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Journal of Organometallic Chemistry 668 (2003) 17–24
Journal of Organometallic Chemistry
www.elsevier.com/locate/jorganchem

Novel redox label for proteins. Electron transfer properties of (η^5-cyclopentadienyl) tricarbonyl manganese bound to bovine serum albumin

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Received 29 July 2002

Abstract

(η^5-Cyclopentadienyl) tricarbonyl manganese (cymantrenyl) derivatives of the protein bovine serum albumin (BSA) with coupling ratios ranging from 7 to 20 were prepared by reaction of cymantrenyl methyl imidate with some of the lysine residues of BSA. Electrochemical measurements based on the AC voltammetric detection of the reduction of cymantrene bound to BSA protein were successfully set up in aqueous solutions and the detection limit of 2×10^{-7} M BSA was achieved. This limit is almost two orders of magnitude lower than that measured by IR spectroscopy. Electrochemical detection of cymantrene label will be used for immunoassay analysis of water-soluble analytes in our future work.

Keywords: Cymantrene; Cymantrenyl methyl imidate; Redox label; Protein detection; Reduction; AC voltammetry; IR spectroscopy

1. Introduction

Immunoassays are very useful analytical methods for quantification of extremely low concentrations of analytes contained in body fluids (serum and urine). Most of the immunological methods use biomolecules labeled by markers detectable by suitable physical methods. Original radioisotopic labels (^{3}H, ^{125}I, ^{131}I) nowadays tend to be replaced by non-radioactive markers such as enzymes or fluorophores. Several immunological assays make use of transition metals or metal complexes. They can be quantified to a very low level by atomic absorption spectroscopy [1,2], colorimetry [3], turbidimetry [4], chemiluminescence [5,6], scanning force microscopy [7], as well as several electrochemical methods [8–12].

A few years ago, we introduced transition metal carbonyl complexes as labels to perform the immunoassay of drugs [13,14]. Fourier transform IR spectroscopy was used as a detection method, because all these complexes display very intense and specific absorption bands in the 1800–2200 cm^{-1} spectral range, owing to the stretching vibration modes of the carbonyl ligands coordinated to the metal. However, the detection must be performed in an organic solvent that limits the choice of analytes to lipophilic molecules of low molecular weight.

In this paper we describe an alternative detection approach of transition metal carbonyl probes based on the electrochemical reduction of cymantrene ([η^5-cyclopentadienyl] tricarbonyl manganese) **1** bound to a protein. The main goal was to develop the electrochemical detection method in aqueous environment that would allow the future immunoassay to be performed on a broader range of analytes. The question whether the conjugate of **1** with bovine serum albumin (BSA) can

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PII: S0022-328X(02)02093-4

be detected electrochemically rather than spectroscopically is addressed in this paper.

2. Experimental

2.1. Materials

All chemical reactions were performed under a dry Ar atmosphere by using standard Schlenk methods. Solvents were previously dried and distilled by classical means. Cymantrene ([η^5-cyclopentadienyl] tricarbonyl manganese) **1** was purchased from Strem chemicals. Other chemicals were obtained from Aldrich. Lipid-free, immunoglobulin-free BSA (grade V) was purchased from Sigma. Aqueous solutions were prepared from demineralized water.

Formyl cymantrene **2** was synthesized according to the literature [15]. Cymantrene (5×10^{-3} mol, 1.02 g) dissolved in THF was reacted with a stoichiometric amount of *n*-BuLi (1.6 N solution in hexanes) at −50 °C. After 15 min, the solution was cooled down to −70 °C and transferred via a cannula to another Schlenk tube containing DMF (6.6×10^{-3} mol). The mixture was warmed up to 0 °C and stirred for 90 min. Hydrolysis was performed by addition of a saturated solution of NH_4Cl. After the classical workup, formyl cymantrene was obtained as a yellow solid (72%). ^{1}H-NMR ($CDCl_3$, ∂ in ppm/Me_4Si): 9.35 (s, 1H, CHO), 5.49 (t, 2H, 2.2 Hz, Cp–$H_{2,5}$), 4.96 (t, 2H, 2.2 Hz, Cp–$H_{3,4}$). IR (CCl_4, ν in cm^{-1}): 2035, 1950 (C≡O), and 1700 (C=O).

Cyanocymantrene **3** was synthesized according to the literature [16]. Briefly, formyl cymantrene (3.56×10^{-3} mol, 0.8 g) and hydroxylamine hydrochloride (3.5×10^{-3} mol, 0.5 g) were allowed to react in formic acid at 80 °C for 30 min. The mixture was hydrolyzed as it was poured into 150 mL of icy water. The resulting brown precipitate was filtered on a fritted glass and washed with water until neutral pH was achieved. The aqueous phase was extracted with Et_2O and the organic layer combined with the precipitate. After workup, cyanocymantrene was obtained as a brown oil (72%). ^{1}H-NMR ($CDCl_3$, δ in ppm/Me_4Si): 5.32 (t, 2H, 2.3 Hz, Cp–$H_{2,5}$), 4.85 (t, 2H, 2.3 Hz, Cp–$H_{3,4}$). IR (CCl_4, ν in cm^{-1}): 2039, 1961 (C≡O) and 2240 (C≡N).

Cymantrenyl methyl imidate **4** was prepared from **3**. Starting material (1.3×10^{-3} mol, 0.3 g) was dissolved in MeOH and 0.26×10^{-3} mol of K_2CO_3 was added. The mixture was stirred for 24 h at room temperature and chromatographed on silica gel. Elution with CH_2Cl_2–C_6H_{14} 2:1 afforded unreacted **3**. Then a second yellow species was eluted by CH_2Cl_2–MeOH 2:1. Recrystallization from CH_2Cl_2–C_6H_{14} at −20 °C afforded cymantrenyl methyl imidate as a yellow solid (50%). ^{1}H-NMR ($CDCl_3$, ∂ in ppm/Me_4Si): 5.32 (broad s, 2H, Cp–$H_{2,5}$), 4.76 (unresolved t, 2H, Cp–$H_{3,4}$), 3.82 (s, 3H, OCH_3). IR (KBr pellet, ν in cm^{-1}): 2020, 1933 (C≡O) 1637 (C=N). MS (DCI–NH_3, m/z) 262 [MH^+].

Labeling of BSA with **4** was performed by the following procedure: 3 mL solutions containing BSA (50 μM) in borate buffer pH 9.0 and **4** (500, 1000, 1500, 2000 or 3000 μM) in MeOH were incubated in the dark. The final percentage of organic solvent was kept below 10%. The protein conjugates were purified by gel filtration chromatography using 10×10^{-3} M NH_4HCO_3 as eluent (10 ml-Kwiksep desalting column, Pierce Chemicals). The protein concentration was assayed by the Folin–Ciocalteus method [16]. The concentration of cymantrenyl amidinium groups was assayed by UV–vis spectroscopy at 337 nm ($\varepsilon = 1500$ M^{-1} cm^{-1}). The coupling ratio (CR) of the conjugates was calculated as a ratio of cymantrenyl amidinium groups to the protein concentration. Conjugate solutions were subsequently freeze-dried on a Speedvac concentrator (Savant) for the electrochemical studies.

2.2. Methods

^{1}H-NMR spectra were recorded on a FT-spectrometer operating at 200 MHz (Bruker). IR spectra were recorded on a MB100 FT-spectrometer (Bomem) equipped with a liquid nitrogen cooled MCT detector. Five microliters of labeled protein solutions at known concentrations were spotted on 6 mm diameter nitrocellulose discs punched into 0.45 μm porosity nitrocellulose membrane sheets (Whatman) and subsequently dried in air. The IR spectrum of these discs was recorded at 4 cm^{-1} resolution (42 scans accumulated) taking as the reference an untreated disc. Mass spectrum was obtained at the ENSCP, Paris. UV–vis spectra were recorded on a uv/mc^2 spectrometer (Safas).

Electrochemical measurements were done using a home-built system for cyclic voltammetry, phase-sensitive AC voltammetry, AC and DC polarography. It consisted of a fast rise-time potentiostat [17] and a lock-in amplifier (Stanford Research, model SR830). The instruments were interfaced to a 486-type personal computer via an IEEE-interface card (PC-Lab, Advan-Tech Model PCL-848) and a data acquisition card (PCL-818) using 12-bit precision. The AC signal for the cell was derived from the internal oscillator of the lock-in amplifier. The amplitude was 10 mV (p–p). Impedance spectra were measured in the range 1 Hz to 100 KHz using a frequency spectrum analyzer (Stanford Research, Model SRS760). A three-electrode electrochemical cell was used. The reference electrode, Ag | AgCl | 1 M LiCl, was separated from the test solution by a salt bridge. The working electrode was a valve-operated static mercury electrode (SMDE2, Laboratorní Přístroje, Prague) with an area 1.13×10^{-2} cm^2. The auxiliary electrode was Pt wire. Oxygen was

removed from the solution by passing a stream of Ar. Potassium chloride (Aldrich) was used as a supporting electrolyte. All aqueous solutions were prepared using nanopure water (Millipore Q system).

3. Results and discussion

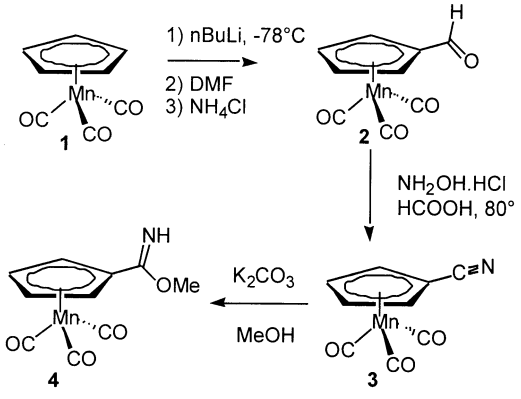
3.1. Synthetic routes towards the preparation of organometallic markers and labeled proteins

3.1.1. Synthesis of cymantrenyl methyl imidate 4

Numerous methods of conjugation of probes to proteins are available [18]. Many of them take advantage of the high nucleophilicity of the amino function borne by the side chain of lysine residues. In the field of transition metal carbonyl probes, we described the synthesis and reactivity of *N*-succinimidyl esters [19,20] and isothiocyanates [21]. Conjugation of these reagents to protein amino groups results in the conversion of positively charged residues into neutral functions: amides and thioureas, respectively. This could lead to a decrease of the solubility of the resulting bioconjugates. Conversely, the reaction with imidoesters with proteins results in the formation of an amidinium function with a pK_a around 11.6, thus preserving the positive charge of proteins at physiological pH [22]. The reaction of imidoesters is also very specific of amino groups [23].

In the course of a preceding work, we described the synthesis of an imidoester containing a [η^5-cyclopentadienyl] dicarbonyl nitrosyl chromium unit [24]. Unfortunately, this compound was shown to undergo rapid decomplexation in aqueous medium, so that this imidoester was not useful to label proteins. For the present study, we switched to [η^5-cyclopentadienyl] tricarbonyl manganese (cymantrene) for its greater stability.

The synthesis of cymantrenyl methyl imidate **4** was achieved in three steps (Scheme 1). The first step consisted in the functionalization of the cyclopentadienyl ligand of cymantrene with an aldehyde group [16]. In the second step, the aldehyde was converted into a nitrile. Finally, the nitrile was transformed into the methyl imidate by the base-catalyzed addition of methanol. The intermediates and the final product were characterized by usual spectroscopic methods. Moreover, the pK_a of cymantrenyl methyl imidate **4** measured by titration with aqueous HCl was equal to 4.8. This value compares well to that of Wood's radioiodination reagent for which pK_a is 5.67 [25]. It is also worth noticing that the protonated form of compound **4** is soluble in water.



Scheme 1. Synthesis of cymantrenyl methyl imidate **4**.

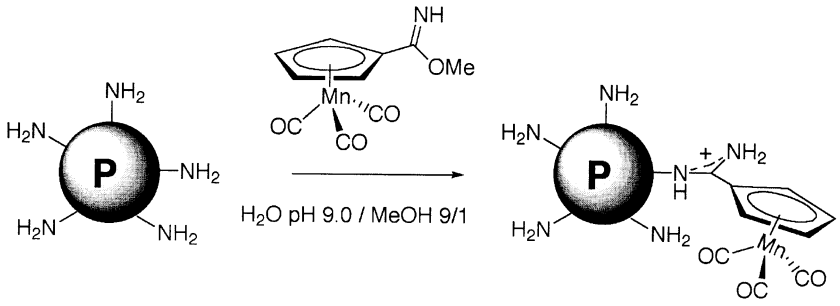
3.1.2. Labeling of BSA and spectroscopic characterization of the conjugates

BSA was selected as a model protein for the labeling assays because it is a readily available and inexpensive protein with a relatively high number of amino functions (60), which should allow the introduction of a high number of cymantrenyl labels. The labeling procedure consisted of treating the protein dissolved in borate buffer pH 9.0 with various amounts of **4** dissolved in MeOH (Scheme 2). The mixtures were incubated 24 h at room temperature and submitted to size exclusion chromatography to separate the unbound cymantrenyl species from the protein conjugates. The resulting protein conjugates were subjected to spectroscopic analyses to determine the number of cymantrenyl labels bound per protein molecule, also called CR. Results are reported in Table 1.

The CR of the resulting conjugates increased with respect to the initial concentration of reagent with yields ranging from 30 to 60%. Starting from the same reagent to protein ratio, the coupling yield was much higher, when the reaction was performed at 26 °C (compare samples 1 and 5). Hence, cymantrenyl methyl imidate **4** appears as an efficient reagent to introduce manganese tricarbonyl moieties onto proteins. For comparison, in the conditions of reaction of sample 3, a cyclopentadienyl rhenium tricarbonyl *N*-succinimidyl ester yielded a CR of 27 [26], whereas a cyclopentadienyl iron dicarbonyl isothiocyanate yielded a CR of 12 [22]. The reactivity of cymantrenyl methyl imidate is thus very close to that of an *N*-succinimidyl ester without the problem of altering the protein net charge.

3.2. Electrochemical characterization of BSA protein labeled by cymantrenyl methyl imidate

The choice of cymantrene as a redox label was made with respect to the requirement to couple its methyl imidate derivative **4** to the protein in aqueous medium. Since only few organometallic compounds can fulfill this requirement, we investigated the potentialities of this particular compound. The electrochemical characterization of **4** was the subject of our previous communication [27]. Cymantrenyl methyl imidate **4** yields a one-electron



Scheme 2. Reaction of **4** with BSA.

Table 1
Characterization of BSA_{CR} conjugates

Sample	$([Cym]/[BSA])_{initial}$	$[BSA]_{final}$ (μM)	$[Cym]_{final}$ (μM)	CR (yield %) [c]
1 [a]	20	37	442	12 (60)
2 [a]	40	37	640	17 (42)
3 [a]	60	39	770	20 (33)
4 [b]	10	38	158	4 (40)
5 [b]	20	37	248	7 (35)
6 [b]	30	35	307	9 (30)

Analyses: see Section 2.
[a] Incubation at 26 °C for 18 h.
[b] Incubation at 4 °C for 48 h.
[c] $CR = [([Cym]/[BSA])_{final}$; $yield = [([Cym]/[BSA])_{final}/([Cym]/[BSA])_{initial}] \times 100$.

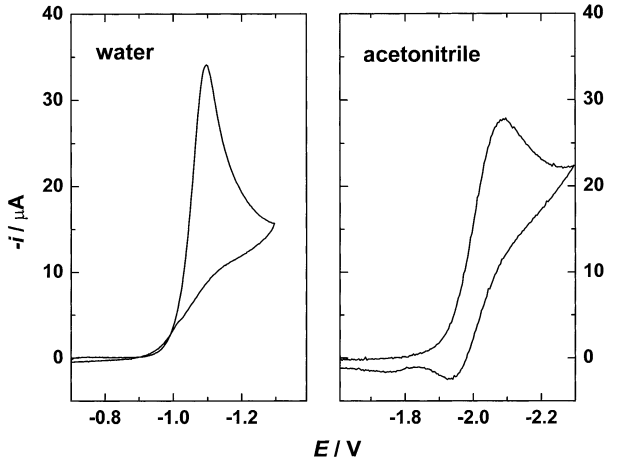


Fig. 1. Cyclic voltammetry of 1×10^{-3} M solution of cymantrenyl imidoester in aqueous and non-aqueous solvent. Left curve: 0.5 M KCl and 2×10^{-4} M HCl in water. Right curve: 0.1 M tetrabutylammonium hexafluorophosphate in acetonitrile. Scan rate was 0.5 V s^{-1}.

reduction at very negative potentials ($E_p = -2.08$ V, Fig. 1) in non-aqueous medium. The electron transfer is not a simple one. The activation of the coordination sphere by uptake of an electron triggers a series of subsequent decomposition reactions [27]. In aqueous medium the reduction of **4** occurs at more positive potentials ($E_p = -1.09$ V, pH 4), but the fast kinetics of the follow-up reactions make the overall redox process completely irreversible (see Fig. 1). At first glance this seems to prohibit the application of **4** as a convenient protein redox label, since it lacks the properties of a simple reversible electron transfer. However, the electrochemical experiments with samples of BSA labeled to different degree with **4** yielded a rather unexpected result: the reduction of BSA-bound **4** occurred at potential −1.72 V (see Fig. 2B). This value lies between those observed in the aqueous (polar) and non-aqueous (non-polar) environments. It will be shown in the following paragraphs that BSA-bound **4** evidently experiences protection by the protein environment as a result of which the complicated follow-up decomposition reactions do not occur and a fast electron transfer process is observed. This is a promising feature for development of an analytical method based on the electrochemical detection.

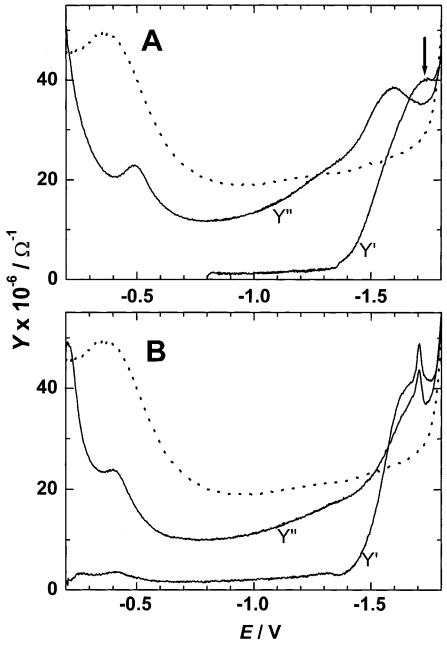


Fig. 2. Real and imaginary component of the cell admittance of 4.8×10^{-7} M BSA (A) and 4.4×10^{-7} M BSA_7 (B) in 1 M KCl aqueous solutions obtained by AC voltammetric measurements. Data were measured at frequency 16 Hz and scan rate 2 mV s^{-1}. Dotted lines represent the imaginary admittance component for 1 M KCl supporting electrolyte.

The choice of the most suitable electrochemical method is subject to several restrictions due to the presence of proteins in solution. Problems stem from the fact that proteins are strongly adsorbed at electrode interfaces [28–32] and many of them catalyze hydrogen evolution in aqueous samples [33–36]. The latter property limits the choice of suitable electrochemical techniques only to those capable to discriminate between two parallel electron transfers: proton reduction and cymantrene reduction. Adsorption of the labeled protein at the mercury electrode could be an advantageous feature for application of methods enhancing the analytical sensitivity by accumulation of the redox-active label at suitably chosen electrode potential. As a consequence, voltammetric methods using the direct current as an analytical signal turned out to be unsuitable. The best results were obtained by using phase-sensitive AC voltammetry, which yields the record of two vector components of the electrode admittance Y. The real admittance component Y' discriminates the processes connected with adsorption, whereas the imaginary admittance component Y'' sensitively reflects the change of the interfacial capacitance due to protein adsorption. Hence, this technique is ideal for simultaneous assessment of the most efficient accumulation regime and for detection of a reversible redox signal in the presence of much larger, but substantially less reversible hydrogen evolution signal.

Fig. 2A shows the interfacial capacitance C ($C = Y''/\omega$) of the aqueous potassium chloride solution in the absence and presence of BSA as a function of the electrode potential. The value of C was considerably lowered after BSA addition in a wide potential range from -0.2 to -1.25 V. The reduction of hydrogen is seen as a maximum on the Y' component (indicated by an arrow in Fig. 2A), which has much smaller counterpart on the imaginary component curve. This indicates that hydrogen evolution catalyzed by a protein is a very slow charge transfer process and the feasibility of separation of both redox signals is realistic provided that the AC frequency is chosen adequately. Labeled BSA shows almost identical suppression of C (see Fig. 2B) and to some extent modified hydrogen reduction peaks. The cymantrene signal is seen as two sharp admittance peaks superimposed over the broad hydrogen signal. These peaks appear at $E = -1.72$ V and are of equal height, confirming the fast electron exchange.

Fig. 3 shows the dependence of the capacitance signal (Y''/ω) on the direction of the voltage scan obtained for 1.5×10^{-6} M solution of labeled BSA with CR = 7. Scan in the negative direction yielded a peak corresponding to fast reduction of the label, whereas no peak was observed, when the mercury drop was formed at -1.8 V and the potential scanned towards more positive values. Such a behavior indicates that the protein-bound cymantrene is reduced only if the protein is adsorbed on

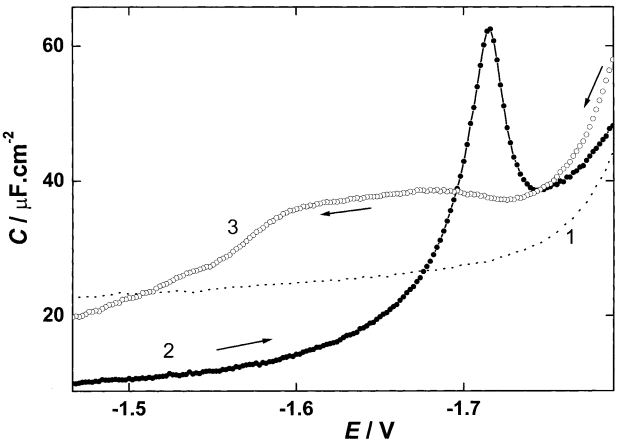


Fig. 3. The dependence of AC signal of labeled BSA_7 on the direction of the voltage scan. Curve 1 is the capacitance of 1 M KCl aqueous solution. The initial potential was -0.8 V for curve 2 and -1.8 V for curve 3. The AC frequency was 16 Hz. Arrows indicate the voltage scan direction at scan rate 2 mV s^{-1}.

the electrode surface. Therefore, we studied the adsorption properties of labeled BSA at different electrode potentials and scan rate regimes. The time frame for BSA accumulation at the mercury electrode surface was obtained from capacitance changes of a fairly dilute solution of BSA as a function of time at different electrode potentials. The final capacitance value was reached within 300 s. Fig. 4 shows the effect of the accumulation time on the reduction signal of the label bound to the protein. Different accumulation times were achieved simply by starting the potential scan at different initial potentials. The peak height at -1.72 V clearly increased with increasing time of protein accu-

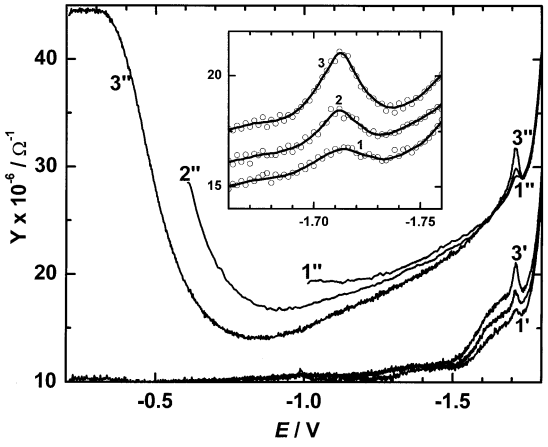


Fig. 4. Dependence of the real and imaginary component of the cell admittance of 5.0×10^{-7} M BSA_{26} in 1 M KCl and 2×10^{-4} M HCl aqueous solution on the accumulation time of protein at the HMDE as obtained by AC voltammetric measurements starting at different initial potentials: -1.0 (curves 1′ and 1″), -0.6 (curves 2′ and 2″), and -0.2 (curves 3′ and 3″). Data were obtained at frequency 16 Hz and scan rate 2 mV s^{-1}. Real admittance curves 1′–3′ are shifted up by 1×10^{-5} Ω^{-1} for clarity. Inset shows the real admittance data in the label reduction potential range.

mulation. The optimum conditions for protein detection using AC voltammetry emerged from these experiments.

Another important feature of the AC method lies in its capability to assess the stability of the label-protein conjugate. The in-phase admittance component of free cymantrenyl methyl imidate showed a peak at −1.12 V (in accordance with the irreversible reduction process detected by cyclic voltammetry in Fig. 1), which was observed in a few circumstances also for the aged labeled-protein samples. Therefore, the AC voltammetric method can be used for the assessment of the label-protein conjugate stability. No signal from free label was observed in the samples used in this experimental work, see Figs. 2 and 4.

The analytical signal used for the construction of calibration curves was obtained from the in-phase admittance after subtraction of the baseline corresponding to the protein-catalyzed evolution of hydrogen. This process was observed in the presence of both labeled and unlabeled BSA. The reduction of proton has a clearly developed peak only in the Y' admittance coordinate, whereas the label has a peak at the same potential in both Y' and Y'' admittance coordinates. This indicates that the hydrogen evolution is a much slower process than the reduction of protein-attached label and the signals can be processed independently. This statement was verified using impedance spectroscopy. This method is similar to AC voltammetry, except that the admittance or the impedance of the system is obtained at many different frequencies. Data can provide quantitative information on the kinetics of a particular process. Impedance analysis of the protein-catalyzed hydrogen evolution was performed on unlabeled BSA. A typical impedance plot is shown in Fig. 5A. Data were analyzed by conventional means [37] and kinetic parameters were obtained directly from the data given in the inset of Fig. 5A. If one assumes a value for the diffusion coefficient 7×10^{-6} cm^2 s^{-1}, the rate constant equals to 1.8×10^{-3} cm s^{-1} for the reduction of proton at the electrode potential $E = -1.6$ V. The same type of analysis led to the value of 9.5×10^{-2} cm.s^{-1} for the reduction rate constant of the cymantrene label attached to BSA at $E = -1.72$ V, see Fig. 7B. Therefore, the reduction of cymentrene bound to the protein proceeds 50 times faster than the protein-catalyzed hydrogen evolution.

3.3. Quantitative determination of proteins labeled with cymantrenyl methyl imidate

3.3.1. IR spectroscopic detection of labeled proteins

Fourier transform IR spectroscopy in a non-aqueous solvent was previously used for the immunoassay of drugs [13,14]. Cymantrene displays two very intense and specific absorption bands in the 1800–2200 cm^{-1} spectral range owing to the three carbonyl ligands coordinated to the manganese. Since water as a solvent

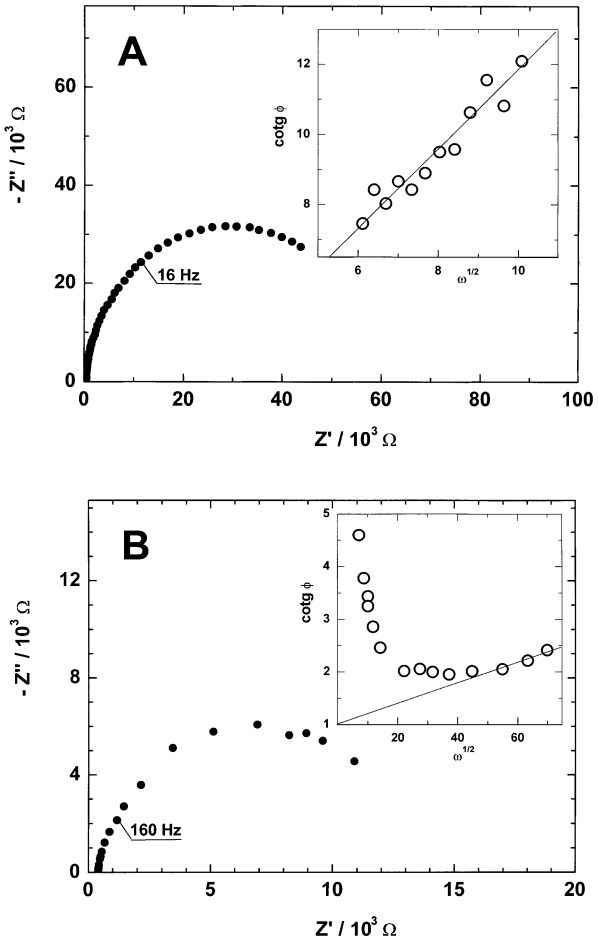


Fig. 5. Impedance spectrum of 4.5×10^{-7} M solution of unlabeled BSA (A) obtained at potential $E = -1.6$ V and 1.53×10^{-6} M BSA_7 (B) in 1 M KCl aqueous solution obtained at potential $E = -1.72$ V. Inset represents the cotangent analysis of the impedance spectrum.

cannot be used in this spectral range, aqueous solutions of cymantrene labeled BSA samples were deposited onto nitrocellulose membranes, air dried and only then the IR spectra were taken. This procedure affected the absorbance signal only at high labeling ratios of the protein. Inset in Fig. 6 shows a typical IR absorption spectrum of the labeled BSA in the region of interest. Peak at 2034 cm^{-1} was chosen for construction of the calibration curves. The IR signal was clearly related both to the concentration of protein at constant coupling ratio and to the CR of the labeled protein. However, the reproducibility of FT-IR measurement deteriorated at higher CR values. The detection limit was calculated from the linear part of calibration curves in Fig. 6. For example, at CR = 8 in the range from 3×10^{-6} to 5×10^{-5} M BSA a detection limit of 8 μM BSA was obtained. Conservatively, it represents the BSA concentration corresponding to three times the standard deviation of the measured absorbance signal $3s_y = 0.0025$. This method was more sensitive than recently reported spectrophotometric [38] and electrophoretic [39] methods.

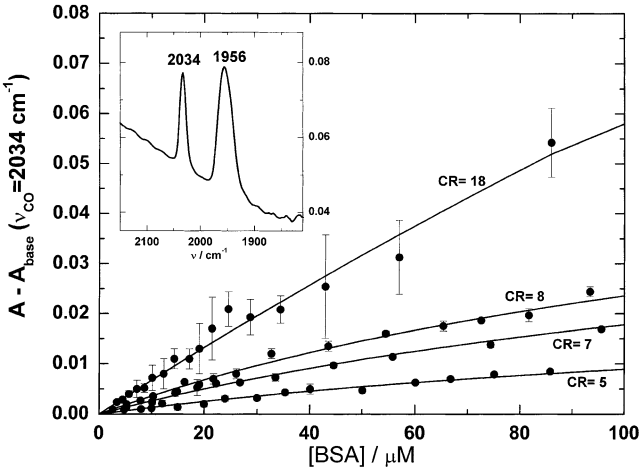


Fig. 6. The dependence of absorbance on the BSA protein concentration at different labeling ratios of cymantrenyl methyl imidate. Absorbance value was taken from the peak maximum at $\nu = 2034$ cm^{-1} (C≡O) after the baseline subtraction, see FT-IR spectrum in the inset. PBS buffered solutions of labeled BSA (pH 7.4) were used.

3.3.2. Electrochemical detection of labeled proteins

The electrochemical method was designed in order to improve the detection limit of BSA labeled with cymantrene derivative. The calibration curve resulting from the plot of the in-phase admittance at −1.72 V subtracted of the hydrogen evolution contribution at this potential versus the concentration of protein is shown in Fig. 7 for two different BSA samples. This curve can be linearized using a log–log plot as shown in the inset of Fig. 7. Data obtained for two different BSA samples were gathered. Linearization follows the Freundlich isotherm expression and is in agreement with our previous finding that the signal is observed only when the protein is allowed to adsorb at the electrode surface,

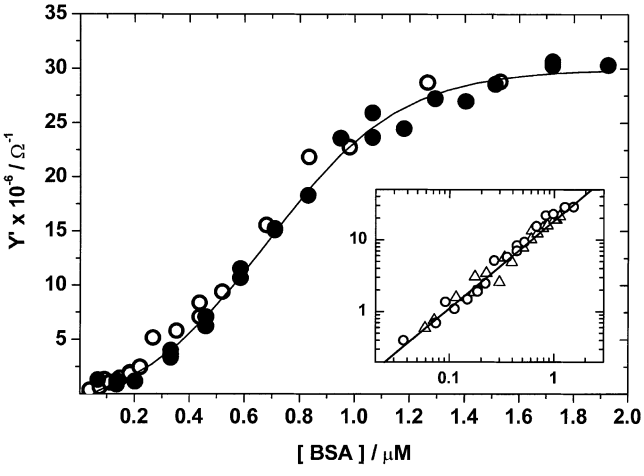


Fig. 7. In phase-component of the cell admittance for different concentrations of BSA_7 (○) and BSA_{20} (●) in 1 M KCl aqueous solution. Data were measured at frequency 16 Hz, $t = 20$ °C and scan rate 2 mV s^{-1}. A linearized double-logarithmic plot based on the Freundlich adsorption isotherm is shown in the inset for BSA_4 (△) and BSA_7 (○) samples.

bringing the electro-active label to a distance appropriate for fast electron transfer. Most importantly, the calibration curve does not seem to depend on the number of labels bound to BSA (CR), conversely to the FT-IR measurements. This should greatly simplify the analytical application of our electrochemical approach. The detection limit was calculated the same way as in the case of the FT-IR method and amounts to 0.2 μM BSA. This method is therefore capable of detecting about concentrations of labeled BSA 50 times smaller than the FT-IR spectroscopic one. Greater sensitivity of the electrochemical detection is obvious at first glance if one compares the BSA concentration ranges used in Figs. 6 and 7. The electrochemical method therefore surpasses in sensitivity the spectroscopic methods mentioned above as well as the recently developed PQC impedance technique [40]. Comparable detection methods are based on the electrochemical detection of suitable labels [41–43] with a similar aim to monitor the immunological interactions. Therefore, the cymantrene label shows a promise as a good label for any future immunoassay applications.

4. Conclusions

A sensitive electrochemical method for detection of proteins was designed and demonstrated. Organometallic label based on cymantrene ([η^5-cyclopentadienyl] tricarbonyl manganese) derivative was attached through the lysine residues covalently to BSA and its redox properties in the aqueous environment were described. Even though cymantrene in its native form does not have desirable properties for design of a good electrochemical detection method, redox properties of the cymantrene derivative covalently bound to the protein are different and allow detection of BSA concentration almost two orders of magnitude lower than the traditional FT-IR method used for cymantrene detection. The reason is a fast reversible one-electron transfer of water-soluble label-protein conjugate. Experimental work towards the design of an immunoassay analysis of water-soluble analytes is in progress. These results will be used towards the future goal to use it in the immunoassay analysis of water-soluble analytes.

Acknowledgements

This work was supported by the Ministry of Education, Youth and Sports of the Czech Republic (grant COST D15/001/98, OC-D15.10) and the grant agency of the Czech Republic (grant 203/02/P082). The CNRS (France) and the Academy of Sciences of the Czech Republic are gratefully acknowledged for supporting the project with exchange grants.

References

[1] M. Cais, Methods Enzymol. 92 (1983) 445.
[2] I. Rémy, P. Brossier, Analyst 118 (1993) 1021.
[3] J.H.W. Leuvering, P.J.H.M. Thal, D.D. White, A.H.W.M. Shuurs, J. Immunol. Methods 62 (1983) 163.
[4] T.C.J. Gribnau, J.H.W. Leuvering, H. van Hell, J. Chromatogr. 76 (1986) 175.
[5] D.R. Deaver, Nature 377 (1995) 758.
[6] M. Motsenbocker, Y. Ichimori, K. Kondo, Anal. Chem. 65 (1993) 397.
[7] A. Perrin, A. Theretz, B. Mandraud, Anal. Biochem. 256 (1998) 200.
[8] F.J. Hayes, H.B. Halsall, W.R. Heineman, Anal. Chem. 66 (1994) 1860.
[9] K. Digleria, A.O. Hill, C.J. McNeill, M.J. Green, Anal. Chem. 58 (1986) 1203.
[10] S. Rapicault, B. Limoges, C. Degrand, Anal. Chem. 68 (1996) 930.
[11] J. Wang, B. Tian, K.R. Rogers, Anal. Chem. 70 (1998) 1682.
[12] W. Guo, J.-F. Song, M.-R. Zhao, J.-X. Wang, Anal. Biochem. 259 (1998) 74.
[13] M. Salmain, A. Vessières, P. Brossier, I.S. Butler, G. Jaouen, J. Immunol. Methods 148 (1992) 65.
[14] A. Varenne, A. Vessières, M. Salmain, P. Brossier, G. Jaouen, J. Immunol. Methods 186 (1995) 195.
[15] N.E. Kolobova, Z.P. Valueva, M.-Ya. Solodova, Bull. SSSR Acad. of Sci., Div. of Chemistry 29 (1990) 2387.
[16] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[17] L. Pospíšil, J. Fiedler, N. Fanelli, Rev. Sci. Instrum. 71 (2000) 1804.
[18] G.T. Hermanson, Bioconjugate Techniques, Academic Press, San Diego, USA, 1996, p. 298.
[19] A. Gorfti, M. Salmain, G. Jaouen, M.J. McGlinchey, A. Bennouna, A. Mousser, Organometallics 15 (1996) 142.
[20] M. Salmain, M. Gunn, A. Gorfti, S. Top, G. Jaouen, Bioconjug. Chem. 4 (1993) 425.
[21] A. Kazimierczak, J. Zakrzewski, M. Salmain, G. Jaouen, Bioconjug. Chem. 8 (1997) 489.
[22] G.R. Bright, B.S. Spooner, Anal. Biochem. 131 (1983) 301.
[23] M.J. Hunter, M.L. Ludwig, J. Am. Chem Soc. 84 (1962) 3491.
[24] S. Blanalt, M. Salmain, B. Malézieux, G. Jaouen, Tetrahedron Lett. 37 (1996) 6561.
[25] F.T. Wood, M.M. Wu, J.C. Gerhart, Anal. Biochem. 69 (1975) 339.
[26] M. Salmain, A. Gorfti, G. Jaouen, Eur. J. Biochem. 258 (1998) 192.
[27] M. Salmain, G. Jaouen, J. Fiedler, R. Sokolová, L. Pospíšil, Collect. Czech. Chem. Commun. 66 (2001) 155.
[28] B. Kuznetsov, G. Shumakovich, Bioelectrochem. Bioenerg. 1 (1973) 345.
[29] M.T. Stankovich, A.J. Bard, J. Electroanal. Chem. 86 (1978) 189.
[30] P. Bernabeu, L. Tamisier, A.D. Cesare, A. Caprani, Electrochim. Acta 33 (1988) 1129.
[31] F. Lacour, R. Torresi, C. Gabrielli, A. Caprani, J. Electrochem. Soc. 139 (1992) 1619.
[32] Y. Mao, W. Wei, H. Peng, J. Zhang, J. Biotechnol. 89 (2001) 1.
[33] G. Dryhurst, K.M. Kadish, F. Scheller, R. Reunberg (Eds.), Biological Electrochemistry, vol. 1, Academic Press, New York, London, 1982, p. 398.
[34] E. Paleček, Topics in Bioelectrochemistry and Bioenergetics, vol. 5, Wiley, London, 1983, p. 65.
[35] H.W. Nürnberg, Bioelectrochemistry, vol. 1, Plenum Publ. Corp, New York, 1983, p. 183.
[36] D.R. Jackson, S. Omanovic, S.G. Roscoe, Langmuir 16 (2000) 5449.
[37] A.J. Bard, L.R. Faulkner (Eds.), Electrochemical Methods—Fundamentals and Applications, John Wiley & Sons, New York, 1980, p.383.
[38] J.H. Francisco, A. Manuel, Z.A. Rosario, Anal. Biochem. 262 (1998) 129.
[39] W.R. Jin, Q.F. Weng, J.R. Wu, Anal. Chim. Acta 342 (1997) 67.
[40] Y. Cai, F. Yin, Q. Xie, Y. Zhang, S. Yao, Microchem. J. 68 (2001) 71.
[41] H.-M. Zhang, Z. Zhu, N.-Q. Li, Fresenius J. Anal. Chem. 363 (1999) 408.
[42] A. Costa García, M.T. Fernández Abedul, P. Tuñón Blanco, Talanta 41 (1994) 1191.
[43] C. Fernández Bobes, M.T. Fernández Abedul, A. Costa García, Instr. Sci. Technol. 29 (2001) 65.