visible. These potential values are similar to those obtained for complexes **7** and **10,** and hence, are attributed to the monosubstituted products formed by substitution of only one chloro ligand in the $[Os(bpy)_2Cl_2]$ precursor by the bidentate ligand **12** (coordination of only one N moiety within the bidentate N2 ligand). The cyclic voltammogram of complex **11** (data not shown) shows two chemically reversible oxidation waves with estimated half-wave potentials ($E_{1/2}$) of ≈−0.03 V and ≈+0.44 V, with the latter redox process assigned to the reversible oxidation of **11** (a weak shoulder at approximately +0.35 V in the peak at +0.44 V is visible, which, again, is attributed to the monosubstituted product).

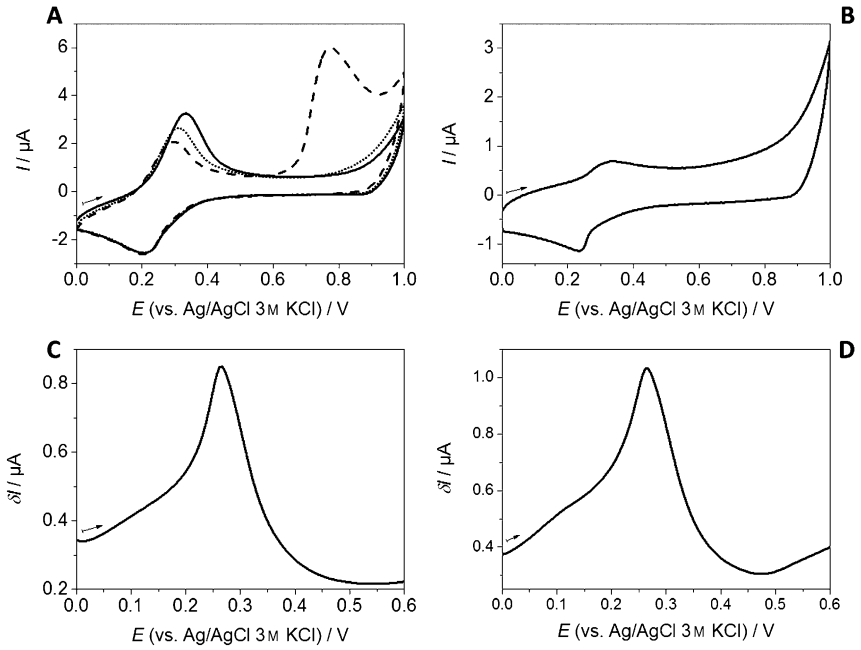


Figure 3. Electrochemically induced polymerization of **7** (A) and voltammetric characterization of the resulting poly(benzoxazine) film **P7** in monomer-free electrolyte by means of cyclic (B) and differential pulse (C, D) voltammetry. A) Cyclic voltammograms of the potentiodynamic deposition of **7** (1.25 mg mL^{-1}) in 1.5 M KCl/water at a GC electrode; dashed line: first potential cycle, dotted line: second potential cycle, solid line: third potential cycle. B,C) Cyclic (B) and differential pulse (C) voltammograms of the corresponding film deposited in (A) in monomer-free 3 M KCl/water. D) Differential pulse voltammogram of a **P7** film deposited on a glassy carbon electrode by applying potential *pulse profile A* (+1.0 V (0.05 s)/0 V (0.5 s); 20 repetitions) in 3 M KCl/water; cyclic voltammograms: $v = 50$ mV s^{-1}; differential pulse voltammograms: pulse amplitude = 25 mV, pulse duration = 0.05 s, interval time = 0.5 s, step potential = 5 mV; arrows indicate scan direction.

The electron-transfer process at lower potentials is ascribed to the oxidation of the remaining $[Os(bpy)_2Cl_2]$. In the cyclic voltammogram of **6** this signal is absent. This demonstrates the advantage of synthetic pathway (a), which leads to an $[Os(bpy)_2Cl_2]$-free product. The redox potentials for complexes **6**, **7**, **10**, and **11** are in good agreement with values obtained for Os complexes bearing similar/identical ligand systems.[16,19,21,22]

Anodic polymerization of monomers 6, 7, 10, and 11 and electrochemical characterization of the corresponding polymer films P6, P7, P10, and P11

The electrochemically induced deposition of the benzoxazine monomers modified with Os complexes (**6**, **7**, **10**, and **11**) under formation of the corresponding polymers (**P6**, **P7**, **P10**, and **P11**) was first investigated by means of cyclic voltammetry (potentiodynamic deposition) in 1.5 M KCl/water at GC disk electrodes in potential windows of −0.2 to +1.0 V (**11**) or 0 to +1.0 V (**6**, **7**, and **10**). Figure 3A shows the first (dashed line), second (dotted line), and third (solid line) potential cycles recorded in a solution containing monomer **7** at a scan rate (v) of 50 mV s^{-1} as an example. In the first cycle, two oxidation processes located at +0.33 V (chemically *reversible*) and +0.77 V (chemically *irreversible*) are visible. The first oxidation corresponds to the Os^{II}/Os^{III} couple in **7**. The second anodic process is attributed to the oxidation of the benzoxazine moiety. The chemically irreversible nature of the benzoxazine oxidation indicates that there is indeed a chemical follow-up reaction coupled to the electron-transfer process, that is, the polymerization of the oxidized benzoxazine species that forms polymer **P7**. In the second potential cycle, only signals of the Os^{II}/Os^{III} couple are detected, and the peak for the oxidation of the benzoxazine monomer is absent. The electrochemically induced polymerization during the first potential cycle leads to the passivation of the electrode surface with respect to further monomer oxidation, and thus, further polymerization, probably owing to hampered diffusion of the large benzoxazine monomers modified with Os complexes to the electrode surface. In contrast, the signal for the Os moiety is still detectable: diffusion of the smaller electrolyte counter ions that are necessary for a successful electron-transfer reaction seems to be possible within the polymer film.

The slight increase in the peak current (I_p) of the oxidation wave at around +0.33 V in the second and third potential cycles may be attributed to the transition from a pure diffusion-controlled electron transfer (ET) reaction (first cycle, no polymer film is present, $I_p \sim v^{1/2}$) to an adsorption-controlled ET reaction (Os species is entrapped in the polymer film, $I_p \sim v$).

For evaluation of the stability of **P7**, the modified electrodes were removed from the monomer solution, rinsed thoroughly with deionized water, and placed in a monomer-free electrolyte solution (3 M KCl/water). The cyclic voltammogram of **P7** shows a chemically reversible oxidation of the Os^{II} moiety with

an oxidation potential of +0.33 V (Figure 3B). The corresponding differential pulse voltammogram (Figure 3C) shows a pronounced peak of maximum current at +0.27 V.

In addition, the formation of **P7** succeeds upon application of a potential pulse sequence (*pulse profile A*) that consists of pulse 1 = +1.0 V/0.05 s followed by pulse 2 = +0 V/0.5 s. The differential pulse voltammogram of **P7** deposited by applying 20 repetitions of *pulse profile A* in monomer-free solution shows a pronounced signal for the oxidation of the Os complex at +0.26 V (Table 1). This value is in good agreement with that obtained for the film deposited under potentiodynamic conditions (Figure 3C).

Furthermore, the values for **P7** are in good agreement with the potential observed for the freely diffusing monomer **7** (+0.33 V). Similar results are obtained for **10**/**P10** (Table 1). Clearly, the potentiodynamic deposition has no significant effect on the redox potential of the Os moiety in **P7** and **P10**. However, for polymers **P6** and **P11**, a shift to more positive potentials by 120 mV (Table 1) is observed, indicating that the ligand sphere of the Os center is affected by the polymerization process. Compared with the monodentate pyridine ligand in **7** and **10**, the bidentate pyridine-imidazole ligand in **6** and **11** seems to be less stable during the polymerization process. The electron spray ionization mass spectrum of the Os precursor **4**, which is used as the Os complex in the synthesis of **6**, shows a peak with an *m/z* ratio that corresponds to a fragment of $\mathbf{4}^+$ in which the aminohexyl group at the imidazole moiety is removed from the parent complex. The $[M]^+$ peak is absent. The N–C bond between the hexyl chain and the N atom of the imidazole ring seems to be labile, and may be attacked during the electrochemically induced polymerization process. This may alter the electronic structure of the coordination sphere, and thus, the potential of the Os complex. Nonetheless, the peaks in the voltammetric curves indicate that an Os complex is still present in the deposited film.

The differential pulse voltammogram of **P11** reveals two oxidation processes in a potential window of −0.2 to +0.8 V located at potentials of −0.03 V ($[Os(bpy)_2Cl_2]$) and +0.56 V (**P11**). Clearly, the remaining $[Os(bpy)_2Cl_2]$ is entrapped within the polymer film during the polymerization process. Thus, for **P11**, both Os species may contribute to the biocatalytic activity within this film. This has to be taken into account upon comparison of the biocatalytic properties of this compound with that of **P6** (see below). For **P6**, signals that correspond to the oxidation of $[Os(bpy)_2Cl_2]$ are absent.

Enzyme immobilization and biocatalytic activity

The codeposition of benzoxazine monomers **6**, **7**, **10**, and **11** to the corresponding poly(benzoxazine) films, simultaneously entrapping GOx under formation of the bioactive layers **P6-GOx**, **P7-GOx**, **P10-GOx**, and **P11-GOx** on the electrode, was performed in different buffer solutions (citrate/phosphate or phosphate buffer) by applying potential *pulse profile A* or *pulse profile B* (pulse 1 = +1.7 V/0.05 s followed by pulse 2 = 0 V/0.5 s, vs. Au pseudo reference) in a 7 mL one-compartment cell (*pulse profile A*) or in 200 μL Au micro-wells (*pulse profile B*). In the case of the potentiodynamic deposition by means of cyclic voltammetry, the electrode was fully blocked after the first potential cycle (Figure 3A). Thus, for the codeposition with GOx, a pulse profile with short pulse durations and only a few repetitions (*pulse profile A* = 2 repetitions; *pulse profile B* = 5 repetitions) was employed. For the investigation of the biocatalytic activity, chronoamperometric measurements in the presence of different glucose concentrations and at applied potentials of +0.4 or +0.5 V were performed.

Figure 4 shows the dependence of the biocatalytic current response for **P6-GOx** (blue triangles), **P7-GOx** (black squares), **P10-GOx** (red filled circles), and **P11-GOx** (green triangles) deposited by applying *pulse profile B* in Au micro-wells in the presence of different glucose concentrations (0–200 mM). The data were obtained through chronoamperometric experiments in phosphate buffer (10 mM, 0.1 M NaCl) at pH 7.4 with an applied potential of +0.4 V. All the films show a biocatalytic activity that correlates well with Michaelis–Menten-type behavior. For each active layer, the apparent Michaelis constant K_M^{app} was estimated from fits based on Michaelis–Menten kinetics, and equals 14.5 (**P7-GOx**, black line), 18.1 (**P10-GOx**, red line), 24.5 (**P6-GOx**, blue line), or 28.5 mM (**P11-GOx**, green line). The corresponding saturation currents are 6.79 (**P7-GOx**), 6.72 (**P10-GOx**), 11.92 (**P6-GOx**), and 14.94 nA (**P11-GOx**).

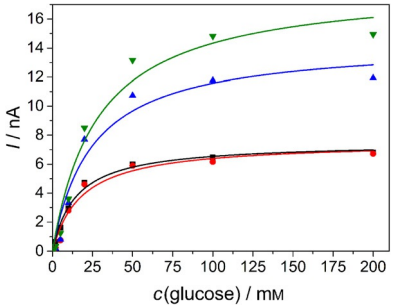
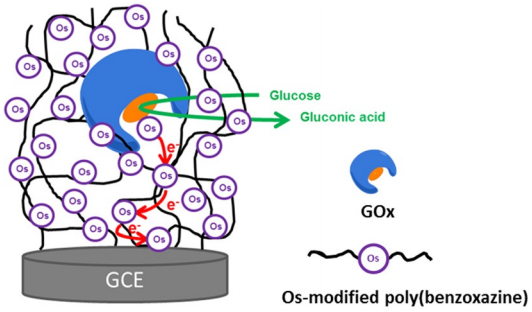


Figure 4. Dependence of the biocatalytic current response on the glucose concentration for **P6-GOx** (blue triangles), **P7-GOx** (black squares), **P10-GOx** (red filled circles), and **P11-GOx** (green triangles) determined in phosphate buffer (10 mM, pH 7, 0.1 M NaCl); data were obtained through chronoamperometric measurements at an applied potential of +0.4 V (vs. Ag/AgCl/3 M KCl/water). Films were deposited onto glassy carbon electrodes that were placed in 200 μL Au micro-wells containing a mixture of the corresponding **BO** monomer and GOx by applying *pulse profile B* (+1.7 V (0.05 s)/0 V (0.5 s), 5 repetitions).

The biocatalytic currents obtained for the films deposited by applying *pulse profile A* show a similar behavior. An increase in the operating potential from +0.4 to +0.5 V has no significant effect on the biocatalytic currents, suggesting that the current response is caused exclusively by the wiring of the enzyme through the polymer-bound Os complexes, as illustrated in Scheme 5.

H_2O_2 potentially formed in a competition reaction for the reduced prosthetic group within the enzyme between the polymer-bound Os complexes and O_2 does not contribute to the observed current response. An increase in the number of potential pulses during the deposition of the films leads to com-



Scheme 5. Schematic illustration of the active layer of the amperometric biosensor obtained from the codeposition of an Os-modified **BO** monomer and glucose oxidase (GOx) onto the surface of a glassy carbon electrode (GCE). Red arrows indicating the electron transport from the enzyme to the electrode surface through the Os-complex-based redox mediators.

plete blocking of the electrode surface, and hence, no significant catalytic current could be detected for films deposited with more than ten repetitions of the potential pulse sequence. The diffusion of the substrate glucose to the active center of the enzyme within the polymer film seems to be prevented completely in this case.

Conclusion

Benzoxazine precursors modified with Os complexes were synthesized by following two different synthetic approaches: the reaction of an amine-modified Os complex with bisphenol A and formaldehyde to generate directly an Os complex containing the benzoxazine monomer; or by the synthesis of benzoxazine monomers bearing mono- or bidentate ligands for the coordination of the Os center through a ligand exchange reaction with $[Os(bpy)_2Cl_2]$. Electrochemically generated poly(benzoxazine) films bearing covalently bound Os complexes that act as redox relays were successfully introduced as a stable immobilization matrix for the wiring of glucose oxidase to electrode surfaces. The enzyme-modified electrodes exhibited the envisaged biocatalytic activity in the presence of glucose. This opens a route for the application of films of poly(benzoxazine)s modified with Os complexes as a novel immobilization matrix for reagentless amperometric biosensors.

Experimental Section

Materials and methods

All chemicals and materials were purchased from Sigma–Aldrich, Merck, J.T. Baker, Acros Organics, VWR, or Fluka, and were of analytical or reagent grade. Deuterated solvents for NMR spectroscopy were purchased from Deutero. Glucose oxidase from *Aspergillus niger* (type X-S, lyophilized powder, 100,000–250,000 units g^{-1}) was purchased from Sigma–Aldrich and stored at −20 °C or +4 °C, respectively.

All ^1H and $^{13}C\{^1H\}$ NMR experiments were performed with a DPX 200 or a DRX 400 NMR spectrometer (Bruker) with resonance frequencies of 200.13 MHz (^1H) and 50.32 MHz (^{13}C) as well as 400.13 MHz (^1H) and 100.62 MHz (^{13}C), respectively, at room temperature or 300 K.

Electron spray ionization (ESI) mass spectrometry measurements were performed with a Finnigan GCQ Iontrap spectrometer in methanol solution. Fast atom bombardment (FAB) mass spectrometry measurements were recorded with a VG Autospec spectrometer.

UV/Vis measurements in water were made with a Cary 60 spectrometer (Agilent Technologies) using polystyrene cuvettes (340–900 nm) with an optical path length of 1 cm.

Electrochemical methods

All electrochemical experiments were conducted at room temperature with an Autolab PGSTAT12 or a CHI 1030 potentiostat in a one-compartment, three-electrode cell. Glassy carbon (GC) disk electrodes with nominal diameters of 3 mm (BASi) were used as working electrodes. The working electrodes were subsequently polished with diamond paste (3 μm) and Al_2O_3 (1 μm, then 0.3 μm, both from Leco Cooperation) by using a Metkon IV polisher (Metkon). After polishing, the electrodes were rinsed with pure water, sonicated for 15 min in water, and dried in air prior to use. The counter electrode consisted of a Pt wire (diameter ≈ 1 mm). A Ag/AgCl/3 M KCl/water system was used as a reference electrode. All potentials quoted are referenced to this reference electrode.

Differential pulse voltammograms (pulse amplitude = 25 mV, pulse duration = 0.05 s, interval time = 0.5 s, step potential = 5 mV) of the benzoxazine monomers modified with Os complexes were recorded in 1.5 M KCl/water by mixing a 3 M KCl/water electrolyte with stock solutions of the respective benzoxazine monomer in a 1:1 ratio (stock solution of the benzoxazine monomers **6**, **7**, **10**, and **11** were prepared in water with different additives to ensure good solubility of the compounds: **6**: + 1 vol% CH_3COOH, 5 mg mL^{-1}; **7**: 5 mg mL^{-1}; **10**: + 1 vol% CH_3COOH and a few drops of MeCN, 2.5 mg mL^{-1}; **11**: 0.1 M NaCl, 5 mg mL^{-1}).

Deposition of the pristine poly(benzoxazine) films onto GC electrodes was conducted in 2.25 M KCl/water by cycling the potential between −0.2 and + 1.0 V or 0 and + 1.0 V, respectively, (potentiodynamic deposition), or by applying a potential pulse profile: + 1.0 V/0.05 s followed by 0 V/0.5 s (*pulse profile A*, 10 to 20 repetitions) with monomer concentrations of 1.25 mg mL^{-1}. After deposition, the modified GC electrodes were rinsed thoroughly with deionized water to remove weakly bound polymer.

Voltammetric characterization of the pristine films was conducted in monomer-free 3 M KCl/water electrolyte by means of cyclic voltammetry (scan rate = 20–50 mV s^{-1}) or by differential pulse voltammetry (pulse amplitude = 25 mV, pulse duration = 0.05 s, interval time = 0.5 s, step potential = 5 mV).

Codeposition of polymers (monomer concentration = 0.63 mg mL^{-1}) and GOx (c = 10 mg mL^{-1}) was conducted in 1.13 mM KCl/0.1 M citrate phosphate buffer (pH 7.4) by mixing 1:1 ratios of the corresponding benzoxazine monomer stock solution (1.25 mg mL^{-1} in 2.25 M KCl/water) and GOx solutions (20 mg mL^{-1} in 0.2 M citrate phosphate buffer, pH 7.4) in a one-compartment, three-electrode electrochemical cell and applying *pulse profile A* (2 repetitions).

In addition, the codeposition was performed using 200 μL Au micro-wells with monomer concentrations of 2.5 mg mL^{-1} (stock solutions of the native monomers were used, including the addi-

tives for enhancing the solubility, see above) and a GOx concentration of 5 mg mL^{-1} (using a 10 mg mL^{-1} stock solution of the enzyme in water, which was freshly prepared prior to use). For the codeposition the Au micro-well plate was used as the counter electrode and as the Au pseudo reference electrode. A potential pulse 1 (+1.7 V/0.05 s) was employed followed by pulse 2 (0 V/0.5 s) (*pulse profile B*, 5 repetitions).

Chronoamperometric measurements with the GOx-modified electrodes in the presence of different glucose concentrations were conducted in a standard one-compartment, three-electrode cell with 7 mL of a 0.2 M citrate phosphate buffer (pH 7.4) solution or with 7 mL of a 0.1 M NaCl/10 mM phosphate buffer (pH 7) solution. An operating potential of +0.4 V (for films deposited by *pulse profile B*) or +0.5 V (*pulse profile A*) was applied. Glucose concentrations were in the range 1–200 mM. For the different concentrations, appropriate aliquots of a glucose stock solution (1 M) were added to the electrolyte. The addition of the glucose solution was performed after a steady-state current was reached in the *I*–*t* curve. Fits of the calibration curves were based on Michaelis–Menten kinetics implemented in the Origin software package (version 9.0).

Synthesis

All synthesis and purification steps were conducted under ambient conditions unless otherwise noted. Detailed protocols for the syntheses of all compounds and the corresponding analytical data are given in the Supporting Information. The preparations of $[Os(bpy)_2Cl_2]$ and the Os complex **5** were described earlier in references [23] and [19], respectively. The syntheses of ligand **12** and Os complex **4** were based on protocols described earlier.[16]

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[1] N. N. Ghosh, B. Kiskan, Y. Yagci, *Prog. Polym. Sci.* **2007**, *32*, 1344–1391.
[2] B. Kiskan, N. N. Ghosh, Y. Yagci, *Polym. Int.* **2011**, *60*, 167–177.
[3] C. Nair, *Prog. Polym. Sci.* **2004**, *29*, 401–498.
[4] F. X. Perrin, J. Pagetti, *J. Appl. Polym. Sci.* **2000**, *75*, 1054–1067.
[5] L. Li, J. He, X. Wan, D. Zhou, G. Xue, Y. Wang, S.-W. Choi, H. Ishida, *J. Adhes.* **2003**, *79*, 351–360.
[6] T. Agag, T. Takeichi, *J. Polym. Sci. Part A* **2007**, *45*, 1878–1888.
[7] T. Takeichi, T. Kano, T. Agag, *Polymer* **2005**, *46*, 12172–12180.
[8] T. Takeichi, T. Kawauchi, T. Agag, *Polym. J.* **2008**, *40*, 1121–1131.
[9] R. P. Subrayan, F. N. Jones, *Chem. Mater.* **1998**, *10*, 3506–3512.
[10] P. Audebert, M. Roche, J. Pagetti, *J. Electroanal. Chem.* **1995**, *383*, 139–143.
[11] K. Nesmerak, I. Nemec, M. Sticha, K. Waisser, K. Palat, *Electrochim. Acta* **2005**, *50*, 1431–1437.
[12] C. Andronescu, S. Pöller, W. Schuhmann, *Electrochem. Commun.* **2014**, *41*, 12–15.
[13] T. J. Ohara, R. Rajagopalan, A. Heller, *Anal. Chem.* **1993**, *65*, 3512–3517.
[14] T. J. Ohara, R. Rajagopalan, A. Heller, *Anal. Chem.* **1994**, *66*, 2451–2457.
[15] D. A. Guschin, J. Castillo, N. Dimcheva, W. Schuhmann, *Anal. Bioanal. Chem.* **2010**, *398*, 1661–1673.
[16] S. Pöller, Y. Beyl, J. Vivekananthan, D. A. Guschin, W. Schuhmann, *Bioelectrochemistry* **2012**, *87*, 178–184.
[17] T. Kothe, N. Plumeré, A. Badura, M. M. Nowaczyk, D. A. Guschin, M. Rögner, W. Schuhmann, *Angew. Chem. Int. Ed.* **2013**, *52*, 14233–14236; *Angew. Chem.* **2013**, *125*, 14483–14486.
[18] D. R. Baker, R. F. Simmerman, J. J. Sumner, B. D. Bruce, C. A. Lundgren, *Langmuir* **2014**, *30*, 13650–13655.
[19] P. Ó Conghaile, S. Pöller, D. MacAodha, W. Schuhmann, D. Leech, *Biosens. Bioelectron.* **2013**, *43*, 30–37.
[20] K. Pakkethati, A. Boonmalert, T. Chaisuwan, S. Wongkasemjit, *Desalination* **2011**, *267*, 73–81.
[21] F. Zhao, K. Sliozberg, M. Rögner, N. Plumere, W. Schuhmann, *J. Electrochem. Soc.* **2014**, *161*, H3035–H3041.
[22] R. Kumar, D. Leech, *J. Electrochem. Soc.* **2014**, *161*, H3005–H3010.
[23] K. Habermüller, A. Ramanavicius, V. Laurinavicius, W. Schuhmann, *Electroanalysis* **2000**, *12*, 1383–1389.

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