Insights into the formation and operation of polyaniline sulfonate/cytochrome c multilayer electrodes: contributions of polyelectrolytes' properties

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The multilayer formation of two different sulfonated polyanilines with cytochrome c is presented and mechanistic aspects of the contributions of the polyelectrolytes' properties to the characteristics of the assemblies are discussed. These two modified polymers, PASA1 and PASA2 are chemically synthesized and differ in the grade of sulfonation, substitution, and the chain length of the polymer. The influence of these properties on the multilayer assembly with cytochrome c is studied in detail by Quartz Crystal Microbalance (QCM) technique and Cyclic Voltammetry (CV). It is shown that the multilayer formation is successful, however, the redox activity of polyanilines itself has to be taken into account. In the case of a strong redox activity (PASA2) voltammetric analysis allows the separation of redox processes addressed to the polyelectrolyte and cyt c. For multilayers with PASA1 as building block electroactivity can be predominantly attributed to cyt c ensuring a high amount of electroactive protein and a low probability for interfering redox reaction, making this system suitable for biosensor applications.

Introduction

In the course of the last years, the layer-by-layer (LBL) electrostatic adsorption technique has been particularly considered as a way to construct thin polymeric architectures, the properties of which can be controlled by the adsorption conditions and number of steps.[1] The simplicity and versatility of such build-up procedure creates great possibilities for its widespread use in biomaterial coating, immobilization of biomolecules, and protection of bioassemblies. The formation of multilayer films by electrostatic self-assembly has been extensively reviewed.[2–8]

One of the main trends in the biological applications of LBL assembly is embedding bioactive proteins into thin films and utilizing their unique functions in opto-electronical devices, sensors, drug delivery, cell seeding and growth, tissue engineering, and implantable materials.[7,9,10] Proteins can be directly integrated into the architecture without any covalent binding by means of a polyelectrolyte, retaining the structure close to that of their native form.[11–17]

Intensive work in the area of bioelectrochemistry during the last two to three decades has certainly been devoted to the metalloprotein cytochrome c (cyt c).[12,18–27] Strategies have been developed for producing cyt c monolayers on coated electrodes with different properties,[19,28–32] and their applications as sensors for superoxide and peroxide have been shown.[33–38] By means of the LBL process the formation of electroactive multilayers on the electrode could also be demonstrated.[16] On the surface of gold electrodes subsequently coated with up to 16 alternating layers of cyt c and poly(anilinesulfonic acid) (PASA) the immobilized protein is fully electroactive, retains its native structure and can be successfully applied as a sensor for superoxide.[39,40] Moreover, on this basis it is possible to construct bi-protein architectures by the combination of cyt c with enzymes such as xanthine oxidase, bilirubin oxidase, laccase, and sulfite oxidase in self-assembled multilayers, so that a biomimetic signal chain from the enzyme substrate *via* the enzyme and cyt c towards the electrode can be established.[16]

Among the variety of different polyelectrolytes, sulfonated polyaniline (PASA) is found to be suitable for the construction of electroactive cyt c-containing multilayers. The formation of intermediate layers as distinguishable units in a multilayer assembly of PASA/cyt c has been confirmed by Atomic Force Microscopy (AFM) measurements.[41] All terminating layers of PASA or cyt c have similar charge and adhesive properties throughout the multilayer film independent of the number of preceeding layers.[41] However, interpenetration of cyt c and PASA layers can be concluded from the slightly exponential growth of the layer assembly. Furthermore, the redox properties and dynamics of cyt c and PASA conversions in the layered structures have been investigated by Surface-Enhanced Resonance Raman Spectroscopy (SERR)[42] It is found that cyt c is kinetically the fast component, and that PASA undergoes a rather slow redox conversion at the modified gold surface.[42] It was assumed that the polyelectrolyte-protein electron transfer might play an active role in supporting long-range electron transport within

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metal-MUA/MU-Cyt-(PASA-Cyt)$_n$ multilayer assemblies (MUA and MU are 11-mercapto-1 undecanoic acid and 11-mercapto-1-undecanol, respectively).[42] But it has also been demonstrated that DNA or modified gold nanoparticles can be used as building blocks for the formation of electroactive multilayered protein architectures.[43,44]

The aim of the present report is a detailed study of the polyelectrolyte influence on the multilayer formation and redox properties of the assemblies formed. Since sulfonated polyaniline is no more commercially available, two different sulfonated polyanilines (PASA1 and PASA2) have been chemically synthesized, the formation of cyt c multilayer assemblies has been investigated by Quartz Crystal Microbalance (QCM) measurements, and characterization of these systems on electrodes has been done by Cyclic Voltammetry (CV). Furthermore, simulations of the voltammetric response are performed to gain a further insight into protein-polyelectrolyte interaction on the surface of the gold electrode. The results are discussed in terms of a possible application of this system in biosensorics.

Experimental section

Chemicals

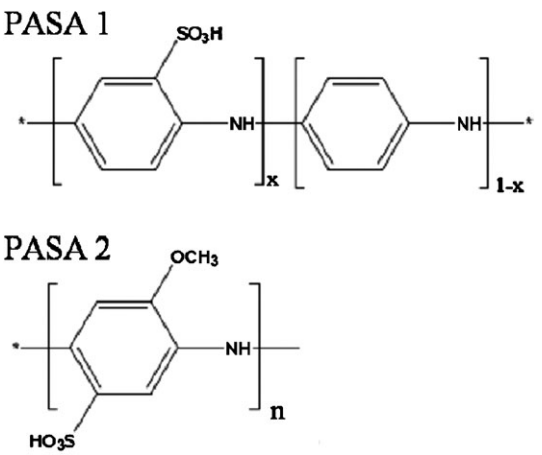
All reagents and solvents were commercially available (Sigma, Acros Organics, and Fluka) and used without further purification. 18 MΩ Millipore water (Eschborn, Germany) was used for all types of measurements. Freshly distilled solvents were used for all syntheses.

PASA1

The starting material-emeraldine base of polyaniline, purchased from Sigma, was firstly reduced in phenyl hydrazine, then washed with ethyl ether, after which the reduced form of polyaniline, leucoemeraldine base, was formed. Upon treating this polymer form with fuming sulfuric acid, the final product was obtained according to a previous report (Scheme 1).[45]

PASA2

2-Methoxyaniline-5-sulfonic acid was polymerized to fully-sulfonated polyaniline poly(2-methoxy aniline-5-sulfonic acid)



Scheme 1 Chemical structures of investigated polymers prepared according to ref. 45 and 46.

upon mixture with ammonium peroxydisulfate under basic conditions. This procedure gave a polymer described in a previous report (Scheme 1).[46]

Determination of molecular weight

The molecular weights of synthesized compounds were determined by Gel Permeation Chromatography (GPC) using water as eluent. UV detector was adopted to detect the molecules. Because standard materials for sulfonated polyaniline were unavailable, the calibration curve of sodium polystyrene sulfonate, which is also a water soluble polyelectrolyte, was used to calculate the weight average molecular weight (M_w) of the sulfonated polyaniline.

Preparation of cytochrome c monolayers

Cyt c monolayers were produced by 2 h incubation of gold wire electrodes, previously modified with 5 mM MUA/MU (1/3) in EtOH, in 0.5 mM potassium phosphate buffer solution (pH 7) containing 20 μM of cyt c.[31]

Preparation of cytochrome c multilayers

The multilayer assembly was carried out by sequential incubations of a cyt c monolayer electrode into polyelectrolyte (0.2 mg/ml) and cyt c (20 μM) solutions.[39] Each of the 10 min long adsorption steps was followed by rinsing the electrodes in 0.5 mM potassium phosphate buffer. The procedure was repeated until desired number of layers was reached.

QCM measurements

A MultiLab 3900 piezoelectric instrument (J. Kitlička, Brno, Czech Republic) was used for the quartz crystal microbalance measurements. A clean gold-covered quartz sensor chip (10 MHz, ICM, USA) was incubated in ethanolic solution containing 5 mM MUA/MU (1/3) for 24 h, then rinsed with water and mounted into the QCM flow system. The solutions containing PASA and cyt c of above given concentrations were successively pumped through the cell for 10 min with 5 min of buffer flow in between, at a flow rate of 20 μL/min.

We estimated the mass increase [Δm (ng)] from the QCM frequency shift [Δf (Hz)] of fixed films by using the Sauerbrey equation.[47] Taking into account the diameter of the electrode (d = 5 mm) and the other technical parameters,[48] this equation can be rewritten as

$$\Delta m = 0.87\ \Delta f \qquad (1)$$

According to (1) a film mass increase can be estimated. Since measurements have been performed in solution, these values are estimates due to unknown amount of bound water.

Electrochemistry

All electrochemical measurements were done in a home-made 1 ml cell using an Ag/AgCl/ 1 M KCl reference (Biometra, Germany) and a Pt-wire counter electrode. The working electrodes were modified gold wires (diameter 0.5 mm) obtained from Goodfellow (Bad Nauheim, Germany). Cyclic voltammetric experiments were carried out with the Autolab PGSTAT 30 device (Metrohm, Netherlands). Scan rates were varied between 5 and 640 mV/s. The potential range has been

chosen between −0.4 and +0.4 V. Data analysis has been performed using GPES (General Purpose for Electrochemical System, Eco Chemie, Utrecht, The Netherlands) software.

Results and discussion

Characteristics of the polyelectrolytes

Some key properties of sulfonated polyanilines are important in terms of their use as polyelectrolytes: the self-doping effect caused by the amount of SO_3^- groups,[49] chain length, and the overall charge of the polymer. Also the methoxylation additionally affects the redox properties and conductivity of polymers.[50,51] That is why we concentrate on two different PASA polymers that differ in the grade of sulfonation, methoxylation, and the molecular weight of the polymer.

In the case of the product obtained by the sulfonation of polyaniline, PASA1, the sulfonation is ~75% and no methoxylation has been performed (see Scheme 1). In contrast, PASA2, obtained by the polymerization of a single modified aniline, is fully sulfonated and methoxylated (Scheme 1). It can be seen that PASA2 has a considerably lower molecular weight than PASA1 (see Table 1).

Assembly of cytochrome c/PASA multilayers

First of all, we have studied whether a successful immobilization with these two polymers can be achieved. For this purpose cationic cyt c and both negatively charged PASA are assembled on negative MUA/MU-modified quartz surfaces by alternate adsorption from pH 5 buffer, where the protein is positively charged. QCM has been used to analyze the adsorption kinetics. Fig. 1a and 1b show QCM measurements of alternate PASA/cyt c adsorption for both different polymers. In the first step, a cyt c monolayer is being adsorbed onto an underlying layer of MUA/MU on gold. The QCM frequency decreases sharply because of rapid adsorption. After that, buffer solution has been pumped through the cell. From the frequency shift it can be seen that in buffer about 50% of the adsorbed cyt c is removed, presumably involving weakly attached molecules. After the washing step, the solutions of polyelectrolyte and protein are consecutively pumped through the cell and further decreases in frequency are monitored.

In the case when PASA1 is immobilized (Fig. 1a), during each washing step an amount of only 20% of deposited material is being removed, leading to a small increase in frequency. Each subsequent step is quite reproducible, and adsorption always reaches saturation during the period of cyt c and PASA solution flow over the quartz crystal surface. For every layer of PASA1 adsorption, QCM frequency (f) decreases by about 31 ± 5 Hz (averaged value based on results

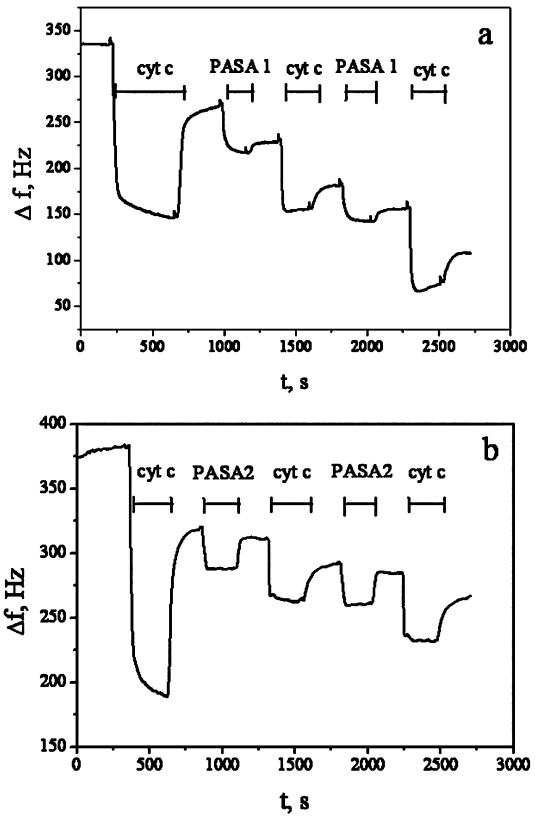


Fig. 1 Frequency shift for three successive deposition rounds of mofified (a) PASA1 and (b) PASA2 and cyt c on a monolayer of a cyt c-modified gold chip (quartz crystal 10 MHz). Experimental conditions: [PASA] = 0.2 mg/ml, [cyt c] = 20 μM, 0.5 mM phosphate buffer, pH 5.

Table 1 Determined molecular weights and degrees of sulfonation (the sulfur-to-nitrogen, S/N ratios) for both polyelectrolytes. M_w is the weight average molecular weight

Polyelectrolyte	M_w, g/mol	S/N
PASA1	6470	~0.75
PASA2	3680	~1

obtained after 6 electrodes have been measured). After each following cyt c adsorption step, f decreases by approximately 53 ± 3 Hz, which corresponds to film mass increases of 27 ± 3 ng for PASA1 layers and 46 ± 5 ng for cyt c layers, respectively. These values include not only deposited material, but also bound water.[52]

In general, the kinetic behavior in the case of PASA2 looks similar. However, during each washing step an amount of about 80% of deposited material is being removed, leading to a smaller overall decrease in frequency with every deposition step than in the former case. The analysis of the measurement gives following results: upon deposition of PASA2 layers f decreases by 7 ± 1 Hz; after each following cyt c step by 20 ± 1 Hz. These values correspond to a mass change Δm of 6 ± 1 ng for PASA2 and 17 ± 1 ng for cyt c. Notwithstanding the similarity of the kinetic behavior, it can be seen that the adsorbed amounts of both polyelectrolyte and cyt c are smaller when PASA2 is being used as a polyelectrolyte for the multilayer assembly. As explanation of this result it should be noted that although PASA2 has a higher content of SO_3^- groups, its polymer chains are significantly shorter, indicating that not only electrostatics is responsible for the polymer and protein immobilization. But in fact, the successful assembly of cyt c and modified PASA can be demonstrated for both kinds of synthesized sulfonated polyanilines.

Voltammetry of Cyt c/PASA1 and Cyt c/PASA2 multilayers

In all experiments with cyt c and the two different PASA films in aerobic solutions at pH 7, we obtain defined and reproducible cyclic voltammograms (CV). A total of 12 electrodes coated with 4 and 6 protein/polyelectrolyte bilayers has been measured for the both modified PASA systems. Fig. 2a and b show CVs for 4 and 6 cyt c/PASA1 and cyt c/PASA2 bilayer systems on a Au-MUA/MU-cyt c monolayer electrode, respectively.

In the case of PASA1 (Fig. 2) a single pair of reduction-oxidation peaks is observed with slightly higher reduction peak heights. According to the formal potential obtained (see Table 2), these results suggest that the electroactive Cyt cFe^{II} in the film is converted to Cyt cFe^{III} on the forward scan to positive potentials, with conversion of Cyt cFe^{III} to Cyt cFe^{II} on the reversed scan. The formal potential (Table 2) is independent on the amount of immobilized bilayers and corresponds to the value of cyt c redox couple at pH 7. The average amounts of electroactive species on the electrode surface, calculated from the integration of both reduction and oxidation peaks, are also summarized in Table 2. It can be seen, that an increase in surface concentration depending on the number of the immobilized bilayers is achieved. This is in agreement with a previous report, where the currently commercially unavailable PASA was immobilized with cyt c.[39] It has to be mentioned here that the electroactive amount of cyt c cannot be directly compared with the deposited mass from QCM experiments, since a different type of electrodes has been used (needle electrodes instead of planar gold) and

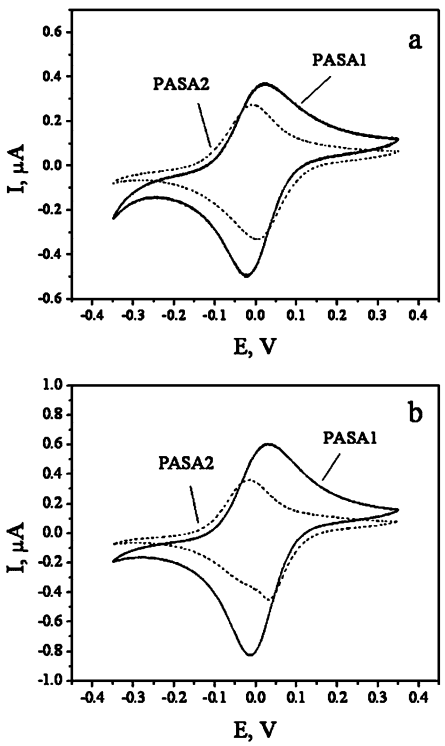


Fig. 2 Cyclic voltammograms of (a) 4 bilayer and (b) 6 bilayer cyt c/PASA electrodes [Au-MUA/MU-cyt c-(PASA-cyt c)$_{4\ or\ 6}$]. All curves are taken at scan rate 100 mV/s in 5 mM potassium phosphate buffer, pH 7.

Table 2 Summary of the electrochemical data for cyt c/PASA multilayer assemblies with two different polyelectrolytes and different numbers of cyt c/PASA bilayers. All electrochemical measurements were done at scan rate 100 mV/s

polyelectrolyte	PASA1		PASA2	
number of bilayers	4	6	4	6
E_f, mV	5 ± 2	7 ± 2	−7 ± 3	−15 ± 5
ΔE, mV	29 ± 7	43 ± 10	−10 ± 3	3 ± 1
$E_{w/2(red)}$, mV	134 ± 14	143 ± 10	161 ± 17	168 ± 20
$E_{w/2(ox)}$, mV	178 ± 21	182 ± 24	145 ± 13	143 ± 15
$\Gamma_{cyt\ c}$, pmol/cm^2	79 ± 8	141± 7	(68)[a] ± 5	(83)[a] ± 5

[a] Evaluated from the integration of the CV peaks and assuming only cyt c redox activity.

the determined mass from QCM measurements also comprises bound water. The peak separations (ΔE) (Table 2) are indications of the quasi-reversible character of electron transfer to the electrode surface from the outer layer through the whole bilayer assembly. The CV peak widths at half-height (Table 2) are larger than the theoretically predicted 0.09 V for ideal surface waves.[18] This may result from a distribution of distances and/or orientations of cyt c and/or interactions between cyt c units in the films.

However, when PASA2 is a part of the multilayer assembly, the shape of the voltammogram looks different (Fig. 2). The potential of the reduction peak in this case is higher than that of oxidation, which is not possible, when a single redox couple is considered. By increasing the amount of the bilayers from 4 to 6, the situation becomes clearer, since two separate reduction peaks appear. This fact is an indication of two redox processes detected on the electrode surface. It is known that the redox process of cyt c can be accompanied by reversible oxidation-reduction of PASA as revealed by SERR analysis.[42] Taking into account the structural differences between PASA 2 and PASA1 (Scheme 1), the presence of an additional electron-donating methoxy group on the aniline ring in PASA2 increases its redox activity.[50,51] The electrochemical conversion of PASA2 obviously occurs close to that of cyt c, so that the superposition of these processes leads to the unusual shape of cyclic voltammograms. That is why the electroactive surface loading for cyt c in these assemblies cannot be quantified correctly by simply analyzing the charge from the peak area. But even when assuming that the whole charge of the reduction process would be originated from cyt c, the obtained surface loadings in the case of PASA2 (Table 2) are lower than those of PASA1, confirming the conclusions from QCM measurements of less protein deposition when PASA2 is used as building block. In general, the amount of electroactive species on the electrode surfaces is in both cases lower than in previously reported multilayer system.[39] But still, successful multilayer assembly can be achieved, comprising electroactive cyt c molecules.

Voltammetry of Cyt c/PASA1 and Cyt c/PASA2 multilayers at lower scan rates

In order to gain a further insight into the redox processes on the surface measurements at low scan rates have been done.

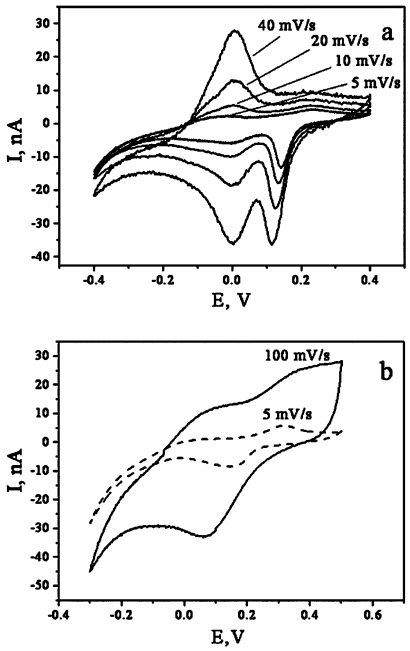


Fig. 3 Cyclic Voltammograms of (a) [PASA2/cyt c]-6-bilayer electrode (Au-MUA/MU-cyt c-[PASA2/cyt c]$_6$) taken at slow scan rates 5–40 mV/s and (b) of an Au-MUA/MU electrode in 0.5 mg/ml solution of PASA2. All measurements were done in 5 mM potassium phosphate buffer, pH 7.

Fig. 3 and 5 demonstrate summarized CVs taken in the scan rate range 5–40 mV/s.

In the case of PASA2 (Fig. 3a), two redox processes can be identified. The first process with a formal potential $E_{f1} = 0$ V is addressed to the redox conversion of cyt c. In the case of the second redox process a clear reduction peak ($E_{red2} = +0.12$ V) appears, but the oxidation peak is rather weak and broadening with increasing scan rates. This process can be addressed to the redox conversion of polyaniline itself. It can be seen that both deposited components react rather independently on the electrode surface. However, taking into account the cathodic movement of the reduction potential of polyaniline (E_{red2}) with increasing scan rates, whereas the peak position of cyt c remains constant, it can be seen that the peak position difference is getting smaller. This fact indicates that the redox conversion of PASA is slower compared to that of cyt c. To support these conclusions, measurements with PASA2 in solution in the absence of cyt c have been performed at a MUA/MU modified gold, demonstrating the presence of pronounced redox processes even at higher scan rates (Fig. 3b). The appearance of these redox processes can be attributed to the convertion of emeraldine to pernigraniline and is in good agreement with already reported data for self-doped polyanilines.[50,51,53–55] At 5 mV/s a peak separation of 152 mV appears, indicating a rather slow rate of conversion at the modified gold.

In order to obtain a distribution of species on the surface, a simulation of all PASA 2/cyt c voltammograms by means of MatLab software has been performed. This species distribution can be seen in Fig. 4. It was possible to calculate the percentage of electroactive species on the surface, resulting in a

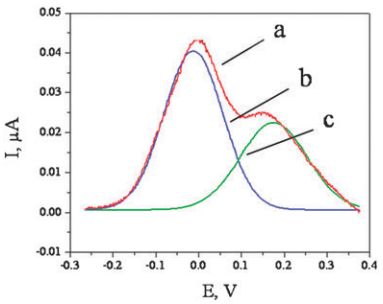


Fig. 4 Anodic part of the CV measured for [PASA2/cyt c]-6-bilayers at 5 mV/s, simulated with MatLab software. The curve (a) represents the measured CV (overall process), the curves (b) and (c) represent two separated processes (cyt c and PASA2 conversion, respectively), calculated from (a).

ratio of cyt c/PASA of about 68/32. Because of the relatively high percentage of PASA2, compared to cyt c, it is clear that the redox conversion of sulfonated polyaniline itself has to be taken into account by considering the redox properties of the whole assembly, successful multilayer formation notwithstanding.

However, in the case of the PASA1/cyt c system measured at low scan rates no distinct redox peaks of polyaniline on the surface can be determined from the voltammetric response. One redox couple with a formal potential $E = 0$ V can be recognized also at lower scan rates (Fig. 5a), although a small shoulder ($E_{red} = 0.08$ V) appears at 5 mV/s. By increasing the scan rates this is disappearing completely, leading to a conclusion that the redox activity of PASA1 itself is rather low.

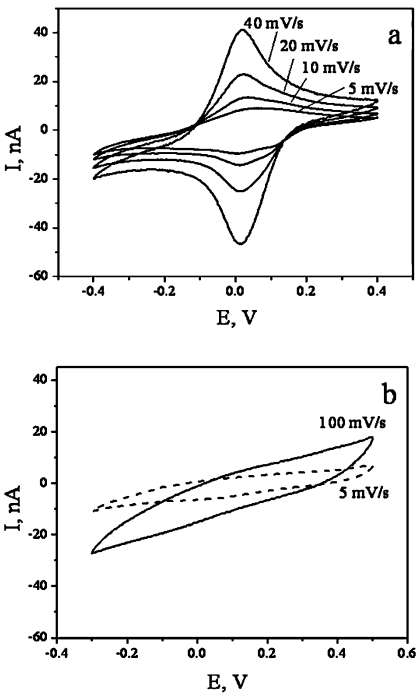


Fig. 5 Cyclic voltammograms of (a) [cyt c/PASA1]-6-bilayer electrode (Au-MUA/MU-cyt c-[PASA1/cyt c]$_6$) taken at slow scan rates 5–40 mV/s and (b) of an Au-MUA/MU electrode in 0.5 mg/ml solution of PASA1. All measurements were done in 5 mM potassium phosphate buffer, pH 7.

In general, the voltammetric behavior of this protein/PASA system is similar to previously reported results.[39] The absence of a redox conversion of polyaniline itself is further supported by measurements of a thiol-modified gold electrode in solution containing 0.5 mg/ml PASA1 (Fig. 5b). No redox activity is found at neutral pH in the potential range investigated. Thus, the conclusion can be made, that the amount of electroactive species calculated for both 4 and 6 PASA1/cyt c bilayer systems (Table 2), can be rather completely addressed to cyt c. Moreover, it was not possible to get separated redox processes by the same kind of MatLab analysis as for the PASA2 system. These results are promising, since the chemically synthesized PASA1 can be used as a polyelectrolyte to build up electroactive protein multilayer architectures without additional side reactions, such as redox conversion of the polyaniline itself. Thus, the idea of increasing the electroactive amount of the recognition element used can be realized by increasing the number of deposition steps by means of the LBL technique. When the redox protein is the only redox-active compound, a desirable precondition is fulfilled to make use of a specific reaction of this protein with an analyte molecule for a specific biosensor. In addition, these results support the idea that a high redox activity of the polyelectrolyte is not necessary for the electron transfer through the assembly. The exchange between the cyt c molecules seems to be the predominant mechanism and the polyelectrolyte acts mainly as a structural support.

Conclusions

In the present report we demonstrate that multilayer formation of chemically modified sulfonated polyanilines used as polyelectrolyte with the redox protein cytochrome c is possible. Both the deposited mass and the amount of electrochemically active species are increased by increasing the number of layers. The use of a multilayer sensor with higher amounts of electroactive cyt c is expected to yield a significantly enhanced sensitivity. However, the redox activity of the polyelectrolyte can significantly contribute to the overall redox activity of the assembly as shown for PASA2. This may cause side reactions when the system would be applied in sensorics. Multilayer assemblies with PASA1, in contrast, show a behavior dominated by the redox protein, which makes them promising for sensor approaches, *e.g.* for superoxide detection. Finally, the integration of sulfonated polyanilines with different chemical properties in an assembly with cyt c is a further contribution to artificial models of biological redox chains leading to bioelectronic functionalities.

Acknowledgements

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