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# Tetraphenylporphyrin as a protein label for triple detection analytical systems

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## Abstract

Porphyrins and metalloporphyrins are promising new protein labels that can be detected using multiple techniques; improving the reliability of the analysis and broadening the range of the linear response. Here, we investigate the potential of 5,10,15,20-tetraphenyl-21H,23H-porphyrin (Tpp) as a hybrid protein label. The electrochemical and optical properties of porphyrin conjugated with bovine serum albumin (BSA), chicken egg albumin (CEA) and immunoglobulin G (IgG) were determined and optimal conditions for Tpp-protein conjugation established. Model conjugates of carboxylated Tpp with BSA and short peptides were characterized using differential pulse voltammetry, UV–Vis spectrophotometry and spectrofluorimetry. These results reveal that Tpp is a promising molecule to be used in a triple detection protein labelling system.

Keywords: Protein modification, Proteins, Visible spectrophotometry, Electrochemistry, Spectroscopy, Chemistry, Analytical chemistry, Biochemistry, Voltammetry

## 1. Introduction

The use of molecules of biological origin with relevant selectivity in the receptor layer of the affinity biosensor allows one to determine distinct proteins in the samples of complex matrices comprising interfering compounds such as carbohydrates, lipids or other proteins. Due to the limited sensitivity of the analytical methods that could ensure the direct determination of proteins, the need to use labels arises. The compound used as a label must exhibit some unique properties – most frequently spectroscopic, which enable its determination using analytical techniques and therefore indirect determination of the coupled biomolecules.

In the affinity biosensors labels are typically conjugated with antibodies, receptor proteins or aptamers [1, 2, 3]. Different labels are used depending on the detection mode. However, a given label is usually dedicated to one detection technique only. For instance, in the optical mode of detection, i.e. spectrophotometric and spectrofluorimetric [4, 5, 6], several types of dyes, fluorophores and quenchers are being applied [7, 8]. The use of quantum dots and nanoparticles has also gained popularity [9]. In the electrochemical detection mode one typically employs redox-active labels, such as methylene blue [10, 11], ferrocen [12, 13], ferricyanides [14, 15] or  $\text{Ru}(\text{bpy})_3^{2+}$  [16],  $\text{Ru}(\text{phen})_3^{2+}$  [17],  $[\text{Fe}(\text{CN})_6]^{3-/4-}$  [18],  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  [19],  $\text{Co}(\text{phen})_3^{3+}$  [20] complexes. There is also a tendency to use enzymes [21] catalytic activity of which allows for indirect determination of the analyte through determination of the respective products of enzymatic reaction. In this case different detection techniques are used, depending on the reaction substrate. Radioactive isotopes, e.g.  $^{111}\text{In}$  or  $^{57}\text{Co}$  [22, 23] are also widely applied, mainly in radioimmunoassays.

Porphyrins and metalloporphyrins are known from their application as ionophores in ion-selective electrodes for determination of several anions [24, 25, 26]. However, they are also promising candidates for labels, since their presence may be monitored *via* triple detection system, improving thereby the reliability of the analysis and broadening the range of linear response. This is possible as porphyrins and metalloporphyrins exhibit both spectroscopic properties [27] (detection based on absorption and fluorescence spectra) and the capacity to undergo redox reactions [28]. Moreover, the latter may occur either within rings of porphyrin or on a metal cation in its coordination center. Finally, the metalation of porphyrins with cations of radioactive isotopes allows for their detection using radiometry. These features make this group of compounds an excellent candidate for hybrid labels of proteins.

To date, porphyrins are widely used as labels of biomolecules in the photodynamic therapy of tumors [29] and sensitizers for cancer detection [30].

In this context, various conjugates of porphyrins with different types of biomolecules were reported. For example, the selective accumulation of porphyrins bound to monoclonal antibodies allowed for detection of cancer cells [31]. In another study conjugates with steroids were created [32] in order to initiate a localized oxidative stress and apoptosis of the cancer cells. Moreover, conjugates with mono- and polynucleotides [33] allowed for selective photo-cleavage of specific strands of DNA. Finally, the metalloporphyrin binding with peptide, forming targeted the ranoctic conjugate, has recently been reported [34]. Apart from therapeutic applications, porphyrins were rarely used in the role of labels. One of the few examples of their analytical applications is a complex of porphyrin with cobalt which was used in a genosensor construction [35].

The aim of our work was to investigate the properties of tetraphenylporphyrin (Tpp) in terms of its potential use as a hybrid label of proteins. The presented study encompasses: *i*) electrochemical and optical characterization of Tpp, *ii*) optimization of the Tpp-protein conjugation reaction, *iii*) characterization of the obtained conjugates. The performed research is a first stage in the design of a Tpp-based affinity biosensor.

The structure of the paper is as follows. In Sections 2.1–2.2 we present the electrochemical and optical characterization of Tpp. Its behaviour under various conditions is analyzed. A special attention is given to examine the impact of proteins on the response of porphyrin. The examined proteins were selected to mimic receptors or surface blocking agents which are commonly used in affinity biosensors and may interfere with the label. The type and range of changes in absorption and fluorescence spectra and the effect on porphyrin redox properties is taken into account.

In Section 2.3 we present the conjugation of porphyrin with model protein and peptides. To this end, a modified system was required in order to enable the bonding of biomolecules. The derivative of Tpp functionalized with carboxyl group was therefore employed to create covalent bond with proteins chains. The obtained conjugates were successfully characterized using gel electrophoresis (SDS-PAGE) and size-exclusion chromatography (SEC). In addition, we conjugated Tpp with short peptides and those conjugates were characterized by HPLC-ESI/MS.

## 2. Materials and methods

### 2.1. Reagents

Dimethyl sulfoxide (DMSO) purchased from Sigma was applied as a solvent in all of the conducted measurements. 5,10,15,20-tetraphenyl-21H, 23H-porphin (Tpp) was purchased from Aldrich and 5-mono(4-carboxyphenyl)-10,15,20-triphenylporphyrin from Frontier Scientific. The tetrabutylammonium salts of

iodide (TBAI), perchlorate (TBAClO<sub>4</sub>), tetraphenylborate (TBATPB) and tetraoctylammonium salt of bromide (TOABr) were employed as supporting electrolytes as received from Sigma. The antibody – immunoglobulin G (IgG) from rabbit serum and proteins – bovine serum albumin (BSA) and chicken egg albumin (CEA) as well as 1-ethyl-3-(3-dimethylpropyl) carbodiimide hydrochloride (EDC), dicyclohexylcarbodiimide (DCC) and N-hydroxysuccinimide (NHS) were obtained from Sigma. Peptides of amino acid sequences: CFADEF and KFADEF were chemically synthesized by Novozym.

## 2.2. Conjugation procedure

The derivative of Tpp containing one carboxyl group in the porphyrin ring was used for conjugation with biomolecules. The covalent bond with amino groups present in side chains of proteins was formed in a two-step reaction. First, the carboxyl group of porphyrin was activated with EDC in DMSO leading to formation of intermediate product. Second, the solution containing protein in 0.1 M carbonate buffer of pH 9.3 was mixed with organic solution of activated porphyrin. The schematic representation of conjugation reaction is presented in Fig. 1.

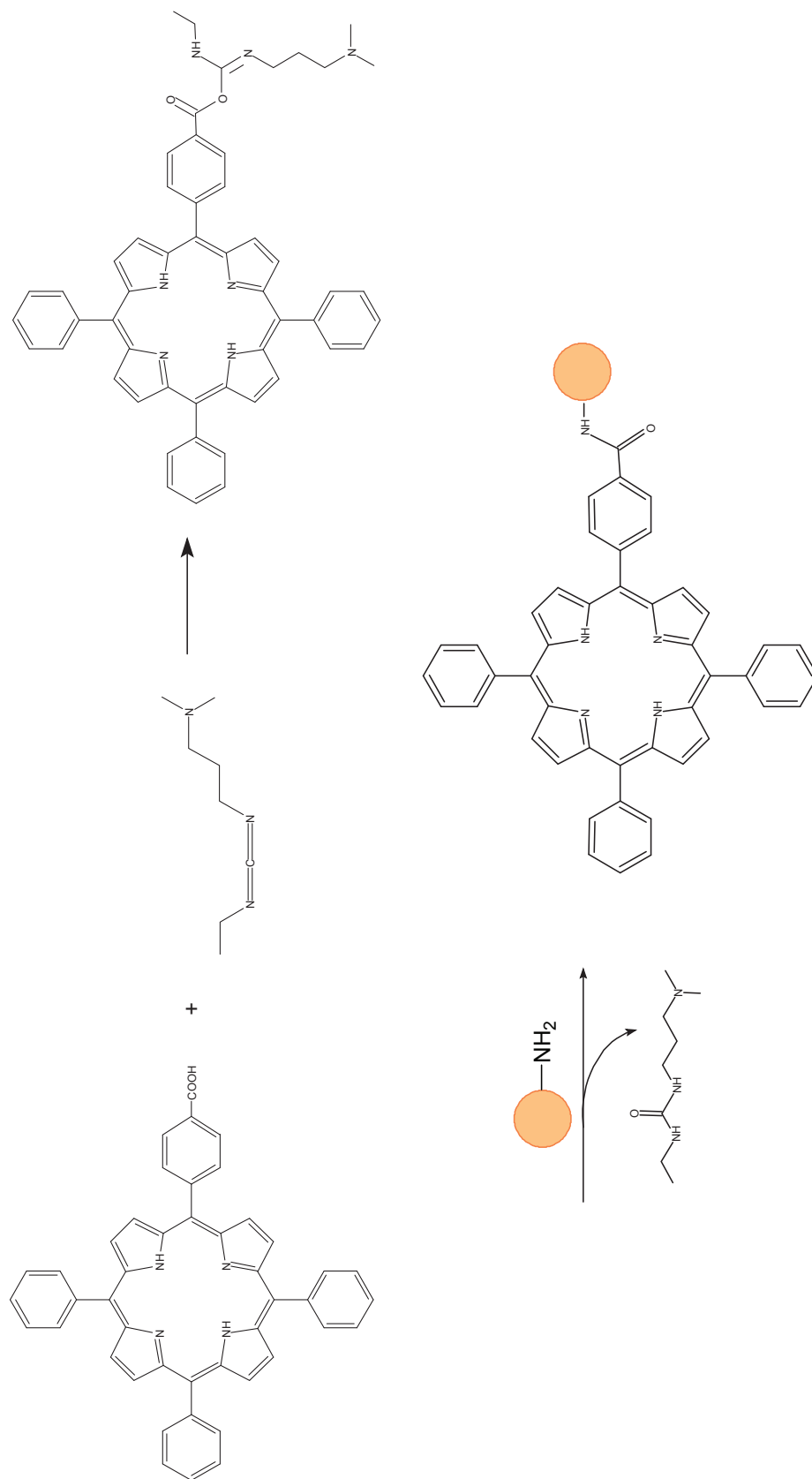
Several parameters were optimized: time of carboxyl groups activation (in the range from 15 min to 5 h), concentration of activator (0.25–6 mg·mL<sup>-1</sup>), time of the conjugation reaction (18 h–48 h) and concentration of protein (1–30 mg·mL<sup>-1</sup>). The largest applied amount of protein corresponded to a 10-fold molar excess of porphyrin with respect to the number of amino groups in protein side chains. The solution obtained after the conjugation was lyophilized using Fisher Bioblock Scientific Alpha 1-2/LDplus freeze dryer at temperature of –50 °C and pressure of 0.04 mbar. The lyophilisate was dissolved in water which allowed for separation of water-insoluble unbound porphyrin. After the centrifugation (carried out for 2 min. in Sprout mini-centrifuge at 2000 × g) the prepared conjugates were characterized using electrochemical and optical techniques, as well as SDS-PAGE and SEC.

The conjugation with peptides was performed according to the same procedure as described for the protein conjugates with maintaining optimal reaction parameters. Two types of peptides were used – CFADEF containing one free amino group and KFADEF with two amino groups. In the first case the porphyrin:peptide ratio of 1:1<sub>mol</sub> was used, whilst in the second one 2:1<sub>mol</sub>. The obtained conjugates were characterized using HPLC-ESI/MS.

## 2.3. Analysis

### 2.3.1. Electrochemical

A three-electrode cell consisting of glassy carbon working electrode, Ag/AgCl reference electrode (containing 1 M KCl as internal electrolyte) and gold-wire



**Fig. 1.** The representation of conducted conjugation reaction: the activation of porphyrin carboxyl group and subsequent formation of peptide bond with amino group of biomolecule. The orange circle stands for protein or peptide.

counter electrode was used throughout the experiments. The measurements were conducted using CHI660A potentiostat (CH Instruments Co., USA). The examination of reference electrode in DMSO using  $\text{Fe}^{2+}/\text{Fe}^{3+}$  system in the form of potassium hexacyano complex revealed some changes in the oxidation peak. The oxidation potential in the solution of DMSO containing 10% of water was shifted by 154 mV towards negative potentials (comparing to 100%-water solution). The potential of reference electrode remained constant during all conducted measurements. The differential pulse voltammetry (DPV) measurements were performed using the parameters of: potential increment = 0.004 V, amplitude = 0.05 V, pulse width = 0.1 s and pulse period = 0.2 s. In order to deaerate the solutions before the measurements, a stream of nitrogen was passed through them for several minutes.

### 2.3.2. Optical

The UV-Vis absorption measurements were carried out in quartz cells of 1 cm optical path using Perkin Elmer – Lambda 25 spectrophotometer. The spectrofluorimetric spectra were recorded on VARIAN – Cary Eclipse spectrofluorimeter. A pure DMSO solution was applied as a blank.

### 2.3.3. HPLC-ESI/MS

The identification of the porphyrin-peptide conjugates was carried out using high-performance liquid chromatography (HPLC) coupled with electrospray ionization mass spectrometry (ESI-MS) system (Agilent Technologies, USA). It comprised an Agilent 1200 series liquid chromatograph equipped with a Zorbax SB-C18 column (4.6 mm  $\times$  150 mm, 3.5  $\mu\text{m}$ ) and an Agilent 6460 LC/MS ESI mass spectrometer as a detector. The analysis was conducted in accordance with the procedure described in [36].

### 2.3.4. SDS – PAGE

BSA and BSA-Tpp samples were suspended in Laemmli denaturing buffer and equivalents of 1  $\mu\text{g}$  of protein were loaded into separate gel wells. Electrophoresis was performed according to the standard protocol in MiniProtean 3 tank (Bio-Rad Laboratories) using 15% (w/v) polyacrylamide resolving gels supplemented with 0.1% (w/v) SDS, 12% (w/v) sucrose and 5% (w/v) polyacrylamide stacking gels containing 0.1% SDS. After separation gels were stained with Coomassie Blue (PageBlue<sup>TM</sup>, Thermo Scientific) according to the manufacturer protocol.

### 2.3.5. Size-exclusion chromatography

BSA and BSA-Tpp samples containing 40  $\mu\text{g}$  of protein were suspended in 25 mM phosphate buffer (pH 6.8) and centrifuged at 10 000  $\times$  g. The supernatant was

transferred into HPLC vials and placed into cooled (4 °C) auto sampler chamber of HPLC system.

Size exclusion chromatography was performed using Dionex ICS-3000HPLC system (Thermo Scientific, USA) and the BioSep-SEC-S3000 (Phenomenex, 5  $\mu\text{m}$   $\times$  400  $\text{\AA}$ , 600  $\times$  7.8 mm) column. The proteins were eluted at a flow rate 0.5 ml min<sup>-1</sup> at 20 °C in isocratic 25 mM phosphate buffer (pH 6.8) containing 100 mM NaCl during 60 min. The column eluate was monitored by PDA detector at 280 and 420 nm with simultaneous spectra recording between 200 and 600 nm.

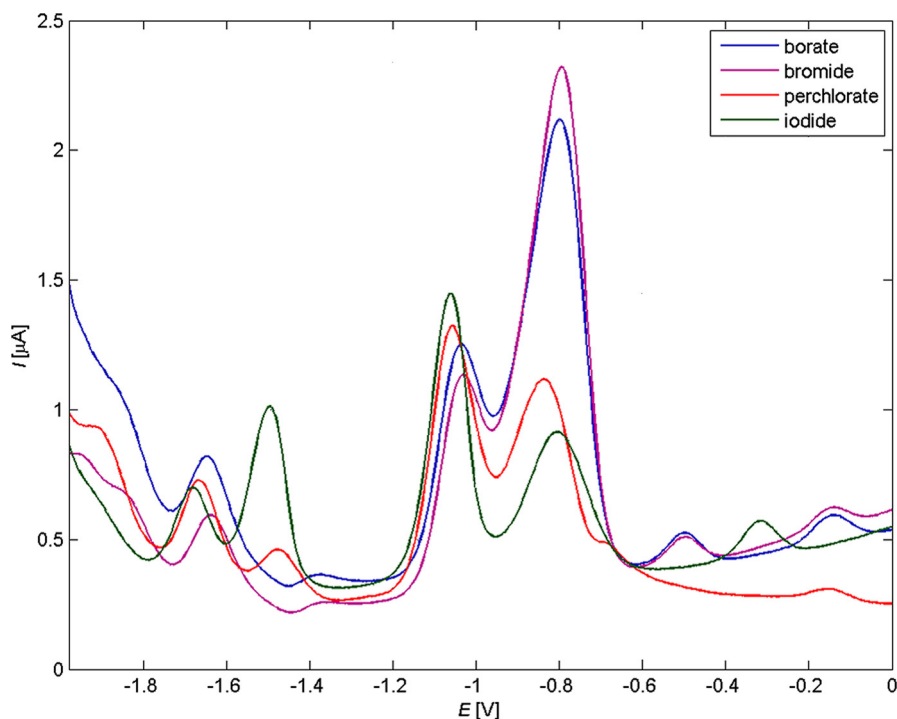
### 3. Results and discussion

#### 3.1. Electrochemical characterization

In the course of presented study, the electrochemical characterization of Tpp was first carried out. The preliminary studies allowed for designation of the optimal analysis parameters, such as working electrode material and the applied solvent (data not shown). In consequence, we carried out all of the experiments using glassy carbon electrode and prepared the samples in DMSO. Aside from the fact that it guarantees well-defined, intense current signals derived from the porphyrin in the wide potential range, DMSO is dedicated to the analysis involving proteins and antibodies. As shown in ref. [37, 38, 39] DMSO is appropriately adjusted for analysis of proteins, as it ensures remaining the activity of biomolecules, which are applied in affinity biosensors or bioassays. Moreover, DMSO is recommended for conjugations and analyses involving proteins if the molecule that is to be attached to the protein is readily soluble in water solutions [40].

The substantial part of electrochemical research concerned the comparison of influence of various supporting electrolytes. To this end, we examined tetrabutylammonium or tetraoctylammonium salts containing different anions: borate, bromide, iodide and perchlorate, as various anions may interact differently with porphyrin rings. Voltammograms obtained for the oxidation of Tpp are presented in Fig. 2. For all electrolytes two well-defined peaks can be observed: the first at -0.80 V corresponding to the formation of the anion radical, and the second one at -1.03 V to the creation of dianion (potential values obtained in the presence of bromide salt).

Depending on the anion in the supporting electrolyte, both shifts in potentials and changes in intensities of signals occurred. For the peak corresponding to dianion formation (-1.03 V) examined ions are set in order: bromide>borate>perchlorate>iodide with respect to the decrease in the potential. This reflects the increase of the interaction strength between the anion



**Fig. 2.** Differential pulse voltammograms representing oxidation of 0.1 mM Tpp obtained on glassy carbon electrode in DMSO solution containing various supporting electrolytes, all at the concentration of 0.01 M.

and  $\pi$ -ring system of porphyrin, which effect had also been observed for other porphyrins [41, 42]. The proportions of signals derived from two forms of porphyrin (anion/dianion) also varies depending on the anion of electrolyte salt, which is related to different values of diffusion coefficients of the formed complexes. The dependence of diffusion coefficients on applied supporting electrolyte has been described in [43]. For bromide and borate, the anion radical formation peak ( $-0.80$  V) has higher intensity, whereas in the case of iodide and perchlorate this proportion is reversed. From the analytical point of view, all applied electrolytes are appropriate as they ensure well-defined analytical peaks and sufficient intensities of signals.

Subsequently, we evaluated the impact of biomolecules. Two types of model albumins of known size and amino acid sequences were employed – bovine serum albumin (BSA) and chicken egg albumin (CEA), as they are widely utilized in the role of surface blocking agents. Moreover, we selected immunoglobulin G (IgG) as an exemplary component of sensor receptor layer.

These studies were performed prior to formation of covalent porphyrin-protein conjugates, in order to verify whether presence of proteins in the sample affects the nature of Tpp response. In the construction of sensors and assays a variety of proteins is present – they may serve as components of the receptor layer,



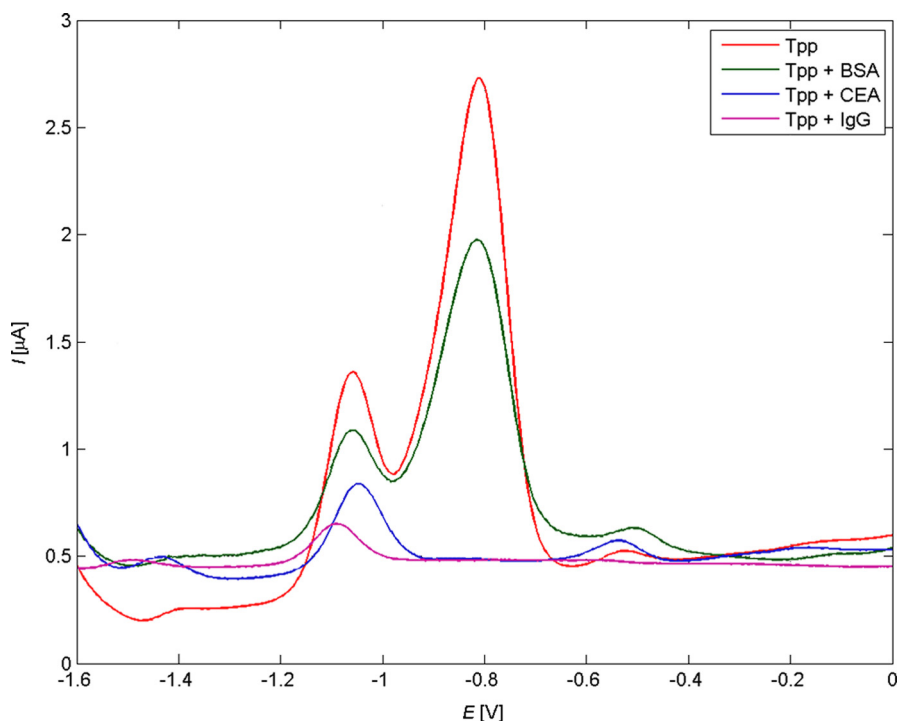
surface blocking agents, analytes or components of a sample matrix. All these proteins may interact with the porphyrin label used in the construction of such assay *via* weak interactions, e.g. van der Waals and hydrophobic interactions or ionic bonds. Above all,  $\pi$ - $\pi$  interactions between porphyrin ring and aromatic moieties of proteins amino acids are expected. As a consequence, the complexation between the protein and porphyrin may occur. Nevertheless, from the analytical point of view the type of interaction is irrelevant – the only important aspect is the total effect on the signals of porphyrin. Therefore, we wish to verify to what extent does the presence of proteins affect the detection of the porphyrin label.

We observed that the presence of examined proteins and antibody affects the electrochemical behaviour of Tpp in different ways. The addition of BSA in the amount of  $4 \text{ mg}\cdot\text{mL}^{-1}$  leads to a decrease of intensities of both peaks (declines of 27% and 21% of signals values for peaks at  $-0.80 \text{ V}$  and  $-1.03 \text{ V}$ , respectively). In the case of CEA or IgG further changes occurred. The peak at  $-1.03 \text{ V}$  decreased by 38% with respect to free Tpp in the presence of CEA, and by 63% in the presence of IgG. Furthermore, the peak of less negative potential disappeared after the addition of either of the two mentioned biomolecules. The absence of anion radical peak indicates destabilization of this form, which can occur as a result of  $\pi$ - $\pi$  interactions between porphyrin rings and phenyl rings of aromatic amino acids present in protein. Depending on the protein structure, the spatial arrangement of protein chains may promote the  $\pi$ -stacking interactions, which lead to one of the porphyrin forms destabilization. The complete disappearance of  $-0.80 \text{ V}$  peak occurs when CEA or IgG is applied. This clearly disqualifies the anion radical signal from the analytical use. Therefore, the  $-1.03 \text{ V}$  peak is suitable for electrochemical detection of Tpp. Voltammograms presenting influence of different biomolecules on Tpp electrochemical characterization are depicted in Fig. 3. The effect of signals reduction was similar regardless of the electrolyte used. Therefore only the representative data for bromide are presented. Moreover, especially in the case of  $-0.80 \text{ V}$  peak, the influence of protein is so significant that the changes resulting from application of different supporting electrolytes are negligible.

The detection limit for Tpp using DPV technique was found to be  $1 \mu\text{M}$  with the linear range of response  $5\text{--}500 \mu\text{M}$ , when considering the signal obtained for dianion formation. It is also noteworthy, that the parameters remained unchanged in the presence of biomolecules at the assumed amount of protein equivalent to the amount of porphyrin in its detection limit (1:1 molar ratio).

### 3.2. Optical characterization

We assessed the influence of proteins on optical properties of tetraphenylporphyrin, reflected in shifts of the absorption or fluorescence

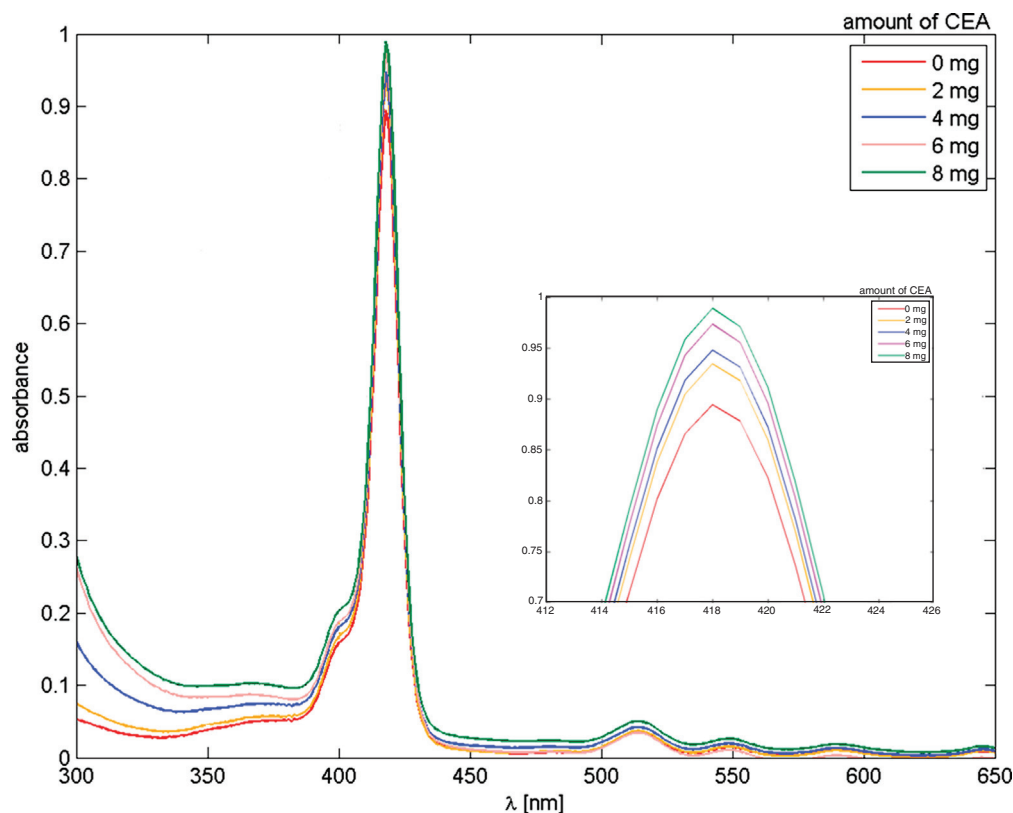


**Fig. 3.** Differential pulse voltammograms representing oxidation of 0.1 mM Tpp in the presence of proteins: BSA, CEA and IgG ( $4 \text{ mg}\cdot\text{mL}^{-1}$ ) obtained on glassy carbon electrode in the DMSO solution containing 0.01 M tetraoctylammonium bromide.

maxima or changes in the signal intensities. The outcomes depend on the concentration of applied protein, as well.

Fig. 4 presents UV–Vis spectra of Tpp with different amounts of CEA. The absorbance maximum, corresponding to the Soret band, is situated at the wavelength of 418 nm and no shifts occur with the changes of proteins concentration. However, the increase in the amount of added protein is followed by the small increase of absorbance intensity, which could be explained by radiation scattering by proteins in solution. These findings are shared by both types of proteins and antibody used. The spectrophotometric detection limit for Tpp was 3.2 nM with the linear range of response: 0.01–20  $\mu\text{M}$ . The addition of equivalent amount of protein did not alter the limit of detection, similarly as it was for electrochemical analysis.

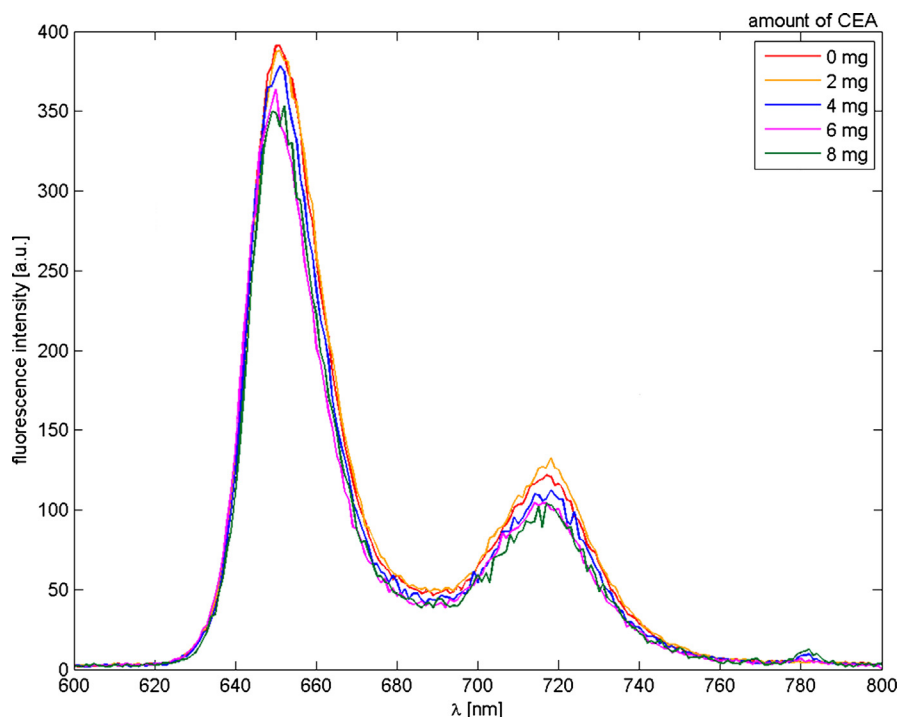
Moreover, in order to verify the possibility of applying Tpp in systems utilizing different detection modes, we examined the impact of salts used as electrochemical supporting electrolytes on obtained absorption spectra. We found that neither of the four supporting electrolytes altered the nature of the spectrum. It is therefore possible to use the same sample in both electrochemical and optical techniques without modification of its composition which minimizes



**Fig. 4.** UV-Vis spectra of 2  $\mu\text{M}$  Tpp with CEA of different concentrations obtained in DMSO solution containing 0.01 M tetraoctylammonium bromide.

its consumption. The only necessary step is a dilution of the sample before spectrometric measurements.

Next we verified the effect of biomolecules on the fluorescence emission. Spectra recorded for various amounts of the added protein are presented in Fig. 5. We observed that the increase of proteins concentration led to the decline of the fluorescence signal for the emission wavelength of 650 nm, regardless of the biomolecules type. A slight spread in results covering 41 units of fluorescence intensity for the emission maximum at the wavelength of 650 nm, and 26 units for the maximum at the wavelength of 716 nm occurred. The differences in the nature of the Tpp spectrum, caused by the increase in the concentration of the protein, are therefore irrelevant and their presence does not prevent the determination of this porphyrin using spectrofluorimetry. Detection limit for this technique was found to be 0.05  $\mu\text{M}$  with linearity of the signal response of 0.1–25  $\mu\text{M}$ . Finally we analyzed samples containing salts needed for electrochemical measurements. Similarly to the spectrophotometric detection, we found that none of the examined electrolytes affects the Tpp fluorescence spectrum.



**Fig. 5.** Emission spectra of 10  $\mu\text{M}$  Tpp with CEA of different concentrations obtained in DMSO solution,  $\lambda_{\text{ex}} = 430$  nm.

### 3.3. Porphyrin conjugates

#### 3.3.1. Protein-porphyrin conjugates

We have performed the conjugation of Tpp with BSA as a model protein to confirm that porphyrins can serve as labels of biomolecules and allow for indirect determination of proteins using several detection techniques. Different conjugation strategies leading to the formation of porphyrin conjugates with peptides or protein have been reported in the literature [44, 45]. Taking into account the physicochemical properties of Tpp and our preliminary studies we have selected the most appropriate reaction conditions. We have employed a carboxylated derivative of Tpp which enabled the formation of covalent bond with amino groups of proteins amino acids.

We established the optimal parameters of conjugation in the following steps. First, the conjugation reaction was conducted either in DMSO or in mixed water-organic solution, and it proved to proceed with the greatest efficiency in mixed medium – porphyrin prepared in DMSO added to protein in carbonate buffer solution. Next, we checked various activating agents: EDC, DCC and NHS. EDC was selected for porphyrin carboxyl group activation.

These findings led to further optimization, taking into account the concentration of the activating agent in the range of 0.25–6  $\text{mg}\cdot\text{mL}^{-1}$ , time of carboxyl

groups activation, from 15 min to 5 h and time of the conjugation reaction, from 18 h to 48 h, each time maintaining the other parameters unchanged. The EDC concentration of  $0.5 \text{ mg}\cdot\text{mL}^{-1}$ , amounting to more than tenfold molar excess with respect to porphyrin, was found to be optimal. Further increase of the activator concentration did not affect reaction efficiency. For studies of conjugation reaction in time we observed similar characteristic. Changing the activation time from 15 min to 2 h led to a gradual increase of the signal intensity with no further growth for longer periods of activation. In the case of time of conjugation reaction, its completion after 24 h gave the most favourable results. Longer incubation period did not alter the signal. The optimal concentration of protein was also evaluated. The highest intensity of the porphyrin absorbance was observed at  $30 \text{ mg}\cdot\text{mL}^{-1}$  of BSA. The efficiency of the conjugation obtained using the optimal parameters amounted to 87.3%. This was estimated on the basis of UV-Vis measurements of Tpp absorbance before and after the conjugation. The efficiencies values obtained for all examined conditions are presented in Table 1. As shown in Section 2.2 no significant effect of the proteins on the Tpp optical response was observed. Therefore, such methodology of efficiency estimation is justified, as the absorbance shall not change after the formation of Tpp-protein conjugates. Such estimation was possible, as the porphyrin becomes soluble in water only after its conjugation with the protein: the Tpp-BSA conjugates were first dissolved in water and then transferred to DMSO. The obtained lyophilisate was therefore dissolved in water to separate unbound porphyrin. The optimization procedure were also confirmed by spectrofluorimetric measurements giving consistent results.

The electrochemical measurements revealed some changes in the nature of Tpp response when its derivative functionalized with carboxyl group was applied. The peak corresponding to dianion formation is slightly shifted to a more negative potential of  $-1.15 \text{ V}$ . The peak related to the anion radical formation is significantly reduced in its intensity and shifted along the potential axis in the opposite direction, to  $-0.60 \text{ V}$ . In BSA-Tpp conjugates the peak at  $-1.15 \text{ V}$  becomes shifted to a less negative potential of  $-1.12 \text{ V}$ . This signal is analytically useful and on its basis the determination of porphyrin label after conjugation is possible *via* differential pulse voltammetry.

We have also confirmed the formation of conjugates by gel-electrophoresis and size-exclusion chromatography coupled with PDA detector. Fig. 6A presents chromatogram obtained for free BSA and BSA conjugated with Tpp. The signal derived from BSA-Tpp is slightly shifted towards shorter retention time, indicating presence of conjugates. However, in the course of conjugation reaction some protein aggregates are additionally formed, as we observe signals representing macromolecular compounds eluted before the monomeric protein. The insert in the upper left corner of Fig. 6A demonstrates the gel

**Table 1.** The values of reaction efficiencies obtained for optimized conjugation parameters estimated on the basis of UV–Vis spectrophotometry (Soret band at 418 nm).

Optimized parameter	Value	Yield [%]
Time of carboxyl group activation <sup>a</sup>	15 min.	34.2
	30 min.	42.4
	1h	71.2
	2h	84.6
	3h	84.4
	4h	83.1
	5h	84.0
Time of conjugation reaction <sup>b</sup>	18h	68.7
	20h	73.6
	22h	76.9
	24h	86.3
	42h	85.9
	45h	86.2
Concentration of EDC <sup>c</sup>	48h	86.1
	0.25 mg/ml	81.7
	0.5 mg/ml	87.3
	1 mg/ml	87.0
	2 mg/ml	87.2
	4 mg/ml	86.8
Concentration of BSA <sup>d</sup>	6 mg/ml	87.0
	1 mg/ml	26.8
	2 mg/ml	34.2
	5 mg/ml	67.9
	10 mg/ml	71.0
	15 mg/ml	68.7
	30 mg/ml	78.5

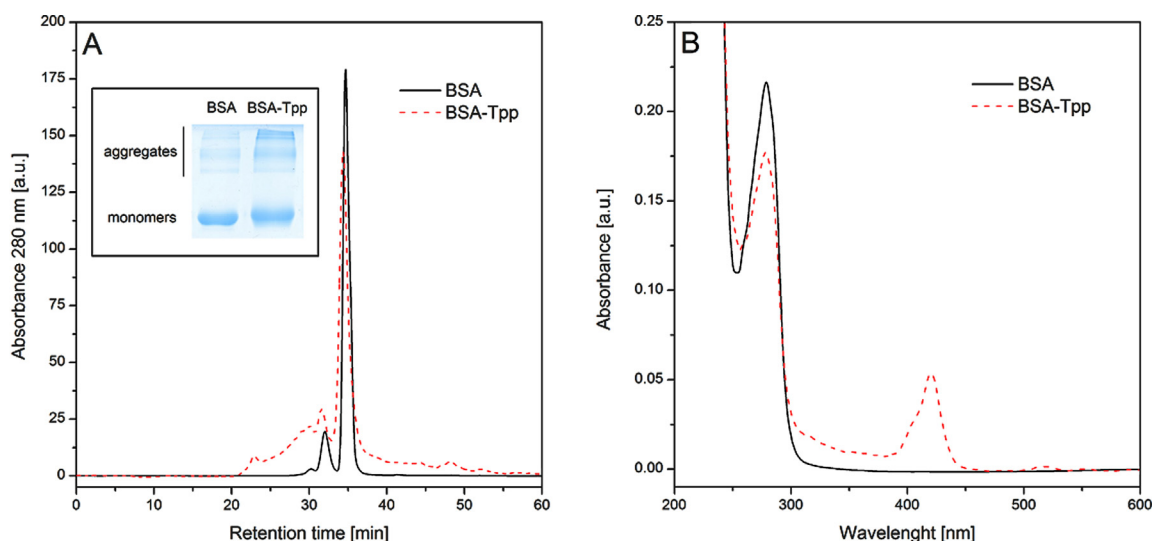
<sup>a</sup>Time of conjugation reaction – 24 h, concentration of EDC - 4 mg/ml, concentration of BSA - 30 mg/ml.

<sup>b</sup>Time of carboxyl group activation – 2 h, concentration of EDC - 4 mg/ml, concentration of BSA - 30 mg/ml.

<sup>c</sup>Time of carboxyl group activation – 2 h, time of conjugation reaction – 24 h, concentration of BSA - 30 mg/ml.

<sup>d</sup>Time of carboxyl group activation – 2 h, time of conjugation reaction – 24 h, concentration of EDC - 4 mg/ml.

electrophoresis separation obtained for both main fractions. The signal derived from conjugate is shifted with respect to the free BSA which additionally confirms SEC results. Fig. 6B shows absorption spectra obtained on-line for the



**Fig. 6.** Confirmation of BSA-porphyrin conjugates formation: A – chromatograms obtained using SEC for BSA and BSA-porphyrin conjugates with insert of gel electrophoresis; B – absorption spectra derived on-line from main chromatographic fractions.

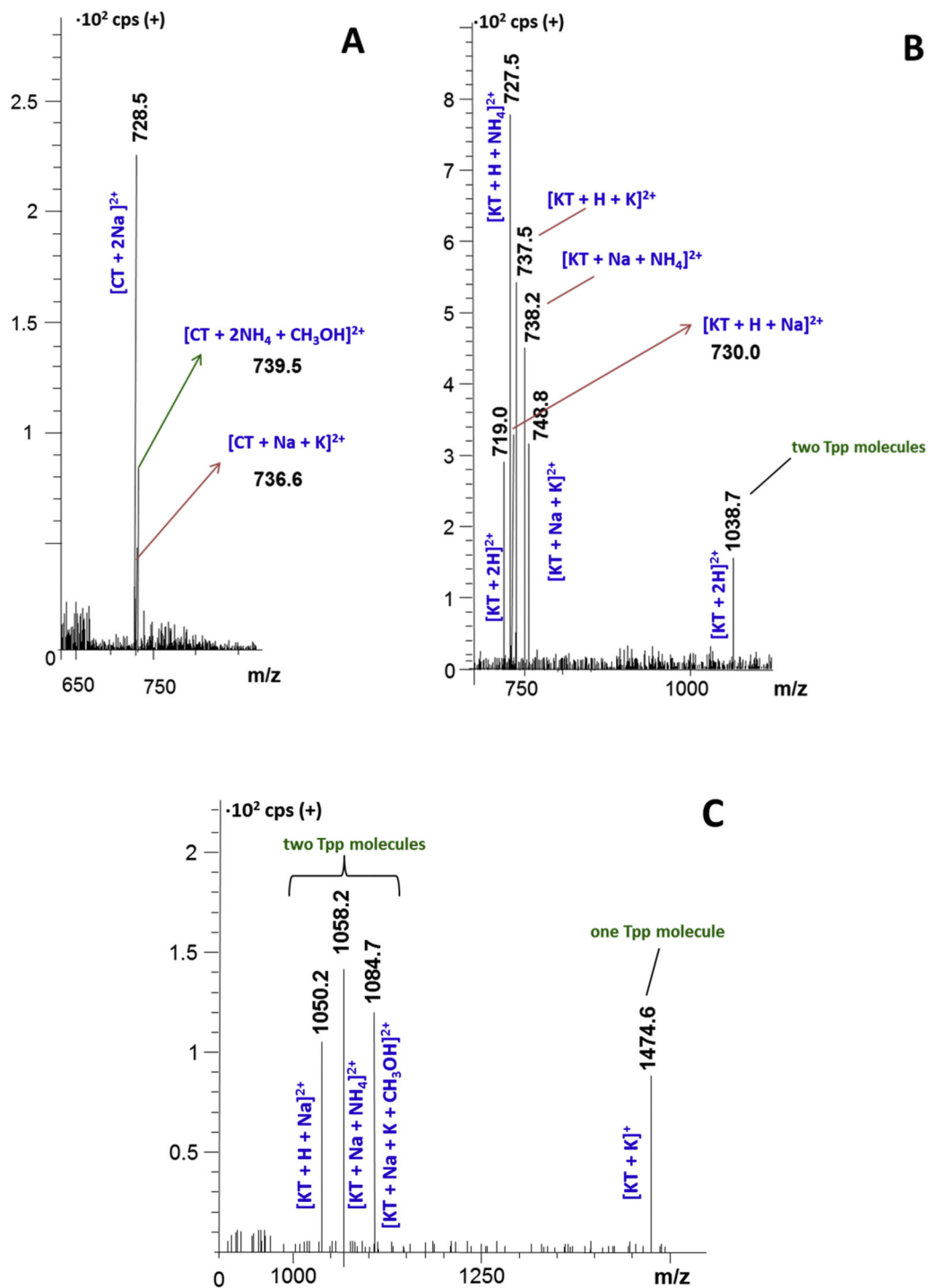
fractions corresponding to BSA and BSA-Tpp monomers. The spectrum registered for conjugate contains absorption peaks characteristic for both protein at 280 nm and porphyrin Soret band at 420 nm.

Having found suitable conditions for the binding reaction, we performed the conjugation of Tpp with IgG – a system suitable for forming a target receptor layer of the sensor. To this end, the optimal parameters for BSA-Tpp conjugation were adapted, obtaining the efficiencies of 75.3% when applying spectrophotometric analysis and 74.9% in case of spectrofluorimetric analysis.

### 3.3.2. Peptide-porphyrin conjugates

We also performed the conjugation of porphyrin with short peptides. To this end, we used peptides of amino acid sequences: CFADEF (Cys-Phe-Ala-Asp-Glu-Phe) containing one amino group (peptide I) and KFADEF (Lys-Phe-Ala-Asp-Glu-Phe) with two amino groups (peptide II). The reaction between the activated porphyrin and peptide was carried out in mixed water:DMSO (1:1<sub>vol.</sub>) solution for 24 h and after that samples were lyophilized. The obtained lyophilisates were dissolved in water, which enabled separation of unreacted porphyrin, as unconjugated carboxylated Tpp itself is insoluble in aqueous media.

The presence of conjugates was confirmed by HPLC-ESI/MS. We found that peptide II formed conjugates with either one or two molecules of Tpp, whilst peptide I contained only one porphyrin bound. The examples of scanning spectra for identification of both peptide-porphyrin conjugates are presented in Fig. 7.



**Fig. 7.** ESI-MS spectra illustrating identification of peptide-porphyrin conjugates (positive ionization mode): A – scanning spectrum of conjugate I registered for chromatographic signal eluted at  $T_R = 25.9$  min. of HPLC separation; B – conjugate II,  $T_R = 25.9$  min.; C – conjugate II,  $T_R = 14.2$  min. CT denotes conjugate I and KT conjugate II.



The porphyrin-peptide conjugates were characterized by UV–Vis spectrophotometry and spectrofluorimetry with detection limits of: 5.0 nM and 0.057  $\mu$ M, respectively (peptide I), and 7.1 nM and 0.060  $\mu$ M, respectively (peptide II). The electrochemical characterization was carried out using differential pulse voltammetry on gold and glassy carbon working electrodes. The detection limits for glassy carbon amounted to: 2.6  $\mu$ M for peptide I and 7.8  $\mu$ M for peptide II and were lower than those obtained for gold electrodes (10.5  $\mu$ M for peptide I and 16.0  $\mu$ M for peptide II).

#### 4. Conclusions

In the course of presented study we verified the possibility of Tpp application as hybrid label of proteins. To this end, characterization of Tpp using electrochemical and optical detection techniques was carried out.

In the preliminary studies we established the optimal conditions for electrochemical analysis of Tpp and examined the proteins effect on the electrochemical signals obtained from the porphyrin. The model proteins were compared – two types of albumins: CEA and BSA, and IgG. Simultaneously, we conducted the spectroscopic characterization of Tpp – its UV–Vis and fluorescence spectra with addition of proteins were registered.

The possibility of Tpp application as proteins label was confirmed. The carboxylated derivative of Tpp was employed to create protein(or peptide)-porphyrin conjugates. First, we optimized the conjugation conditions using BSA as model protein, and subsequently we conjugated porphyrin with IgG, mimicking a target receptor layer system. The formation of conjugates was confirmed by three target techniques – electrochemistry, spectrophoto– and spectrofluorimetry, and additionally by HPLC-ESI/MS for peptide conjugates and SDS-PAGE and SEC for protein conjugates.

Moreover, we checked the possibility of labeled peptides application as selective receptors of analytes. The preliminary studies revealed that porphyrin conjugated with peptide may be successfully determined electrochemically, when peptide is immobilized on gold electrode using its thiol group.

Finally there exists a possibility of applying the optimized conjugation procedure established in this work to bind proteins with metalated Tpp derivatives containing manganese(III) (Mn-tpp) and tin(IV) (Sn-tpp) cations in the coordination center. The characteristics of both Mn-tpp and Sn-tpp and the possibilities of their determination were presented elsewhere [46, 47].

The results revealed that Tpp meets all the criteria imposed on labels of biomolecules. The application of more than one detection technique contributes to the improvement of the reliability of the analysis and broadening of the range

of linear response. In the future we plan to apply Tpp in a model immunoassay as an immunoglobulins label.

## Declarations

### Author contribution statement

Kamila Konopińska: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Mariusz Pietrzak: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Radosław Mazur: Performed the experiments; Analyzed and interpreted the data.

Elżbieta Malinowska: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

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### Competing interest statement

The authors declare no conflict of interest.

### Additional information

No additional information is available for this paper.

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