

1 **Ultrasensitive electrochemical determination of the cancer biomarker**

2 **sPD-L1 protein based on BMS-8 modified gold electrode**

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26 **Abstract**

27

28 Soluble form of Programmed Death - Ligand 1 (sPD-L1) is one of the immune
29 checkpoint proteins which can be detected in the sera of patients with many types of cancer.
30 Taking advantage of the BMS-8 compound properties to create a strong complex with PD-L1
31 protein, we established a novel biosensing interface detecting sPD-L1. This work describes
32 the chemical modification of gold electrode with BMS-8 compound which interacts with PD-
33 L1 protein. The results show that we can confirm the presence of the sPD-L1 protein in the
34 concentration range of 10^{-18} to 10^{-8} M using electrochemical impedance spectroscopy (EIS)
35 with a limit detection (LOD) of 1.87×10^{-14} M for PD-L1 (S/N=3.3) and at the concentration
36 of 10^{-14} M by cyclic voltammetry (CV).

37 Additionally, the high-resolution X-ray photoelectron spectroscopy (XPS), contact
38 angle, and surface free energy measurements were applied to confirm the successful
39 functionalization of electrode. Moreover, we investigated the selectivity of the obtained
40 electrode for other proteins, Programmed Death - 1 (PD-1), Cluster of Differentiation 160
41 (CD160), and the B- and T-Lymphocyte Attenuator (BTLA) in a concentration of 10^{-8} M.

42 Differentiation between of PD-L1 and PD-1 was achieved on the basis of study of
43 frequency dispersion of capacitance effect at the surface of the modified Au electrode with
44 BMS-8 after incubation in at various concentrations of PD-L1 and PD-1 protein in the range
45 of 10^{-18} to 10^{-8} M. The significant differences are observed in the heterogeneity of PD-L1 and
46 PD-1 measured at the same concentrations of both proteins. The results of quasi-capacitance
47 studies demonstrate that BMS-8 strongly and specifically interacts with PD-L1 protein.

48

49

50 **Keywords:** cysteamine, sPD-L1 protein, gold electrode modification, cyclic voltammetry
51 (CV), Electrochemical Impedance Spectroscopy (EIS).

52 **1. Introduction**

53

54 Every year the number of cancer cases is increasing and only in 2018, 18 million new
55 cases were diagnosed [1]. The cancer treatments are more effective if applied in the early
56 stages of the disease. In tumor diagnosis, many different methods are used, i.e. imaging tests,
57 genetic testing, and measurements of tumor biomarkers [2]. The last of them is the simplest
58 and the least invasive. The early stage of cancer is often correlated with low levels of
59 molecular biomarkers which are difficult to detect. The series of cancer-related biomarkers
60 are used for early diagnosis and are connected with specific cancer, e.g. carbohydrate antigen
61 125, prostate-specific antigen, alpha fetoprotein, carbohydrate antigen 15-3, carbohydrate
62 antigen 19-9, carcinoembryonic antigen [3–5].

63 Programmed death - ligand 1 (PD-L1) protein and its receptor, programmed death - 1
64 (PD-1), are transmembrane, immune checkpoint proteins responsible for the negative
65 regulation of the immune system. PD-L1 also occurs in the soluble form secreted into the
66 serum (sPD-L1) by monocytes, macrophages, and DC [6,7] and is often overexpressed by
67 tumor cells. Moreover, larger amount of soluble form of PD-L1 protein is detected in the sera
68 of patients with malignant melanoma [7], renal carcinoma, nasal natural killer/T-cell
69 lymphoma [8,9], diffuse large B-cell lymphoma [10], myeloma [11], and hepatocellular
70 carcinoma [10]. High level of sPD-L1 impacts overall survival and is associated with the
71 increased mortality in cancer patients [6,9,12]. It is reported that tumor-secreted sPD-L1 is
72 biologically active and able to deliver immunosuppressive signals to lymphocyte T sPD-L1
73 may be a potential biomarker for anti-PD-1/anti-PD-L1 therapy [7,13].

74 Currently, the diagnostic tests for PD-L1 which were approved by the Food and Drug
75 Administration rely on immunohistochemistry (IHC). PD-L1 expressed in the tumor tissue is
76 detected by antibodies. IHC-based tests have multiple complicating factors. Among other,

77 different IHC tests use a variation of anti-PD-L1 antibodies with diverse percentage ratio cut-
78 off of the PD-L1 expression level for each test [14,15]. PD-L1 expression is heterogeneous in
79 the tumor tissue and the binding sites for antibodies are limited, what combined with the
80 analysis of biopsies specimens embedded in parafilm (FFPE) provides poorly conclusive
81 results. IHC-approved tests cannot be compared one to another and require standardization
82 and validation [16–18]. Additionally, enzyme-linked immunosorbent assays (ELISA) using
83 sPD-L1 are developed but as in case of IHC test they have different detection range and apply
84 different types of antibodies to detect sPD-L1 protein [19–27]. The comparison of different
85 ELISA tests used for sPD-L1 detection is presented in table S1 in Supporting Information file.
86 This situation provides a burning need to develop reliable diagnostic tests for PD-L1 protein
87 detection what was the aim of our studies.

88 In the presented study, we developed the electrochemical biosensor for the detection of
89 sPD-L1 protein and we performed a series of experiments confirming its effectiveness and
90 sensitivity. BMS-8 (Bistol-Myers Sqibb – compound 8; 1-[[3-bromo-4-[(2-methyl [1,1'-
91 biphenyl]-3-yl)methoxy]phenyl]methyl]-2-piperidinecarboxylic acid) molecule was used as a
92 ligand covering surface of gold electrodes [23,24]. The interaction between BMS-8 and PD-
93 L1 was confirmed by co-crystal structure (PDB: 5J8O) and thoroughly tested using Structure-
94 activity relationship by nuclear magnetic resonance spectroscopy (SAR-by-NMR) approach
95 while NMR excluded BMS-8 interaction with PD-1 [28]. The electrochemical studies using
96 gold electrodes modified with BMS-8 enabled the detection of PD-L1 protein at various
97 concentrations in the range of 10^{-18} to 10^{-8} M by EIS technique and at the concentration of
98 10^{-14} M by CV. We used the high-resolution X-ray photoelectron spectroscopy (XPS) to
99 confirm the modification of gold electrodes. The electrodes were also characterized by the
100 contact angle and surface free energy (SFE) measurements. The selectivity of presented test



- 101 towards other immune checkpoint proteins: PD-1, cluster of differentiation 160 (CD160), and
- 102 the B- and T-lymphocyte attenuator (BTLA) has been also investigated.

103 2. Materials and methods

104

105 2.1. Chemicals and reagents

106 All solvents and reagents were used without further purification. 0.1 M phosphate
107 buffer solution (PBS), pH 7.0 was obtained according to the procedure described in [29]. 0.01
108 M of PBS was prepared from tablets purchased from Sigma-Aldrich, dissolved in ultrapure
109 water, and adjusted to pH 7.0 using 0.1 M hydrochloric acid and pH electrode. N-
110 hydroxysuccinimide (NHS), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC),
111 cysteamine, bovine serum albumin (BSA) were purchased from Sigma-Aldrich. Ethanol,
112 dimethyl sulfoxide (DMSO), potassium ferricyanide $K_3[Fe(CN)_6]$, potassium ferrocyanide
113 $K_4[Fe(CN)_6]$ were purchased from POCh (Poland). The BMS-8 was synthesized as described
114 previously [24,30].

115 PD-1 and PD-L1 proteins were expressed and purified as described previously [30].
116 The recombinant human BTLA and CD160 proteins were purchased from Novoprotein, USA
117 (company product code: C563) and ACROBiosystems, USA (company product code: BY5-
118 H5229), respectively.

119

120 2.2. EIS and CV measurements

121 All electrochemical measurements were performed on MultiAutolab M204
122 potentiostat (Metrohm, Netherlands) using three electrode system. The modified gold
123 electrodes (1.6 mm diameter) were used as working electrodes, Ag/AgCl (0.1 M NaCl) was
124 used as a reference electrode, and platinum wire was used as an auxiliary electrode.

125 The cyclic voltammetry measurements were conducted in the solution consisting of
126 the equimolar amounts of 1 mM $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$ dissolved in 0.1 M PBS, pH
127 7.0. Before each measurement, the solution was purged with nitrogen to remove oxygen. All

128 cyclic voltammograms were recorded in the potential range of - 0.6 V to 0.8 V with the scan
129 rate of 100 mV/s.

130 The electrochemical impedance spectroscopy analyses were performed to evaluate
131 BMS-8/Au sensor efficiency and selectivity. All measurements were performed at room
132 temperature in the same conditions and solutions as in CV measurements using Nova 1.1
133 software. The analysis was carried out using Frequency Response Analyzer (FRA)
134 implemented in MultiAutolab M204 potentiostat. The measurements were carried out in the
135 potentiostatic mode at formal potential. The perturbation amplitude was 10 mV. The studied
136 frequency range was set between 10 kHz and 0.1 Hz in the descending order. The EIS data
137 were analyzed using dedicated software with NelderMead algorithm developed in LabView
138 environment [31].

139

140 *2.3. X-Ray Photoelectron Spectroscopy (XPS) measurements*

141 X-Ray Photoelectron Spectroscopy (XPS) analysis was carried out using Escalab
142 250Xi spectroscope (ThermoFisher Scientific, United Kingdom). The spectroscope was
143 equipped with Al K α monochromatic X-Ray source, 250 μ m spot diameter. The applied pass
144 energy was 15 eV. Charge compensation was controlled through low-energy Ar⁺ ions
145 emission by means of a flood gun, with the final calibration made with reference to the gold
146 substrate (BE +84.0 eV) [32]. Deconvolution procedure was performed using Avantage
147 software provided by the manufacturer. XPS analysis were performed using gold on glass
148 substrates (11 mm \times 11 mm) (Arrandee, Werther, Germany) modified in the same way as
149 electrodes for electrochemical measurements.

150

151 *2.4. Contact angle and surface free energy (SFE) measurements*



152 The contact angle and surface free energy were measured using Drop Shape Analyzer
153 — DSA100 by Krüss. The contact angles of drops of four different liquids (water, formamide,
154 glycerol, and diiodomethane) were measured to determine the surface free energy. The image
155 of a 4 μL drop of the probe liquid deposited using a syringe was captured by a CCD camera
156 connected to a graphics card. The measurements were repeated 20 times. After the digital
157 image analysis, the average contact angle was deduced using the Young-Laplace method from
158 the angles measured at both sides of the drop in equilibrium. The total surface free energy γ_s
159 and its dispersive γ_d and polar γ_p components of the surfaces were determined by the Owens,
160 Wendt, Rabel, and Kaelble (OWRK) method from the contact angles of the four liquid drops.
161 In addition, the polar components were expressed as their acid γ^+ and basic γ^- components by
162 the van Oss and Good method; γ^+ and γ^- reflect the donor and acceptor characters
163 of the surface [33–36].

164 *2.5. Modification of bare Au electrode by cysteamine and BMS-8*

165 The bare gold electrodes before each modification by cysteamine were polished with
166 1 μm and then with 0.05 μm alumina slurry. Afterward, the electrodes were rinsed twice with
167 distilled water and then with 0.01 M PBS, pH 7.0, and dried in a stream of nitrogen. All
168 electrodes before modification were electrochemically tested by CV and EIS measurements.

169 In the first step of modification, the electrodes were covered by formation of
170 self-assembled monolayer (SAM) of cysteamine at the electrode surface. The gold electrodes
171 were immersed in 5 mL of 0.018 M cysteamine solution dissolved in 99.8 % of ethanol for
172 12 h at 4 °C. Subsequently, the gold electrodes were thoroughly rinsed with ethanol, then by
173 0.01 M PBS, pH 7.0, and water to remove the residual amount of cysteamine. Subsequently,
174 the electrodes after drying in the stream of nitrogen were used for the modification with
175 BMS-8.



176 The procedure for the modification of the gold electrodes with BMS-8 consisted of
177 two steps. In the first step BMS-8 was dissolved in 2 mL of DMSO to obtain 5 mM solution.
178 The obtained solution was then added to the 2 mL vessel of the previously prepared mixture
179 of 0.1 M of EDC, 0.05 M of NHS and 100 μ M of trimethylamine in DMSO. Secondly, after 1
180 h the gold electrodes previously modified with cysteamine were placed in the
181 EDC/NHS/BMS-8 mixture for 16 h at room temperature. Described procedure of BMS-8
182 immobilization is characterized by very high reproducibility on gold electrodes as well as on
183 various gold substrates.

184

185 *2.6. Preparation of the modified Au electrodes for the electrochemical detection of PD-L1* 186 *protein*

187 The modified gold electrodes after incubation in BMS-8 solution were rinsed
188 thoroughly with 0.01 M PBS, pH 7.0 and deionized water. Then, the electrodes were dried in
189 a stream of nitrogen. In order to investigate the influence of blocking the nonspecific binding
190 sites occurring on the surface we tested two approaches, the electrodes were incubated in
191 10 μ L of 1 % BSA solution in 0.01 M PBS, pH 7.0, for 30 min and the step of incubation in 1
192 % BSA was omitted. In the case of measurements without using BSA electrode were
193 incubated in solution containing 2 mM 1-hexanethiol for 30 min. Then, the electrodes were
194 incubated in various concentrations of PD-L1 protein - in 10^{-18} to 10^{-8} M concentration range
195 and in 10^{-14} M in the case of EIS and CV measurements, respectively. The deposition process
196 was performed by dropping 10 μ L of protein in 0.01 M PBS, pH 7.0 onto the electrode
197 surface and incubation for 1 h. Modified electrodes were rinsed with deionized water and 0.01
198 M PBS, pH 7.0 before each measurement. Additionally, the test of influence of 0.01 M PBS,
199 pH 7.0 (incubation for 1h) on the electrode was performed.



200 3. Results and discussion

201

202 3.1. Preparation of the electrode sensitive towards PD-L1 protein

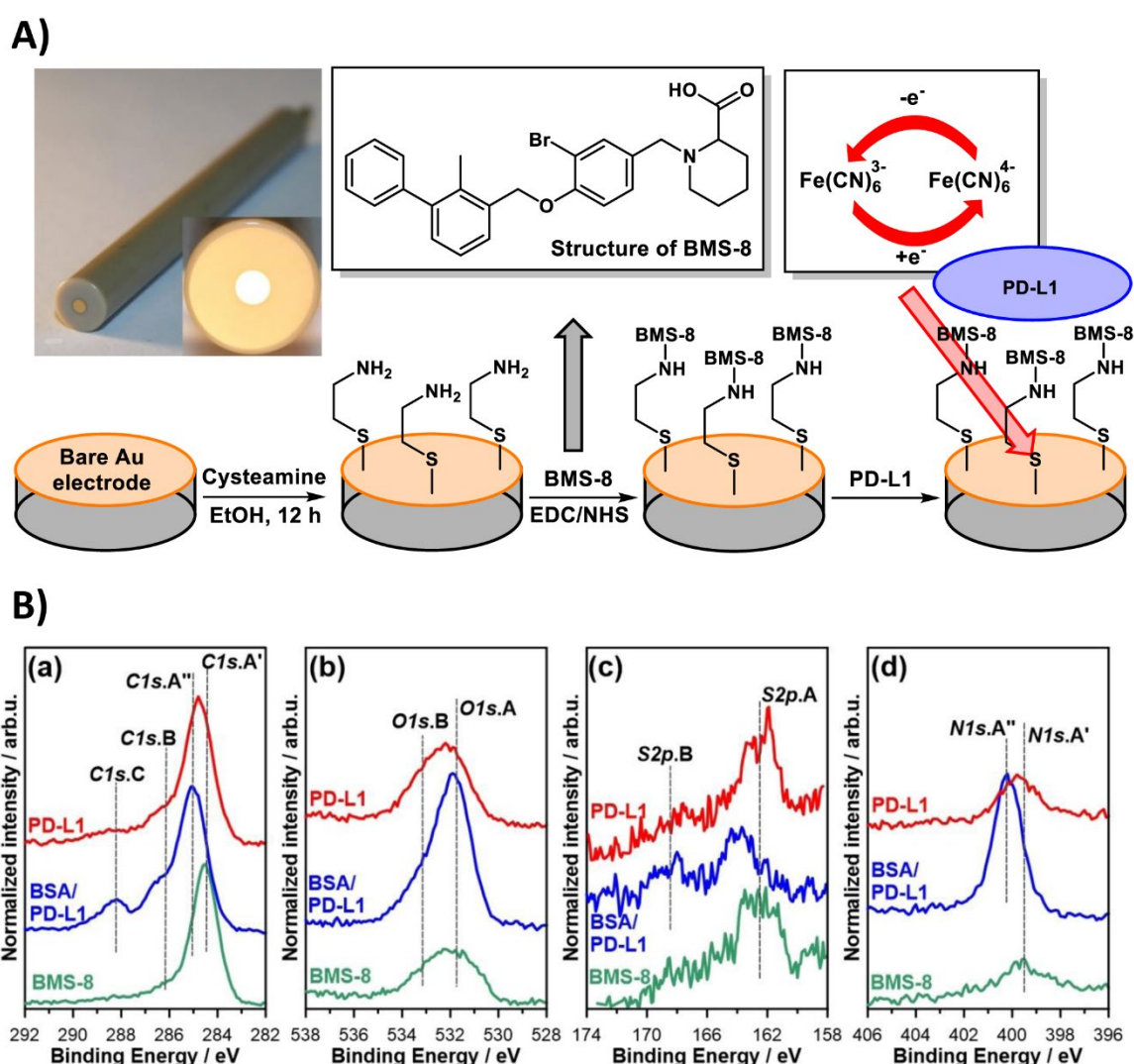
203 The detection of PD-L1 protein was conducted by anchoring of BMS-8 onto the gold
204 electrode surface, previously modified with cysteamine. The modification procedure of the
205 first monomolecular layer used in this work was described previously [37]. This procedure
206 was modified by change of the solvent from water to ethanol. The modification of the gold
207 electrodes was performed in 18 mM solution of cysteamine in 99.8 % ethanol during 12 h.
208 Many authors have performed the incubation of electrodes in aqueous solution during 4 or
209 more hours using various concentrations of cysteamine in aqueous solution [38–43]. There are
210 also some reports of the gold electrode modification in the ethanolic solution [44,45]. The
211 previous work proved that there are no significant differences in the formation of monolayer
212 for the cysteamine dissolved in water or ethanol [46]. The modification of electrodes with
213 BMS-8 was performed in the anhydrous conditions using DMSO due to the better solubility
214 of BMS-8 in this solvent. The chemical reaction was carried out by prior activation of BMS-8
215 carboxylic group performed in the mixture of EDC/NHS [47].

216 The carboxylic group of BMS-8 forms an amide bond with the amine group of
217 cysteamine anchored on the electrode. It is worth noting that the carboxylic group of BMS-8
218 is not essential for the interaction of the compound with PD-L1 protein [30]. Therefore, BMS-
219 8 anchored onto the electrode surface by amide bond maintains its activity.

220 During performed experiments the non-specific binding spots occurring on the surface
221 of the electrode were not blocked by BSA, however the influence of 1% BSA in 0.01 M PBS,
222 pH 7.4 on the electrode behavior after modification was also tested. Electrodes obtained in
223 this procedure were subsequently used to examine various concentrations (in the range of 10^{-18}
224 10^{-18} to 10^{-8} M) of PD-L1 protein. Each step of electrode preparation and chemical structure of



225 BMS-8 is shown in Figure 1 A. The detection of PD-L1 protein concentration is based on the
 226 modulated charge transfer kinetics, in presence of the redox species, after PD-L1 is anchored
 227 on the functionalized electrode surface. All steps of the modification and detection of
 228 examined proteins were characterized by changes in EIS and CV measurements. The same
 229 procedure as described above was performed for the study of interaction of obtained
 230 electrodes with PD-1 protein in the range of concentration from 10^{-18} to 10^{-8} M and BTLA
 231 and CD 160 in the concentration of 10^{-8} M.



232 Fig 1. A) The picture of gold electrode and the procedure of its modification by cysteamine
 233 and BMS-8. B) High-resolution XPS spectra obtained in the binding energy range of: (a) *C1s*,
 234 (b) *O1s*, (c) *S2p*, and (d) *N1s* photopeaks.
 236

3.2. XPS measurements during each step of gold electrode modification process

Figure 1B presents the results of the high-resolution XPS analysis conducted on the surface of functionalized Au electrodes in the energy range of *C1s*, *O1s*, *S2p*, and *N1s* peaks. The analysis was also carried for *Au4f* peak doublet, which served as a reference for the peak calibration and the indicator of the acquired functionalization thickness.

The primary component reported in *C1s* spectrum (Fig. 2a) — *C1s.A* — is located at 284.6 ± 0.1 eV for BMS-8 and PD-L1 samples but exhibits even more positive shift towards 285.1 eV for BSA+PD-L1 samples. The peaks located at this binding energy range are typically attributed to various aliphatic hydrocarbon species but can also originate from adventitious carbon contamination due to air exposure. The second notable component — *C1s.B* — is shifted versus the primary *C1s* component by approx. +1.6 eV and originates from C-O and C-N bonds found in hydroxyls, esters, amines, and others. The last component *C1s.C* was observed at approx. 288.2 eV in an energy range most often associated with carboxyl functional groups. The contribution of the last component is distinctly more prominent for BSA/PD-L1 samples, where its share in total carbon content is approx. 25% versus 15% for PD-L1 samples and 7% for BMS-8. The details of the peak decomposition are summarized in Table 1. The applied model is consistent with numerous literature reports [48–52].

Table 1. Results of high-resolution XPS analysis and peak deconvolution

	<i>C1s</i>			<i>O1s</i>		<i>S2p</i>		<i>N1s</i>	<i>Au4f</i>
	<i>C1s.A</i>	<i>C1s.B</i>	<i>C1s.C</i>	<i>O1s.A</i>	<i>O1s.B</i>	<i>S2p_{3/2}.A</i>	<i>S2p_{1/2}.B</i>	<i>N1s.A</i>	--
BE / eV	284.6*	286.2	288.2	531.6	532.8	164.5	167.7	399.7**	84.0
BMS-8	41.2	7.6	3.5	3.5	3.7	2.8	1.1	3.5	33.1
BSA/PD-L1	40.2	11.1	11.3	7.0	7.3	1.8	1.0	9.1	11.2
PD-L1	36.7	10.1	5.7	4.0	5.9	2.6	0.9	4.5	29.6

* *C1s.A* peak was equal 285.0 eV for both BSA/PD-L1 samples.

** *N1s.A* peak was equal 400.2 eV for both BSA/PD-L1 samples.

260 The presence of carbon-oxygen bonds, which are characteristic for organic
261 compounds, was further confirmed based on *O1s* peak analysis (Fig. 2b) where the presence
262 of C-O/OH and C=O bonds is reflected in the photopeaks located at *O1s.A* = 531.6 eV and
263 *O1s.B* = 532.8 eV, respectively. The lowest amount of oxygen in the BMS-8 sample
264 corresponds to the smallest share of *C1s.B* and *C1s.C* peaks during *C1s* spectra analysis
265 [53,54]. XPS analysis carried out in the binding energy range of *N1s* photopeak (*N1s.A*)
266 resulted in the observation of the significant differences between BSA/PD-L1 and the
267 remaining samples. The *N1s* peak position is shifted by +0.5 eV with respect to BMS-8 and
268 both PD-L1 samples. The position of this peak is most commonly attributed to N-H and N-C
269 bonds in amines [55,56]. The energy shift most likely originates from the different number of
270 carbon atoms adjacent to nitrogen. Indeed, the amount of nitrogen in BSA/PD-L1 samples
271 was over twice higher than in PD-L1 samples. Higher nitrogen content might also be the
272 reason of the *C1s.A* component energy shift (*C1s.A'* and *C1s.A''*). Each analyzed sample
273 contained between 2.5 and 4 at.% of sulfur present in two chemical states. The primary state
274 marked as *S2p.A* is located in the binding energy characteristic for thiols and other organic
275 forms of sulfur, while the second component (*S2p.B*) was significantly smaller and shifted
276 towards BE range typical for sulfates. The XPS analyses allow to bring a conclusion
277 regarding successful electrode surface functionalization with studied proteins.

278 Finally, the XPS analysis also provides coarse information about the electrode
279 functionalization thickness. The photoelectrons are emitted only from approximately 5-10 nm
280 depth underneath the interface. Two conclusions can thus be drawn. First, the
281 functionalization thickness did not exceed 10 nm in any case, a conclusion drawn based on
282 the presence of *Au4f* peak doublet for metallic gold in the analyzed surface chemical states.
283 Second, the functionalization is thinner in the case of BSA/PD-L1 samples.

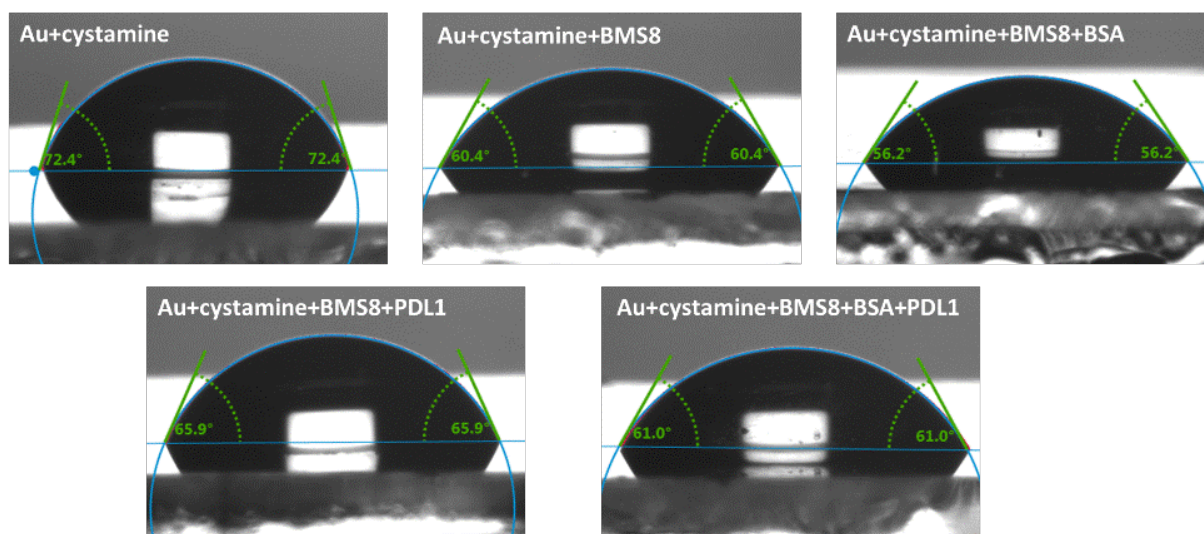
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285 3.3. Surface wettability measurements

286 In order to assess the hydrophobic and hydrophilic character of the modified surface at
287 the different steps of the modification process of the gold electrodes, contact angles of water
288 drops deposited on the surfaces were measured (Fig 2, Fig. 3a). For gold electrodes the water
289 contact angle decreased after modification with cysteamine for about 10°. Further
290 modification with BMS-8 led to a decrease in the contact angle for about 12°. The decrease of
291 contact angle on the modified Au electrodes revealed an increase of hydrophilic character of
292 the surface because of the functional groups present in the modified layer. Deposition of BSA
293 on the modified layer leads to another decrease in the contact angle for about 5°. In both cases
294 (with or without the BSA) after exposure to the PD-L1 protein an increase in the contact angle
295 is observed, hence the hydrophobicity of the layer increases (Fig 2, Table S2).

296

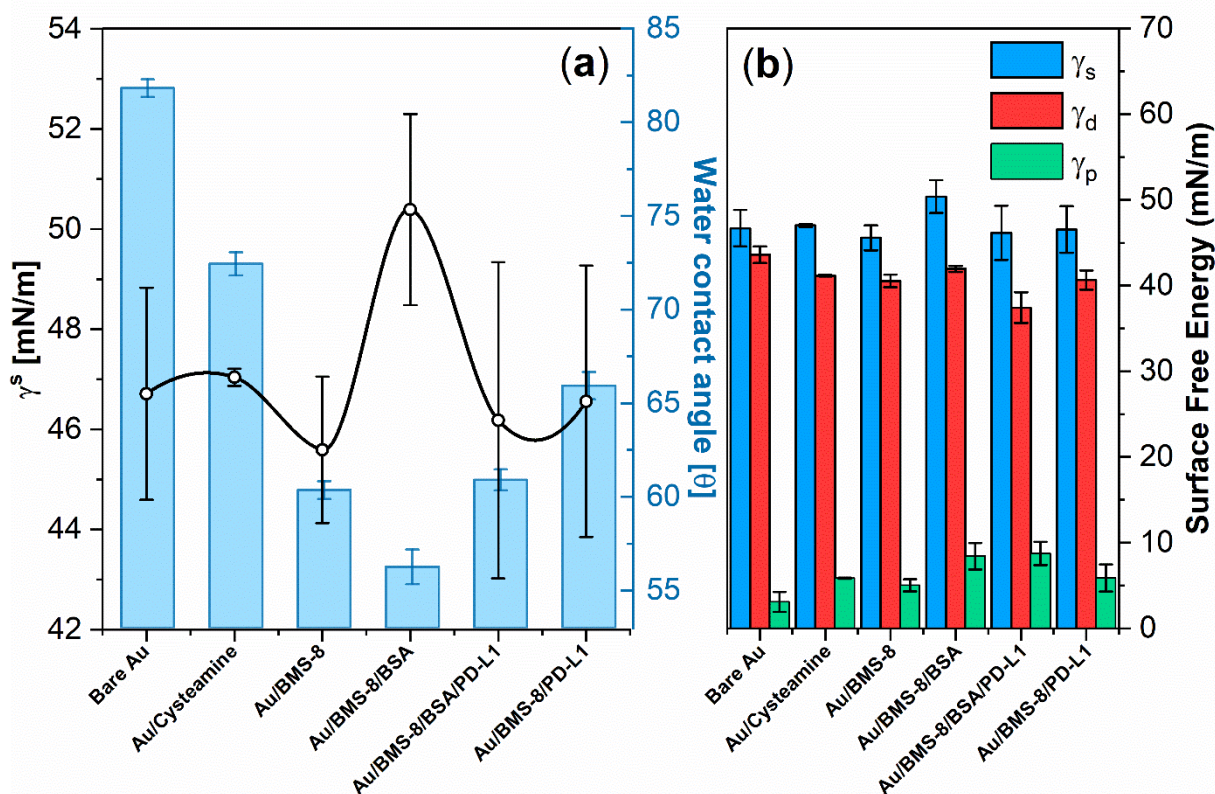


297
298 **Fig. 2.** Photos of the water contact angle measurements for each step of gold electrode
299 modification for PD-L1 sensing.

300
301 Considering the changes in SFE energy in relation to the subsequent stages of
302 modification, the total free energy does not change significantly as to the value, while the
303 changes in the chemical structure of the layer also change the SFE value (Fig. 3b). The total

304 SFE and its polar component increased as a result of the incorporation of more functional
305 polar groups due to the modification process. Changes in the free energy of the surface are
306 mainly the effect of the change of the polar component, while the dispersion part remains
307 essentially unchanged.

308 Already the first stage of modification with cysteamine causes the increase of the
309 acid-base component from 3.09 mN/m to 5.86 mN/m. Furthermore, the acidic and basic
310 elements of the polar component undergo a complete change. In the case of an unmodified
311 electrode, the acid component has a much higher share. The modification process not only
312 lowers their values but also reverses the proportions — the basic component is now dominant.
313 This is probably consistent with the presence of amino groups exhibiting basic properties on
314 the electrode surface. The largest increase in the basic component is observed in the case of
315 BMS-8/BSA and BMS-8/BSA/PD-L1 samples. This observation is consistent with the results
316 obtained from the XPS measurements which indicated these samples as containing the most
317 nitrogen atoms in the form of different types of amino groups.



318

319 Fig. 3.a) Water contact angle and b) SFE energy γ_s diagram with uncertainties for each step of
 320 the modification of the gold electrode for PD-L1 sensing.

321

322 This variability affects the hydrophilicity of the surface and thus the observed contact angle.

323 The most hydrophilic surfaces are observed for BMS-8/BSA and BMS-8/BSA/PD-L1

324 samples. Their water contact angle (WCA) decreased from 81.82 for unmodified gold

325 electrode to 56.26 and 60.90 for BMS-8/BSA and BMS-8/BSA/PD-L1, respectively.

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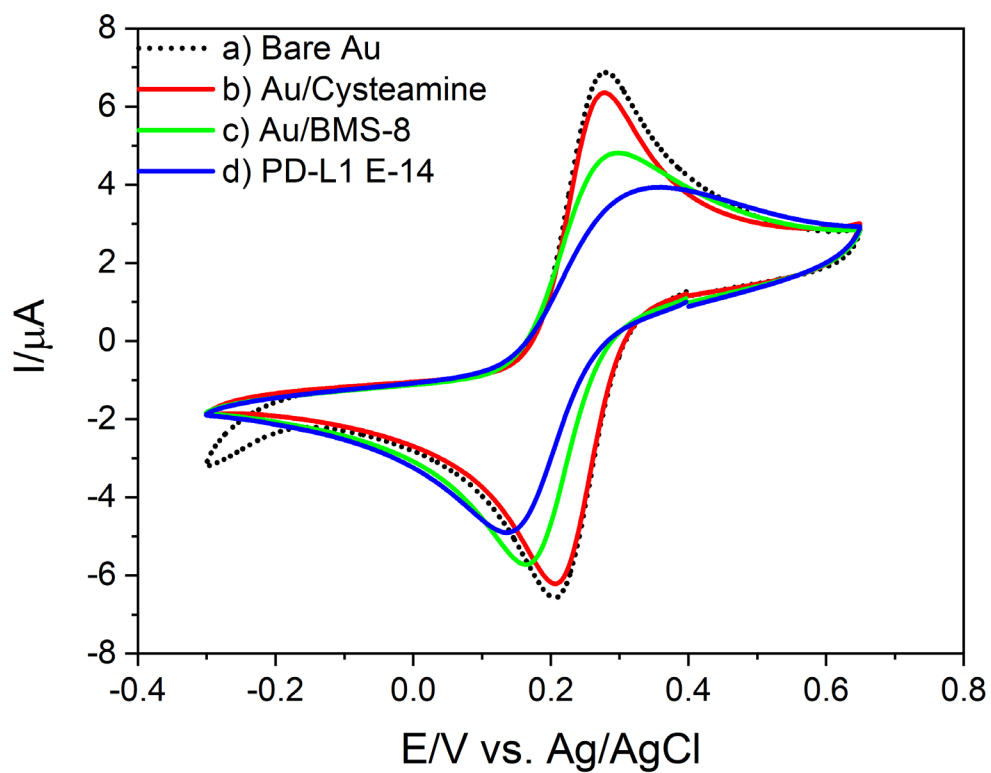
327 3.4. Electrochemical measurements

328 The cyclic voltammetry measurements were performed during each step of electrodes
329 modification in 0.1 M of PBS, pH 7.0 containing 1 mM $\text{Fe}[(\text{CN})_6]^{3-/4-}$. The results of CV
330 measurements show two reversible peaks for bare Au electrodes, where the ratio of anodic
331 peak to cathodic peak current i_A/i_C is close to 1 with the peak-to-peak separation (ΔE) of 67
332 mV (Fig. 4. black dotted line). After modification by cysteamine, the peak-to-peak separation
333 ΔE decreased to 64 mV and is similar to those calculated for bare Au electrodes. Therefore, a
334 conclusion may be drawn that cysteamine modification of Au electrodes does not influence
335 significantly its electrochemical behavior, which was also confirmed in our measurements by
336 EIS technique. The voltammogram is shifted towards more negative potential after
337 modification, a feature observed before [44].

338 The modification with BMS-8 caused the peak currents to decrease and the increase of
339 peak-to-peak separation to 119 mV. In the next step, such modified electrode was incubated
340 with PD-L1 protein in a concentration of 10^{-14} M. The changes of obtained voltammograms
341 are significant. The peak current values decreased, and the peak-to-peak separation increased,
342 ΔE to 180 mV (Fig. 4), as an effect of PD-L1 anchoring at the modified electrode surface and
343 affecting the charge transfer kinetics by the redox species. This particular behavior of the
344 modified electrodes is probably the consequence of two competitive factors: partial blockage
345 of active sites at the electrode and electrostatic interactions between PD-L1 protein and
346 negatively charged ions $\text{Fe}[(\text{CN})_6]^{3-/4-}$ present in the examined solution [55,56].

347 The experiment was performed using gold electrode modified by cysteamine with
348 BMS-8 without incubation with 1% BSA in 0.01 M PBS due to the small changes caused by
349 BSA observed in electrode response in previous experiments (see Fig. S1). Above experiment
350 directly confirm that gold electrode modified by BMS-8 is highly sensitive to PD-L1 protein
351 present in solution.





352

353 **Fig. 4.** Cyclic voltammograms of the redox reaction of 1 mM $\text{Fe}[(\text{CN})_6]^{3-/4-}$ in 0.1 M PBS, pH
 354 7.0 solution obtained at a) Bare Au b) Au/Cysteamine c) Au/BMS-8 d) Au/BMS-8/PD-L1
 355 electrode, scan rate 100 mV/s.

356 Next, the electrochemical impedance spectroscopy measurements have been
357 performed to determine the capability of the obtained electrodes for detecting PD-L1 at very
358 low concentrations. Additionally, the electrodes modified with BMS-8 were used as a control
359 test during PD-1 detection. The impedance approach offers significantly higher sensitivity for
360 the determination of electrode kinetics changes in comparison with CV measurements.
361 Therefore, it was selected for the evaluation of both PD-L1 and PD-1 concentrations in the
362 same range of 10^{-18} to 10^{-8} M. Figure 4 presents the EIS impedance spectra for the bare Au
363 electrodes in 0.1 M PBS pH 7.0 solution containing 1 mM $\text{Fe}[(\text{CN})_6]^{3-/4-}$, after consecutive
364 surface modification steps and after incubation in solution containing PD-L1 protein in
365 concentration range of 10^{-8} to 10^{-18} .

366 The shape of the impedance spectra is characterized by the semicircle at the high-to-
367 moderate frequency range and very distinctive feature in the form of solid line inclined at 45°
368 at low-frequency range. The discussed feature should be associated with the diffusion-related
369 impedance and testifies for the co-occurrence of the diffusion control in the charge-transfer
370 process. On the other hand, it is clearly visible in the inset of Fig. 5 that the next electrode
371 modification steps influence the electrode's charge transfer resistance as observed through the
372 increase of the high-frequency semicircle.

373 The electric equivalent circuit (EEC) was selected based on the obtained impedance
374 studies. The EEC with abbreviated notation $R(Q(RW))$ is composed of R - solution resistance
375 and a parallel connection of the constant phase element (CPE), imitating the heterogeneities at
376 the electrode surface and charge transfer resistance R_{CT} with Warburg diffusion resistance W
377 (Fig. 5). The impedance of the CPE is given with eq. (1)

$$378 \quad Z_{CPE} = \frac{1}{Q(j\omega)^\alpha} \quad (1)$$

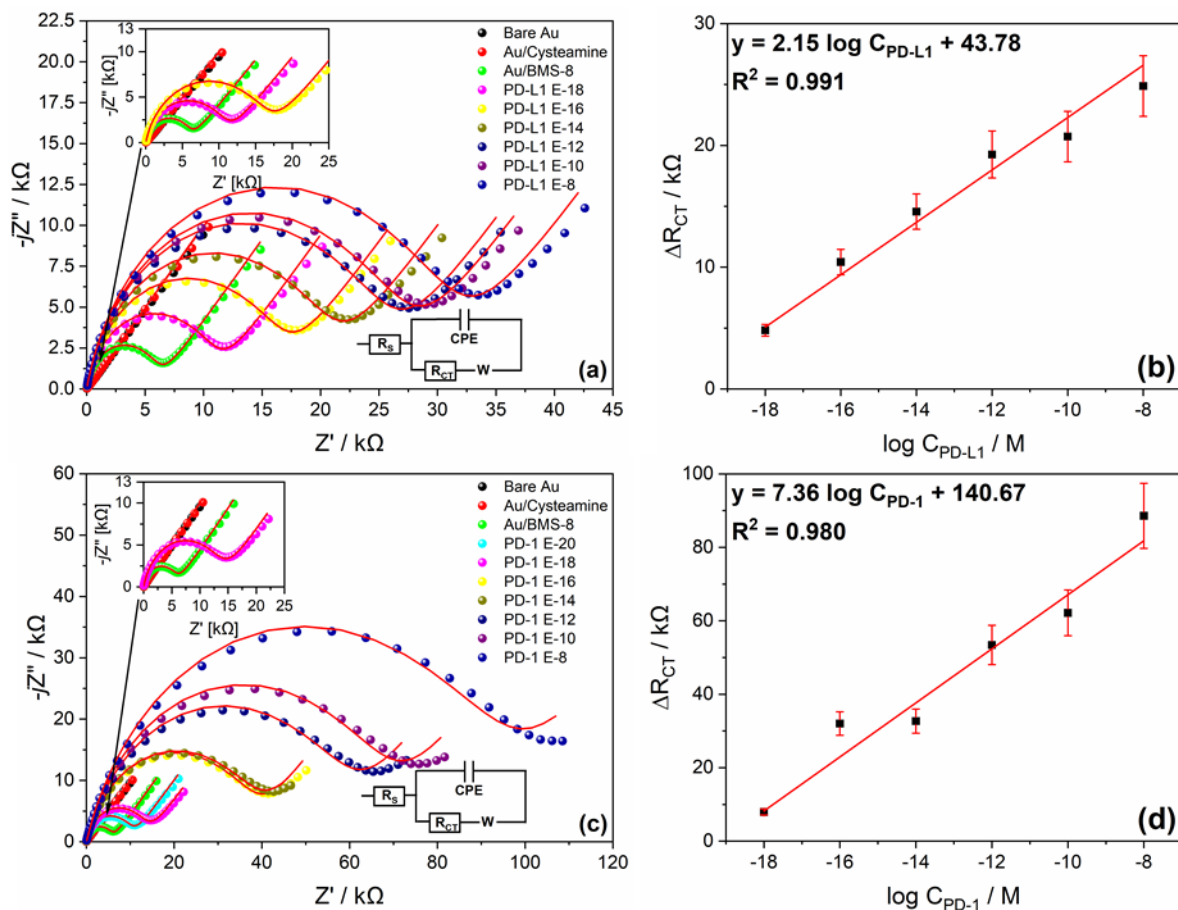
379 where Q is the quasi-capacitance in the presence of frequency dispersion of capacitance, CPE
380 exponent α is the heterogeneity factor, j is the imaginary number and ω is the angular

381 frequency [57–62]. CPE exponent α is often considered to be the surface heterogeneity factor
382 ($0 < \alpha < 1$). The closer to unity α approaches, the more closely the CPE resembles a pure
383 double-layer capacitance, and if α approaches 0, the CPE behaves more like a resistor. The
384 above-defined heterogeneity may be introduced by numerous features, including non-uniform
385 site-specific charge transfer kinetics due to electrode polycrystallinity, 2D adsorption of
386 macromolecules or contaminants, and resultant interspace regions but also electrode material
387 geometry and porosity [63–69]. The utilization of such electric equivalent circuit is explained
388 due to the large molecular mass of examined proteins and is widely used for the gold
389 electrode modified by cysteamine and other organic molecules [70–74]. The EEC allowed
390 obtaining a very good fit as represented by χ^2 distribution about 10^{-4} . The more detailed
391 analyses of are summarized in Table S3 in Supporting Information file.

392 The impedance spectra for both: bare Au electrode and Au after modification with
393 cysteamine reveal nearly straight line at 45° (Fig. 5a inset), a feature characteristic for a mass
394 diffusion limiting the electron transfer process. The electrode modification through a chemical
395 reaction between electrode-terminating amine functional groups with the carboxyls within
396 BMS-8 molecule caused the appearance of a distinctive capacitive semicircle on the
397 impedance spectra, indicating the formation of the adsorbed layer, which influence the
398 interfacial electron transfer.

399 The BMS-8 surface functionalization process occurs with different efficiency for
400 various electrodes, differing in BMS-8 anchoring density, resulting in differences in layer
401 thickness, and subtle Au pretreatment conditions, etc. These features have a non-negligible
402 influence on the kinetics of the charge transfer through the electrode interface. Therefore, in
403 order to efficiently verify the effect of anchoring the PD-L1 molecule on the modified
404 electrode surface, it is essential to perform the experiment on a single electrode, to be able to
405 observe the relative changes of R_{CT} parameter. The relative change of the charge transfer

406 resistance was calculated according to the equation: $\Delta R_{CT} = R_{CT(PD-L1)} - R_{CT(BMS-8)}$, where
 407 $R_{CT(BMS-8)}$ is a value of R_{CT} of electrode modified by BMS-8 and $R_{CT(PD-L1)}$ is R_{CT} after
 408 incubation in different concentration of PD-L1. The obtained results are shown in Fig. 5b.



409
 410 **Fig. 5.** The EIS impedance spectra of BMS-8 functionalized electrode in the absence and the
 411 presence of a) PD-L1 and c) PD-1 protein in 0.01 M PBS, pH 7.0 at various concentrations
 412 ratio from 10^{-18} M to 10^{-8} M. In the inset enlarged the comparison of obtained spectra for a)
 413 PD-L1 and c) PD-1 concentration of 10^{-18} M. Points represent experimental results while solid
 414 red line represent data calculated using EEC.
 415 The calibration curve for the ΔR_{CT} changes resulting from b) PD-L1 and d) PD-1 protein
 416 exposure of the electrode as a function of logarithmic concentration of PD-L1.
 417

418 It is clearly visible that tested BMS-8 deposited at the Au electrode surface is highly
 419 sensitive, differentiating impedance characteristics of the electrode even at the lowest studied
 420 concentration of PD-L1. These results indicate that studied protein binds to the BMS-8
 421 molecules, anchored on the Au electrode. The increasing protein concentration causes
 422 inhibition of the charge transfer process, resulting in R_{CT} increase. The observed behavior

423 corresponds with the formation of the functionalized organic layer affecting the charge
424 transfer kinetics. The EIS studies revealed that the R_{CT} of the above-functionalized electrode
425 increases over 2.5 times in the presence of PD-L1 protein in the vicinity in the concentration
426 of 10^{-18} M to 10^{-8} respectively (Table S4).

427 Performing the above-described R_{CT} normalization allowed us to form the calibration
428 curve, thus offering the detection tool of ultra-small PD-L1 concentrations on the modified
429 Au surface, based on electrochemical impedance measurements. The electrochemical
430 response of the BMS-8 modified gold electrode was linear in the entire range of the PD-L1
431 from 10^{-8} M to 10^{-18} M. The calculated regression equation was: $\Delta R_{CT} = 2.15 \log C[\text{PD-L1}] +$
432 43.78 with the correlation coefficient of 0.991. The detection limit (LOD) was estimated to be
433 1.87×10^{-14} M and 2.93×10^{-14} M for PD-L1 and PD-1 (S/N=3.3) respectively, while the limit
434 of quantification (LOQ) was calculated to be $5,67 \times 10^{-14}$ M for PD-L1 and $8,87 \times 10^{-14}$ M for
435 PD-L1.

436

437 ***3.6. Specificity and selectivity of the impedance PD-L1 assay***

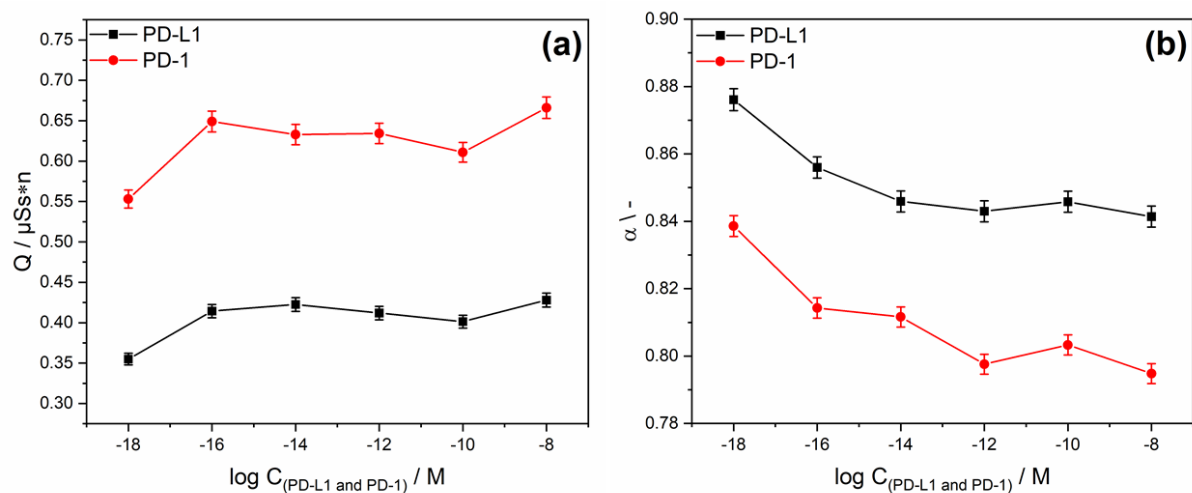
438 In order to examine the selectivity of the above-presented approach, the same
439 experiment was performed towards the detection of the PD-1 protein. Obtained results
440 confirm that the presence of PD-1 protein in the analyte has a visible effect on the charge
441 transfer, increasing R_{CT} (Fig. 5c). The results of impedance analyses of PD-1 protein detection
442 are summarized in Table S3. The data plotted in Fig. 5d reveals that the ΔR_{CT} changes
443 recorded for the Au/BMS-8 were linear in the range of analyzed PD-1 concentrations, similar
444 as in the case of PD-L1 protein. The estimated regression equation was $\Delta R_{CT} = 7.36 \log$
445 $C[\text{PD-1}] + 140.67$, with the correlation coefficient equal to 0.980 (Fig. 5d). BMS-8
446 functionalized electrode response to PD-1 is unexpected given the fact that BMS-8 was shown

447 not to interact with PD-1 [28]. Likely the surface modification process itself provides anchor
448 points for PD-1.

449 The performed analyses directly indicate the linear trend of ΔR_{CT} change as a function
450 of target protein concentration for both studied proteins. However, there is also a significant
451 difference in the slope of the linear function. The charge transfer resistance through the BMS-
452 8-modified Au electrode increases over 2.5 times in the analyzed concentration range of PD-
453 L1, and over 5.0 times in the same range of PD-1 protein. Thus, a conclusion should be drawn
454 that analysis of this one parameter allows for an ultrasensitive quantitative analysis but does
455 not allow for qualitative analysis distinguishing between PD-1 and PD-L1 proteins.

456 There are, however, other parameters obtained based on the impedance analyses. An
457 important feature should be observed when analyzing the changes of the quasi-capacitive
458 parameter with the concentration change of either PD-1 or PD-L1 molecules, the results of
459 which are presented in Fig. 6.

460



461
462 **Fig. 6.** a) The relationship of a) quasi-capacitance in the presence of frequency dispersion of
463 capacitance (Q) and b) heterogeneity factor (α) as a function of logarithmic concentration of
464 PD-L1 and PD-1 proteins.

465

466 The quasi-capacitance parameter Q_{DL} increases with analyte concentration for both
467 studied proteins PD-L1 and PD-1, and this effect is strongly correlated with the decrease in
468 CPE exponent α , indicating a slight decrease in electrode homogeneity when more and more
469 target proteins are anchored on the electrode surface. This is an expected and valuable result,
470 confirming the correct selection of the EEC.

471 The values of the quasi-capacitance of the modified Au electrode exposed to the
472 studied proteins differ significantly. The heterogeneity introduced by the PD-L1 molecule is
473 significantly smaller than the heterogeneity introduced by the PD-1 molecules (higher α
474 values for PD-L1). BMS-8 interacts more specifically with PD-L1 than with PD-1. More
475 homogeneous surface distribution of the adsorption layer in case of PD-L1 may be caused by
476 formation of homodimer on the electrode surface. Inducing dimerization of PD-L1 protein
477 after interaction with BMS-8 was confirmed by X-ray [30]. On the other hand, experimental
478 data indicate that BMS-8 compound does not bind to PD-1 protein [75]. PD-1 protein also
479 interacts with the electrode, but as a result, the electrode surface is more heterogeneous. Most
480 likely, the interaction of the PD-1 with a modified electrode is probably non-specific and
481 random.

482 The higher the protein concentration the lower the electric homogeneity at the
483 electrode/electrolyte interface. However, the homogeneity level obtained in the case of
484 anchored PD-L1 proteins at the highest studied concentration 10^{-8} M is unattainable for PD-1,
485 even at the lowest studied concentrations. These differences translate into significant
486 differences between quasi-capacitance of PD-L1 and PD-1 films at the modified electrode
487 surface and demonstrate that BMS-8 strongly and specifically interacts with PD-L1 protein,
488 offering possible routes for PD-L1 assay selectivity in presence of other proteins. The
489 comprehensive impedance analysis allows to qualitatively distinguish PD-L1 and PD-1



490 proteins and provide ultrasensitive quantitative information regarding target protein
491 concentration.

492 These results are in good agreement with previous studies by the Holak and co-
493 workers, who show that BMS-8 leads to dissociation of PD-1/PD-L1 complex and induction
494 of PD-L1 protein dimerization [28,76]. The interaction of BMS-8 with PD-L1 partially
495 overlaps the hydrophobic interaction surface between PD-1 protein and its ligand, PD-L1.
496 Furthermore, the formation of the homodimer limits access of the PD-1 receptor to the
497 binding site of PD-L1 protein [75–77].

498 Besides of PD-L1 and PD-1 examination, the electrochemical response of other
499 proteins was also investigated to evaluate the electrochemical behavior of the modified
500 electrode on the selectivity of protein detection. For this purpose, we selected CD160, and
501 BTLA proteins that belong to the immunoglobulin-like proteins superfamily (IgSF), the same
502 as PD-L1 and PD-1 [78,79]. It is worth noting that the interaction studies of the BMS-8
503 molecule with the PD-1 protein have been carried out and the results have shown that BMS-8
504 binds to PD-L1 but not to PD-1 [28]. In vitro NMR measurements presents that BMS-8 is
505 capable of dissociating the PD-1/PD-L1 interaction in the stoichiometric concentration [75].
506 The same studies for CD160 and BTLA were not performed. In presented studies, the EIS
507 impedance of selected proteins in 0.01 M PBS, pH 7.0 at concentration of 10^{-8} M was
508 measured. The impedance results in the form of Nyquist plots are shown in Figure S4.

509 Presented studies show that the functionalized electrodes bind the CD160 and BTLA
510 proteins to the anchored BMS-8 but with significantly less potency than the PD-L1 and PD-1
511 proteins (Fig. S4). The results of the impedance analysis using $R(Q(RW))$ EEC are
512 summarized in Table S5.



513 3. Conclusions

514 Summarizing, this work is focused on designing new assay capable to detect cancer marker,
515 sPD-L1 protein, in low concentration using EIS and CV. It describes the development of the
516 new electrode functionalization, which is capable of PD-L1 detection in PBS solution. The
517 applied approach utilizes the reaction of BMS-8 with cysteamine anchored at gold electrode
518 surface. Performed high-resolution XPS, contact angle, and surface free energy studies
519 confirmed each successful step of the electrode modification. Cyclic voltammetry confirmed
520 the detection of PD-L1 protein in the concentration of 10^{-14} M, while the electrochemical
521 impedance spectroscopy performed at various concentrations in the range of 10^{-18} to 10^{-8} M.
522 We have proved the efficient both PD-L1 as well as PD-1 detection through change in charge
523 transfer resistance R_{CT} even at its lowest concentration of 10^{-18} M. Subsequently, it should be
524 noted that the changes in the electric parameters with PD-L1 and PD-1 concentration show a
525 linear trend, significantly enabling quantitative analysis with the low detection limit of $1.87 \times$
526 10^{-14} for PD-L1 M and 2.93×10^{-14} M for and PD-1 respectively. While offering ultrasensitive
527 protein detection, the R_{CT} analysis does not allow for selective PD-L1 or PD-1 protein
528 determination, since the assay is affected by both proteins. Likely, interaction with PD-L1 is
529 BMS-8 specific while that with PD-1 is guided by less defined surface effects at the
530 functionalized electrode. We claim that the selectivity of the proposed assay may be based on
531 quasi-capacitance parameter analysis. Smaller decrease of electrode homogeneity for PD-
532 L1/BMS-8 interaction in comparison to PD-1/BMS-8 can be explained by dimerization of
533 PD-L1 protein induced by BMS-8 which not occur in case of PD-1/BMS-8. The constant
534 phase element parameters Q and α show that it is possible to differentiate PD-L1 from PD-1
535 protein and it will be investigating further in more complex mixtures e.g. animal or human
536 serum. Our studies confirmed that immune checkpoint proteins CD160 and BTLA anchor at
537 the electrode with significantly less potency than the PD-L1.

538

539 **Acknowledgments**

540 This work was financially supported by Polish National Science Centre (NCN) under
541 Grant No. 2015/17/D/ST5/02571 (J.R.), Grant No. 2017/25/B/NZ1/00827 (G.D.) and by
542 Polpharma Scientific Foundation Grant No. 3/XVIII/2019 (S.R.-M.).

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