Supporting Information for: Spectroelectrochemical Investigation of a Flavoprotein with a Flavin-Modified Gold Electrode

Spectroelectrochemical Setup

The spectroelectrochemical cell applied in this work has been described already in detail [1]. A schematic illustration is shown in Figure 8. Measurements have been carried out under Argon atmosphere. Counter- and reference electrode (not shown) are placed in the bulk solution. A transparent gold minigrid working electrode is fixed between two quartz slides with a distance of about 100 µm. The protein solution forms a thin layer between the quartz slides due to capillary forces. A partial area of this layer is sampled. The cell is mounted in the compartment of a PERKIN-ELMER Lambda 9 UV/Vis/NIR-Spectrometer.

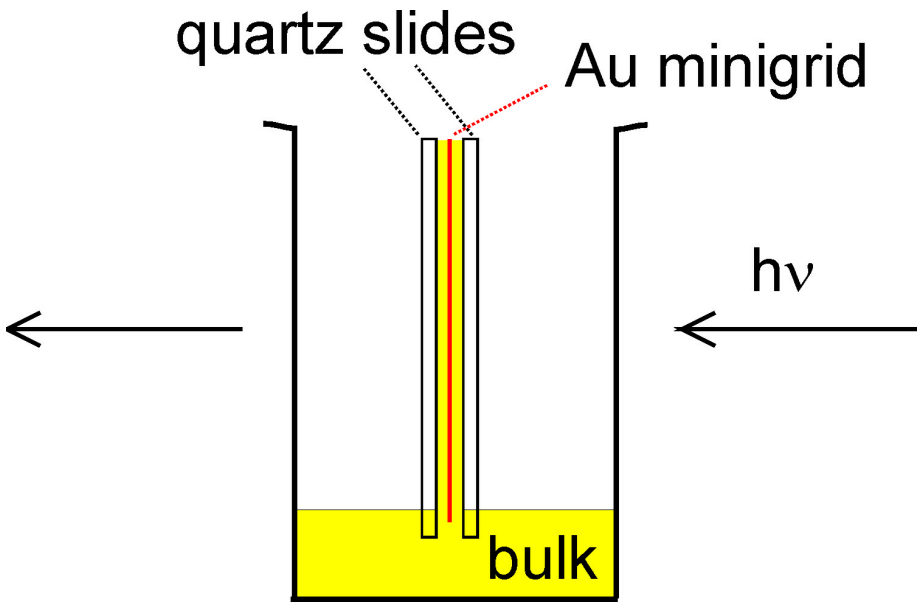


Figure 8. Schematic illustration of the spectroelectrochemical cell applied in this work. A transparent gold minigrid working electrode is fixed between two quartz slides with a distance of about 100 µm.

Electrode Surface Modification

Searching for proper conditions to form the amide bond, we dissolved $CofC_6$ and 3,3‘-dithiodipropionic acid di-(*N*-succinimidyl ester) in DMSO as shown in Figure 9 and stirred over night after addition of NEt_3 to bind the TFA.

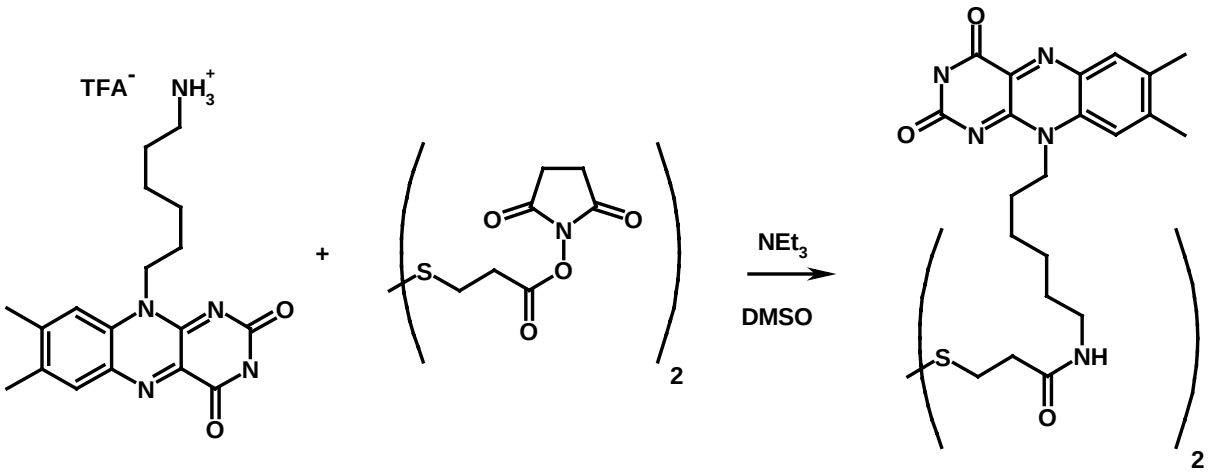


Figure 9. Coupling of $CofC_6$ and 3,3‘-dithiodipropionic acid di-(*N*-succinimidyl ester)

The product was precipitated by addition of water, then collected, and dried. Two dimensional NMR-analysis allowed the assignment of all ^{13}C signals of the pure coupling adduct as illustrated in the experimental part.

Next, we had to establish a procedure for immobilization of the thiol monolayer. After cleaning with H_2SO_4/H_2O_2 (2:1), a gold electrode was immersed in a mixture of activated and non-activated carboxylic acid in DMSO with an overall disulfide concentration of about 100 mM. This assembly was allowed to stand at least for 12 hours up to 2 days at room temperature. With lower incubation time we obtained less satisfactory results. The electrode was rinsed carefully with water and immersed into a solution of $CofC_6$ at a concentration of 1 mM. After addition of 2-3 drops of NEt_3 the coupling was allowed to proceed for 12 hours. Thereafter, the electrode was thoroughly cleaned by rinsing with water and used for electrochemical measurements. For all measurements reported in this paper a ratio of 1:10

between activated and non-activated carboxylic acid was used. The same immobilization procedure also worked successfully on a platinum electrode (data not shown).

We also carried out direct adsorption of 3,3‘-dithiodipropionic acid di-{*N*(10)-[6-(aminohexyl] flavin} amid at gold electrodes and investigated these electrodes by cyclic voltammetry resulting in less good results (The corresponding CVs showed a relatively broad unresolved reductive signal with more than one maximum, data not shown). This observation might be explained by non-specific adsorption of the flavins e.g. by their imide substructure.

Synthesis of the Cofactor

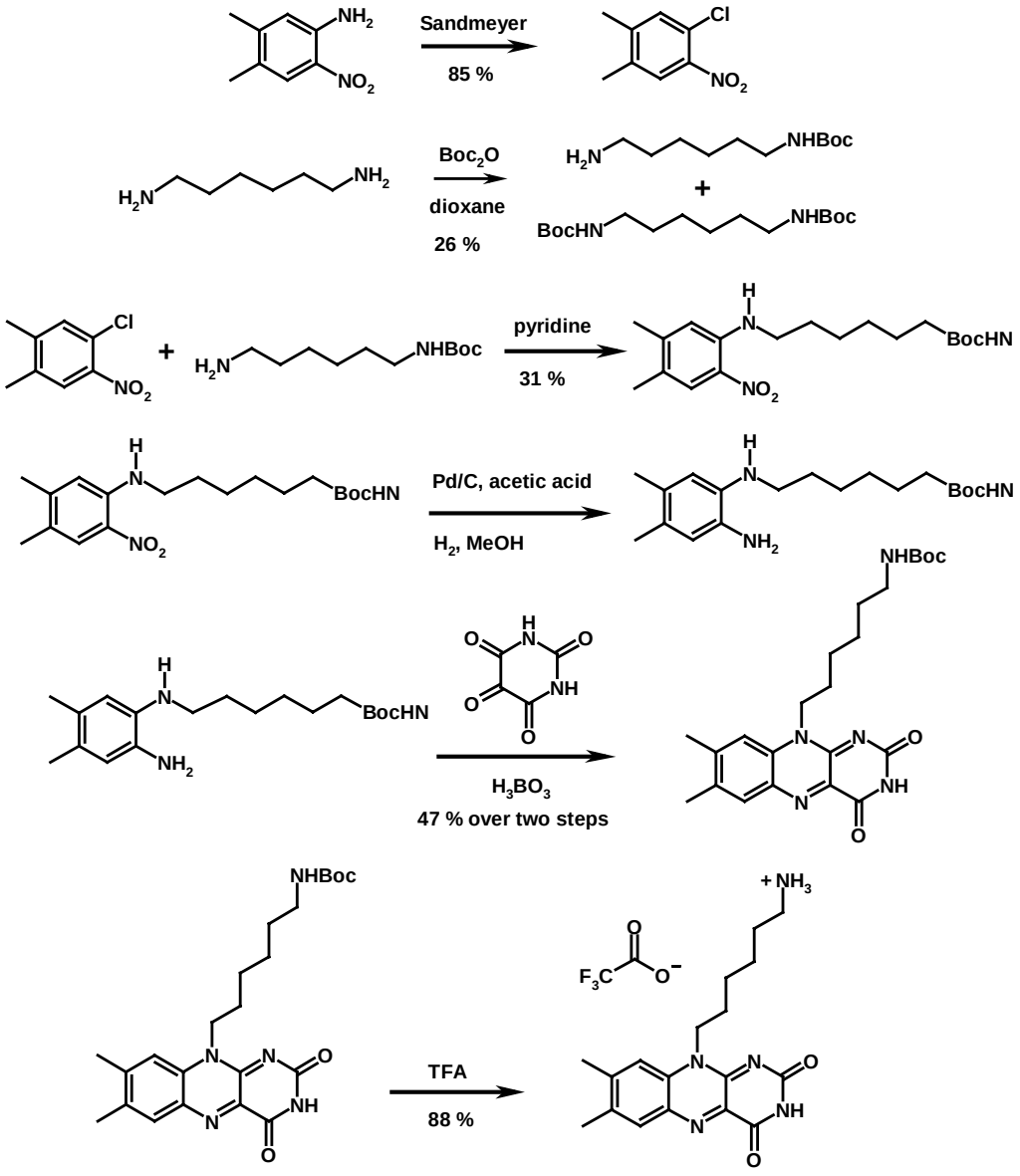


Figure 10. Reaction scheme leading to flavin cofactor $CofC_6$ with hexyl amino chain.

The reaction scheme in Figure 10 leads to flavin derivatives bearing an alkyl amino group at N(10) and was carried out similarly to the synthesis of the homologue ethyl amino derivative[2]. The preparation required prior protection of the amino group finally used for amid bond formation at the electrode.

1-Chloro-2-nitro-4,5-dimethylbenzene was synthesized from 4-amino-5-nitro-o-xylene by a Sandmeyer reaction[3]. We obtained the mono-protected diamine by reacting the diamine directly with Boc_2O but more versatile procedures are known as well[4,5]. The product had to be separated from diprotected diamine by extraction at different pH. In the next step, we performed a nucleophilic aromatic substitution with the mono-protected diamine on 1-Chloro-2-nitro-4,5-dimethylbenzene. The resulting 1-alkylamino-2-nitro-4,5-dimethylbenzene was reduced and immediately reacted with alloxane. The obtained flavin was deprotected with trifluoroacetic acid (TFA). All reaction steps have been carried out only once, hence yields are not optimized.

Experimental Part

1-Chloro-2-nitro-4,5-dimethylbenzene

1-Chloro-2-nitro-4,5-dimethylbenzene was synthesized from 4-amino-5-nitro-o-xylene according to the procedure in Lit.[3]. Yield: 8.13 g; 73 %; 43.8 mmol; (Lit: 9.45 g, 85 %) m.p.: 59-60 °C; (Lit: 61-62 °C) **$C_8H_8ClNO_2$; 185.61 g/mol**

N-Boc-1,6-hexanediamine

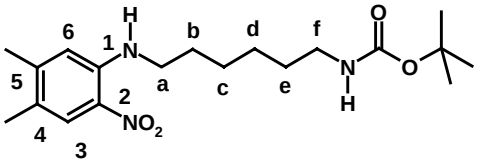
A solution of Boc_2O (4.30 g, 0.02 mol) in 1,4 dioxane (14.3 ml) was added dropwise within 2 h at room temperature to a solution of hexylene-diamine (5.31 g, 0.04 mol) in 1,4-dioxane (21.4 ml). The reaction mixture was stirred for another 20 h at room temperature, whereupon water (20 ml) and ether (30 ml) are added. After vigorous stirring for a few minutes the ether phase was isolated, extracted with aqueous hydrochloric acid (10 ml, pH 4.7), washed with water (8 ml) dried over anhydrous magnesium sulfate and conentrated *in vacuo* to give the diprotected diamine. **$C_{16}H_{32}N_2O_4$; 316,5 g/mol**

The combined aqueous extracts from above were made alkaline (pH 12) with 6 N sodium hydroxide and extracted with ether (4 times 30 ml).The combined etherous phase was washed with concentrated aqueous sodium chloride (80 ml), concentrated to 12 ml by evaporation and mixed with water (8 ml). The pH of the vigorously stirred mixture is adjusted to 5.3 with 6 N hydrochloric acid, whereupon the ether is evaporated in vacuo, and the residual aqueous solution lyophilized to give the hydrochloride of *N*-Boc-1,6-hexanediamine. Crystallization from ethyl acetate/ethanol (4:3) gives hydrochloride of *N*-Boc-1,6-hexanediamine as white crystals. Yield: 1.3 g; 5.12 mmol; 26 %; m.p.: 153°C (Lit.[5]: 153-154°C)
$C_{11}H_{24}N_2O_2$ * HCl = 252.78 g/mol

1-[(^{t}Butyl)oxycarbonylaminohexyl]-2-nitro-4,5-dimethylbenzene

The mono-Boc protected hexylene diamine (1.86 g; 8.6 mmol) was dissolved in pyridine (4.8 ml). This solution was slowly added to a solution of 1-Chloro-2-nitro-4,5-dimethylbenzene **1** (1.6 g; 8.6 mmol) in pyridine (16 ml). The reaction mixture was stirred for 48 h at 90 °C and subsequently concentrated *in vacuo*. The residual material was re-crystallized from ethanol. The product was obtained as intensively

red-colored needles. Yield: 1.04 g; 31 %; 2.85 mmol; m.p.: 93 °C **$C_{19}H_{31}N_3O_4$; 365.47 g/mol** EA: calc.: **C:** 62.44 **H:** 8.55 **N:** 11.50 found: **C:** 62.45 **H:** 8.49 **N:** 11.38 CI-MS (NH_3): MH^+: 366(19%), 365(100%)



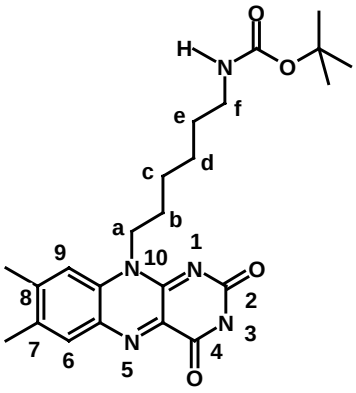
^{1}H-NMR (600 MHz, $CDCl_3$): δ = 1.39(2H, m, H_d), 1.44(9H, s, ^{t}Bu), 1.46(2H, m, H_c), 1.51(2H, m, H_e), 1.72(2H, m, H_b), 2.17(3H, s, CH_3 at C_4), 2.27(3H, s, CH_3 at C_5), 3.12(2H, m, H_f), 3.27(2H, t, J = 7.0 Hz, H_a), 4.52(1H, s, br, NH), 6.61(1H, s, H6), 7.92(1H, s, H3), 7.95(1H, s, br, NH); determination was done by HMBC, HSQC and COSY. ^{13}C-NMR (150.9MHz, $CDCl_3$): δ = 18.6(CH_3 at C_4), 20.7(CH_3 at C_5), 26.5(C_d), 26.8(C_c), 28.4((CH_3)$_3$), 29.0(C_b), 30.1(C_e), 40.5(C_f), 43.0(C_a), 79.1(C(CH_3)$_3$), 114.1(C_6), 124.3(C_4), 126.5(C_3), 129.7(C_2), 144.3(C_1), 147.3 (C_5), 156.0 (N-C=O); determination was done by HMBC, HSQC and COSY.

1-[6-(^{t}Butyl)oxycarbonylamino-hexyl]-2-amino-4,5-dimethylbenzene

A suspension of 10% Pd/C catalyst (21.7 mg) in acetic acid (2.2 ml) was added slowly to a solution of 1-[(^{t}Butyl)oxycarbonylaminohexyl]-2-nitro-4,5-dimethylbenzene; (0.710 g, 1.94 mmol) in methanol (30 ml). The suspension was stirred for 20 h at room temperature in an H_2 atmosphere. The reaction mixture was filtered through Celite under inert-gas atmosphere. The product is very sensitive to oxidation. Therefore, the next reaction step was carried out immediately without further purification. **$C_{19}H_{33}N_3O_2$; 335.49 g/mol**

***N*(10)-[6-(^{t}Butyl)oxycarbonylamino-hexyl] flavin**

Alloxan monohydrate (2.1 g, 13.1 mmol) and boric acid (3.7 g, 60.8 mmol) were added to the filtrate containing 1-[6-(^{t}Butyl)oxycarbonylamino-hexyl]-2-amino-4,5-dimethylbenzene under inert-gas atmosphere and stirred for 12 h at room temperature. The reaction mixture was diluted with $CHCl_3$ (108 ml) and extracted with water (3 times 70 ml). The organic phase was separated and dried over magnesium sulphate. After removing the solvent, the orange product was obtained by column chromatography on silica gel ($CHCl_2$/MeOH 15:1 → 10:1). Yield: 399 mg; 47 %; 0.904 mmol m.p.: pyrolysis at 236 °C **$C_{23}H_{31}O_4N_5$; 441.53 g/mol** ESI-MS: MH^+: 442(100%); 443(28%); MNa^+: 464(12%)



^{1}H-NMR (600 MHz, DMSO-d6): δ = 1.32(2H, m, H_d), 1.36(9H, s, ^{t}Bu), 1.40(2H, m, H_e), 1.45(2H, m, H_c), 1.70(2H, m, H_b), 2.40(3H, s, CH_3 at C_7), 2.52(3H, s, CH_3 at C_8), 2.91(2H, m, H_f), 4.56(2H, m, H_a), 6.77(1H, m, NH), 7.77(1H, s, H_9), 7.89(1H, s, H_6), 11.28(1H, s, NH-Ar). determination was done by

HMBC, HSQC and COSY. ^{13}C-NMR (150,9MHz, DMSO-d6): δ = 18.7; 20.6; 25.8; 26.0; 26.4, 28.2, 29.3, 40.0, 44.0, 77.3; 116.0; 130.7; 131.0; 133.8; 135.7; 137.1; 146.5; 150.0; 155.5; 155.6, 159.9.

_N_(10)-[6-(aminohexyl] flavin • TFA, CofC$_6$ • TFA

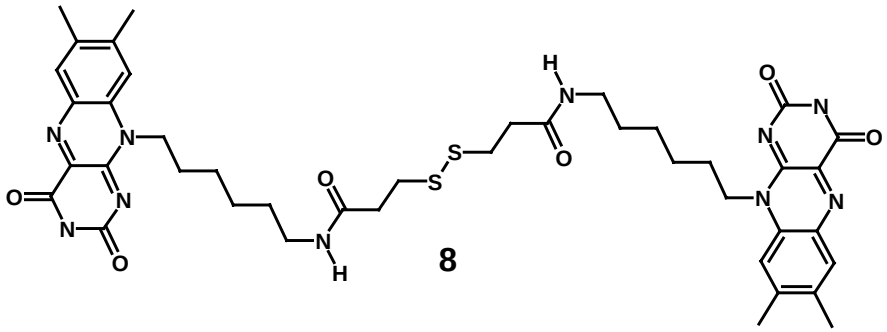
A solution of _N_(10)-[6-(^{t}Butyl)oxycarbonylamino-hexyl] flavin (360 mg, 0.815 mmol) was stirred in TFA/water (8.2 ml, 95:5) for 3 h at room temperature. The reaction solution was evaporated *in vacuo* and the product was precipitated through addition of diethyl ether. Compound CofC$_6$ was obtained as the TFA salt in form of a yellow powder. Further purification was done by precipitation (the product was dissolved in a small amount of MeOH/CH_2Cl_2 and poured into a stirred solution of Et_2O). Yield: 325 mg; 0,714 mmol; 88 %; m.p.: pyrolysis at 177 °C **$C_{20}H_{24}F_3N_5O_4$; 455.43 g/mol**

E.A.: calc.: **C:** 52.75 **H:** 5.31 **N:** 15.38 found: **C:** 52.54 **H:** 5.25 **N:** 14.71 EI-MS(PI) : M^+(amine): 342(6%), 341(38%) additional peaks: 269(38%), 256(24%), 244(27%), 243(100%), 242(31%). EI-MS (high resolution, PI): calc.: 341.1852 found.: 341.1848 Δ = 1.1 ppm

^{1}H-NMR (400 MHz, DMSO-d6): δ = 1.45(4H, m, H_d and H_c), 1.58(2H, m, H_e), 1.73(2H, m, H_b), 2.40(3H, s, CH_3 at C_7), 2.52(3H, s, CH_3 at C_8), 2.81(2H, m, H_f), 4.58(2H, m, H_a), 7.72(3H, s, br, NH_3^+), 7.80(1H, s, H_9), 7.91(1H, s, H_6), 11.32(1H, s, NH-Ar). determination was done by HMBC, HSQC and COSY. ^{13}C-NMR (100.6 MHz, DMSO-d6): δ = 18.7(CH_3 at C_7), 20.5(CH_3 at C_8), 25.2(C_d), 25.3(C_c), 26.0(C_b), 26.7(C_e), 38.7(C_f), 43.8(C_a), 115.9(C_9), 117.1(q, $J_{CF3\text{-}19F}$ = 301.02 Hz, CF_3), 130.6, 131.0(C_6), 133.8, 135.8, 137.0, 146.6, 149.9, 155.8, 157.8(q, $J_{COO(CF3)\text{-}19F}$ = 31.18 Hz, COO), 159.9.

3,3‘-dithiodipropionic acid di-{_N_(10)-[6-(aminohexyl] flavin} amid

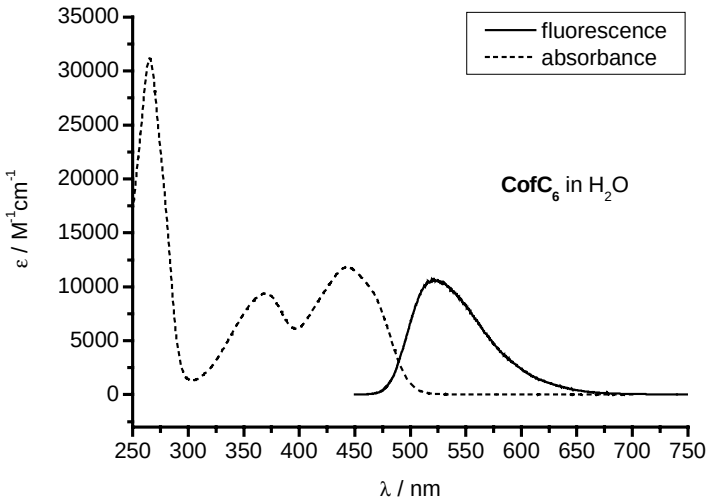
CofC$_6$ • TFA (2 eq, 9.1 mg, 20 µmol) and 3,3‘-dithiodipropionic acid di-(*N*-succinimidyl ester) (1 eq, 4.04 mg, 10 µmol) were dissolved in DMSO (10 ml) and stirred over night after addition of NEt_3 (3 drops). The product was precipitated by addition of water, filtered off, and dried *in vacuo*. NMR analysis showed pure coupling adduct.



^{1}H-NMR (600 MHz, DMSO-d6): δ = 1.35(2H, m, H_d), 1.42(2H, m, H_e), 1.46(2H, m, H_c), 1.71(2H, m, H_b), 2.39(3H, s, CH_3 at C_7), 2.43(2H, t, J = 7.2 Hz, H_g), 2.51(3H, s, CH_3 at C_8), 2.86(43(2H, t, J = 7.2 Hz, H_h), 3.05(2H, m, H_f), 4.56(2H, m, H_a), 7.75(1H, s, H_9), 7.88(1H, s, H_6), 7.90(1H, t, br, J = 5.4 Hz, NH-amid), 11.30(1H, s, NH-Ar); determination was done by HMBC, HSQC, COSY and ROESY. ^{13}C-NMR (150.9 MHz, DMSO-d6): δ = 18.8(CH_3 at C_7), 20.6(CH_3 at C_8), 25.8(C_c), 26.0(C_d), 26.4(C_b), 28.9(C_e), 34.0(C_h), 35.0(C_g), 38.4(C_f), 44.0(C_a), 115.9(C_9), 130.7(C_{9a}), 131.0(C_6), 133.8(C_{5a}), 135.7(C_7), 137.0(C_{4a}), 146.6(C_8), 149.9(10a), 155.7(C_2), 159.9(C_4), 169.7(C-amid); determination was done by HMBC, HSQC, COSY ,ROESY, and comparison with chemical shifts of ^{13}C-enriched flavin derivatives[6,7].

UV/Vis and Fluorescence Spectra

The UV/Vis- and Fluorescence Spectra of $CofC_6$ are shown in Figure 11. These spectra look like typical



absorption[8,9] and emission[8,10] spectra of flavin cofactors. The absorption spectrum shows three maxima at 265 nm (31160 $M^{-1}cm^{-1}$), 370 nm (9350 $M^{-1}cm^{-1}$), and 442 nm (11850 $M^{-1}cm^{-1}$). The fluorescence spectrum shows a maximum at 520 nm.

Figure 11. UV/Vis and fluorescence spectra of $CofC_6$ in H_2O.

Spectroelectrochemical Investigation of $CofC_6$

To gain more information about the reduction of $CofC_6$ we carried out spectroelectrochemical measurements. As the concentration of a $CofC_6$ monolayer immobilized on a transparent gold minigrid is too low to show any absorption in our experimental setup, we had to investigate the reduction of free $CofC_6$ in solution under thin-layer conditions. Primary cyclovoltammetric measurements under semi-infinite conditions showed that $CofC_6$ adsorbs on bare electrode materials, like gold, platinum and glassy carbon (data not shown). Therefore spectroelectrochemistry of dissolved $CofC_6$ was measured at a transparent gold minigrid with a $CofC_6$-modified surface as working electrode.

Figure 12 shows the spectroelectrochemistry of $CofC_6$ at pH 7 (0.2 M phosphate buffer, 0.2 M Na_2SO_4) with potential steps of 20 mV. Upon reduction of $CofC_6$, the spectrum of the neutral oxidized flavoquinone changes to a spectrum that is characteristic for a monoprotonated flavohydroquinone[8].

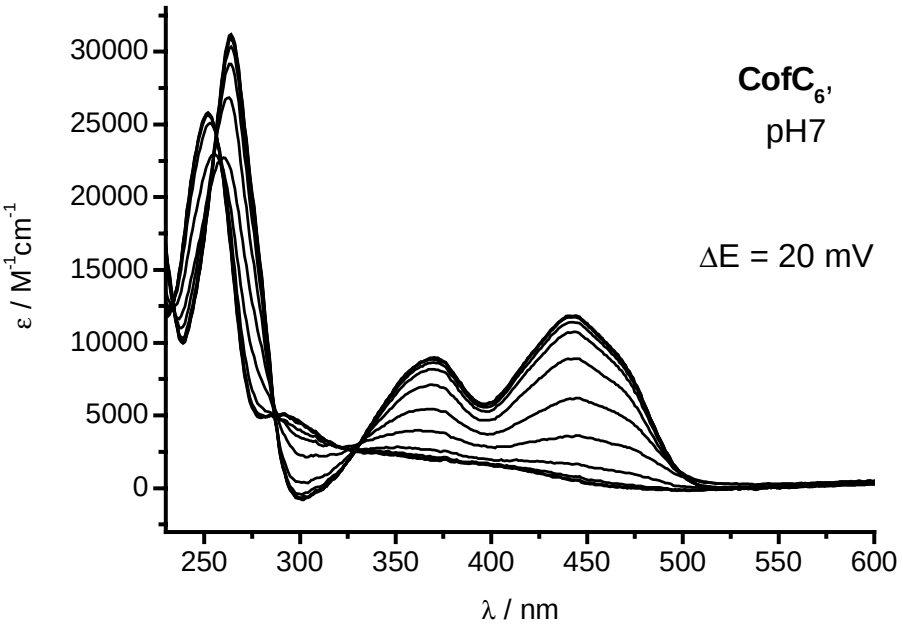


Figure 12: Spectroelectrochemistry of $CofC_6$ at pH 7.0 at a concentration of about 1 mM (0.2 M phosphate buffer, 0.2 M Na_2SO_4). A $CofC_6$-modified gold minigrid served as working electrode. The reductive potential was increased in steps of 20 mV.

The pK_a of flavohydroquinone[11] is 6.2, resulting in a theoretical ratio of 6.3 to 1 between mono- and diprotonated flavohydroquinone at pH 7. Therefore, we can not exclude that this species is also present at low concentration. The existence of isosbestic points indicates that, in the time scale of the experiment, only these two species are involved. Even with smaller potential steps, no additional band was observed that could indicate the existence of a flavosemiquinone. These results are in line with the proposed ece-mechanism. After the reduction was complete, the potential was switched again to the

oxidative region and the measured spectrum did not differ from the initial one, indicating complete reversibility of the observed process.

References

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