

**Phospholipid-functionalized gold electrode for cellular membrane interface studies -
interactions between DMPC bilayer and human cystatin C**

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21 **Abstract:** This work describes the electrochemical studies on the interactions between V57G mutant of
22 human cystatin C (hCC V57G) and membrane bilayer immobilized on the surface of a gold electrode. The
23 electrode was modified with 6-mercaptohexan-1-ol (MCH) and 1,2-dimyristoyl-sn-glycero-3-
24 phosphocholine (DMPC). DMPC was used as a membrane mimetic for monitoring electrochemical changes
25 resulting from the interactions between the functionalized electrode surface and human cystatin C. The
26 interactions between the modified electrode and hCC V57G were investigated by cyclic voltammetry and
27 electrochemical impedance spectroscopy in a phosphate buffered saline (PBS) containing $\text{Fe}(\text{CN})_6^{3-/4-}$ as a
28 redox probe. The electrochemical measurements confirm that fabricated electrode is sensitive to hCC V57G
29 at the concentration of 1×10^{-14} M. The incubation studies carried out at higher concentrations resulted in
30 insignificant changes observed in cyclic voltammetry and electrochemical impedance spectroscopy
31 measurements. The calculated values of surface coverage θ_R confirm that the electrode is equally covered
32 at higher concentrations of hCC V57G. Measurements of wettability and surface free energy made it
33 possible to determine the influence of individual structural elements of the modified gold electrode on its
34 properties, and thus allowed to understand the nature of the interactions. Contact angle values confirmed
35 the results obtained during electrochemical measurements, indicating the sensitivity of the electrode
36 towards hCC V57G at the concentration of 1×10^{-14} M. In addition, the XPS spectra confirmed the
37 successful anchoring of hCC V57G to the DMPC-functionalized surface.

38

39 **Keywords:** human cystatin C, DMPC, phospholipid, membrane, 6-Mercaptohexan-1-ol, electrode
40 modification

41 **1. Introduction**

42 An amyloid is an insoluble, aggregated form of a protein or peptide, which assumes a fiber-like
43 shape [1]. The process of accumulation and deposition of amyloid is a hallmark of amyloid diseases, a
44 group of pathological states exhibiting various symptoms, e.g., Parkinson's or Alzheimer's disease. Up to
45 date about forty amyloid-forming peptides and proteins were described [2]. Among them we find human
46 cystatin C (hCC), a small size (120 amino acid-long) inhibitor of cysteine proteinases [3]. This protein with
47 physiologic isoelectric point of pH 9.3 [4] can be found in all human body fluids at physiologically relevant
48 concentrations [5]. Despite the physiological relevance of the wild-type hCC as a regulator of the activity
49 of inter- and intramolecular cysteine proteases, its Leu⁶⁸→Gln mutant is prone for accumulation, causing a
50 dominant hereditary disorder called hereditary cystatin C amyloid angiopathy, a disease characterized by
51 brain strokes and death of patients at a young age [6].

52 Even though the processes leading to amyloidogenic diseases are not crystal clear, it is known that
53 protein oligomerization is a key feature of the amyloid formation. Research indicates that biological
54 membranes have a great impact on the process of amyloidogenic proteins' oligomerization [7–9]. Up to
55 date two possible mechanisms of protein oligomerization were proposed [10]. The first one assumes that
56 the protein oligomerizes in extracellular matrix. The second, that the membrane is the interface which
57 facilitates and accelerates the whole process. Both ideas involve different oligomeric states of a protein and
58 finally lead to the formation of fibrils [10]. An annular oligomer is one of the oligomeric states on the route
59 to the fibril formation. It may form channels in biological membranes disturbing their integrity, therefore it
60 is indicated as a potential cause of toxicity of amyloidogenic proteins [10]. The formation of annular
61 oligomers was observed for different amyloidogenic proteins *i.e.* amyloid β peptide [11], immunoglobulin
62 light chain [12] and human cystatin C [13]. The interaction of toxic forms of hCC (the protein of our
63 interest) with biological membranes is an interesting aspect of studies on amyloidogenic diseases and may
64 be crucial in the context of describing the mechanisms causing them.

65 The studies on interactions between proteins and membranes are not a trivial task. Biological
66 membranes are complex multi-component structures, therefore to facilitate experimental measurements
67 natural membranes are often substituted with model structures including micelles, bicelles and liposomes
68 [14,15]. Different methods involving the use of natural and model membranes have been developed. Among
69 others we find surface plasmon resonance (SPR) [16], isothermal titration calorimetry (ITC) [17], nuclear
70 magnetic resonance (NMR) spectroscopy [15], Fourier transform infrared spectroscopy (FTIR) [18],

71 hydrogen-deuterium exchange (HDX) mass spectrometry [19] or molecular dynamics (MD) simulations
72 [20]. These techniques include also such interesting concepts as monitoring of resonance frequency and
73 energy dissipation on lipid covered quartz crystal (microbalance) resulting from a contact/interaction with
74 a protein [21] or the use of anisotropy of nuclear interactions (solid-state NMR) to determine protein
75 structure within solid or semi-solid lipid membrane structures [22]. Some of the methods applied for
76 protein-membrane interactions present such a broad range of application or particular design, that they can
77 be applied for studies on soluble proteins as well as protein aggregates and fibrils (*i.e.*, amyloid fibrils),
78 which often prove to be tricky study objects due to their low solubility. These techniques comprise *i.a.* total
79 internal reflection ellipsometry (TIRE) [23], Förster resonance energy transfer (FRET) [24], fluorescence
80 imaging [25], quartz crystal microbalance applications [26] or MD simulations [27]. The technique which
81 seems to be exploited the most in the field of protein-membrane interactions, regardless of the oligomeric
82 state of the studied molecule, is atomic force microscopy (AFM). It allowed, *e.g.*, to visualize aggregation
83 of α -synuclein on a surface of phospholipid bilayers and (combined with computational modeling) to
84 present a possible model of aggregation of this amyloidogenic protein [28]. AFM imaging was also applied
85 to visualize the formation of annular oligomers (amyloid fibril precursors) forming transmembrane
86 channels [29].

87 All the mentioned techniques shine bright in some aspects of application and falter in others. One
88 of the greatest disadvantages of many mentioned above (*e.g.*, NMR, ITC, SPR) is their high cost, resulting
89 from relatively high amount of protein/membrane material required for the measurement and/or high cost
90 of the measurement itself. Therefore, here the application of electrochemical impedance spectroscopy (EIS)
91 and cyclic voltammetry (CV) as methods for studies on protein-membrane interactions based on V57G
92 mutant of hCC and the DMPC membrane mimetic is presented.

93 The hCC protein was previously detected with electrochemical methods including cyclic
94 voltammetry (CV) and differential pulse voltammetry (DPV) on screen printed electrode modified with
95 papain (cysteine protease) [30] or carbon electrode functionalized with graphene oxide-chitosan (GO-Chit)
96 and with anti-cystatin C antibody [31]. The hCC was also detected with CV and DPV methods on glassy
97 carbon electrode covered with aminoferrocene (Fc), graphene oxide (GO) polyethyleneimine (PEI) film
98 and anti-cystatin C antibody, without the use of redox probes during electrochemical measurements [32].
99 The above mentioned methods were also used to detect hCC on synthetic plastic antibody for hCC designed
100 with the molecularly imprinted polymer (MIP) technique on a carbon screen-printed electrode [33]. The
101 EIS method was previously used for hCC detection only using the electrochemical immunosensor based on
102 interdigitated electrode (IDE) modified with polypyrrole/carbon nanotube nanohybrid film and
103 monoclonal antibodies anti-CysC [34].

104 However, to our knowledge, the EIS technique has not yet been suggested before for studies on
105 interactions between hCC and membrane surface or protein-membrane interactions, exploiting a gold
106 electrode. Nevertheless there are reports concerning the modification of gold electrodes for the purpose of
107 detecting proteins in bacterial membrane extracts with the EIS and CV techniques [35]. Additionally, the
108 interactions between amyloid β monomers (A β Ms) and amyloid β oligomers (A β Os) and a floating bilayer
109 lipid membrane (fBLM) using gold electrodes were previously studied with EIS and IR spectroscopy
110 techniques [36].

111 In this study a gold electrode modified with the 6-Mercaptohexan-1-ol (MCH) and DMPC
112 membrane mimetic was applied for monitoring electrochemical changes resulting from the interaction
113 between the functionalized electrode surface and V57G variant of hCC at concentrations ranging from $1 \times$
114 10^{-14} M to 1×10^{-6} . The electrochemical changes were monitored with EIS and CV techniques. The CV
115 results indicate that the most significant current changes were evident after incubation in a solution
116 containing hCC V57G at the concentration of 1×10^{-14} . The EIS measurements confirmed the CV data. The
117 most significant changes in charge transfer resistance were observed for the hCC V57G solution at the
118 concentration of 1×10^{-14} M. The incubation of electrode in hCC V57G solutions at higher concentration
119 caused insignificant changes observed in electrochemical impedance spectra. Additionally, the calculated
120 surface coverage θ_R confirmed that the electrode is coated in a similar manner for incubation studies in
121 solutions at concentrations ranging from 1×10^{-12} M to 1×10^{-6} M. The modification of electrode surface
122 and interactions between DMPC and hCC V57G were studied with the high-resolution X-ray photoelectron
123 spectroscopy (XPS) and contact angle and surface free energy measurements.

124 **2. Methods and materials**

125 *2.1. Materials and reagents*

126 All solvents and reagents were used as received without further purification. 6-mercaptohexan-1-
127 ol (MCH), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$ were
128 purchased from Sigma-Aldrich.

129 *2.2. Expression and purification of human cystatin C*

130 The DNA of hCC V57G variant was obtained with site-directed mutagenesis as previously
131 described [3]. Plasmid DNA (pHD313 vector [37]) including hCC gene coupled with signal peptide
132 delivered from *E. coli* OmpA protein (responsible for secretion of hCC into the periplasmic space),
133 temperature-sensitive λ cI 857 repressor, λ PR promoter and ampicillin resistance gene was expressed in *E.*
134 *coli* BL21(DE3) competent cells according to earlier described-protocol [3].



135 2.3. DMPC phospholipid bilayer preparation

136 The lyophilized DMPC powder was suspended in PBS buffer (Sigma Aldrich). The phospholipid
137 suspension was subjected to 15 incubation cycles involving 30 min incubation in ultrasound bath with
138 heating (313 K) and 30 min incubation in 277 K. This procedure allowed to obtain stable, homogenous,
139 and transparent solution of double-layered lipid bilayers. For the purpose of the experiment, 10 mM DMPC
140 liposome stock solution was prepared and used for the preparation of a dilution series.

141 2.4. Functionalization of gold electrodes

142 The gold electrodes of 1.6 mm diameter used for all measurements were purchased from (Mineral,
143 Poland). Before each modification, the electrodes were carefully polished using polishing pad saturated
144 with micro polish alumina powder (1.0 μm ; Buehler, USA). Then the electrodes were washed, dried in a
145 stream of air, and placed in the vessel containing 1 mL of 1 mM MCH dissolved in absolute ethanol in
146 order to obtain self-assembly monolayer on electrode surface. After 16 h the electrodes were washed with
147 ethanol and dried. Afterwards, the electrode was modified with a layer of DMPC via incubation of its
148 surface in 10 μL of 1 μM solution of DMPC for 1 h. The DMPC concentration used for electrode
149 modification was higher than the critical micelle concentration (CMC) value (6nM), to ascertain the
150 formation bilayer of DMPC on the electrode. The electrodes obtained in above manner were incubated in
151 the hCC V57G solution at ascending concentrations ranging from 1×10^{-14} M to 1×10^{-6} M for 50 minutes.
152 After each electrochemical measurement the electrodes were rinsed with a 0.01 M PBS solution, pH 7.4.

153 The gold electrodes of 11 mm x11 mm used in an XPS, contact angle and surface free energy
154 experiments were purchased from (Arrandee, Germany). Before use the electrodes were incubated in a
155 concentrated sulfuric acid for 4 minutes, washed with water and ethanol and modified according to the same
156 procedure as described above for gold electrodes of 1.6 mm diameter.

157 2.5. X-ray photoelectron spectroscopy measurements

158 X-ray photoelectron spectroscopy (XPS) analyses were carried out using the Escalab 250Xi
159 spectroscope (ThermoFisher Scientific) utilizing AlK α X-ray spot, diameter 500 μm . The pass energy was
160 set up to 20 eV. The low-energy electron and low-energy Ar⁺ ion bombardment were used for charge
161 compensation with final peak calibration using adventitious C1s (284.6 eV). Peak deconvolution was
162 performed in Avantage v.59921 provided by spectroscope manufacturer.

163 2.6. Contact angle and surface free energy measurements

164 The Drop Shape Analyzer – DSA100 by Krüss was used to determine the contact angle and surface
165 free energy of investigated samples. The contact angles of drops of four different liquids (water, formamide,

166 glycerol, and diiodomethane) were measured to determine the surface free energy. The image of a 4 μ L
167 drop of the probe liquid deposited using a syringe was captured by a camera and after the digital image
168 analysis, the average contact angle was deduced using the Young-Laplace method from the angles measured
169 at both sides of the drop in equilibrium. The measurements were repeated 20 times. The total surface free
170 energy γ_s and its dispersive γ_d and polar γ_p components of the surfaces were determined by the Owens,
171 Wendt, Rabel, and Kaelble (OWRK) method from the contact angles of the three liquid drops (water,
172 formamide and diiodomethane) [38–40].

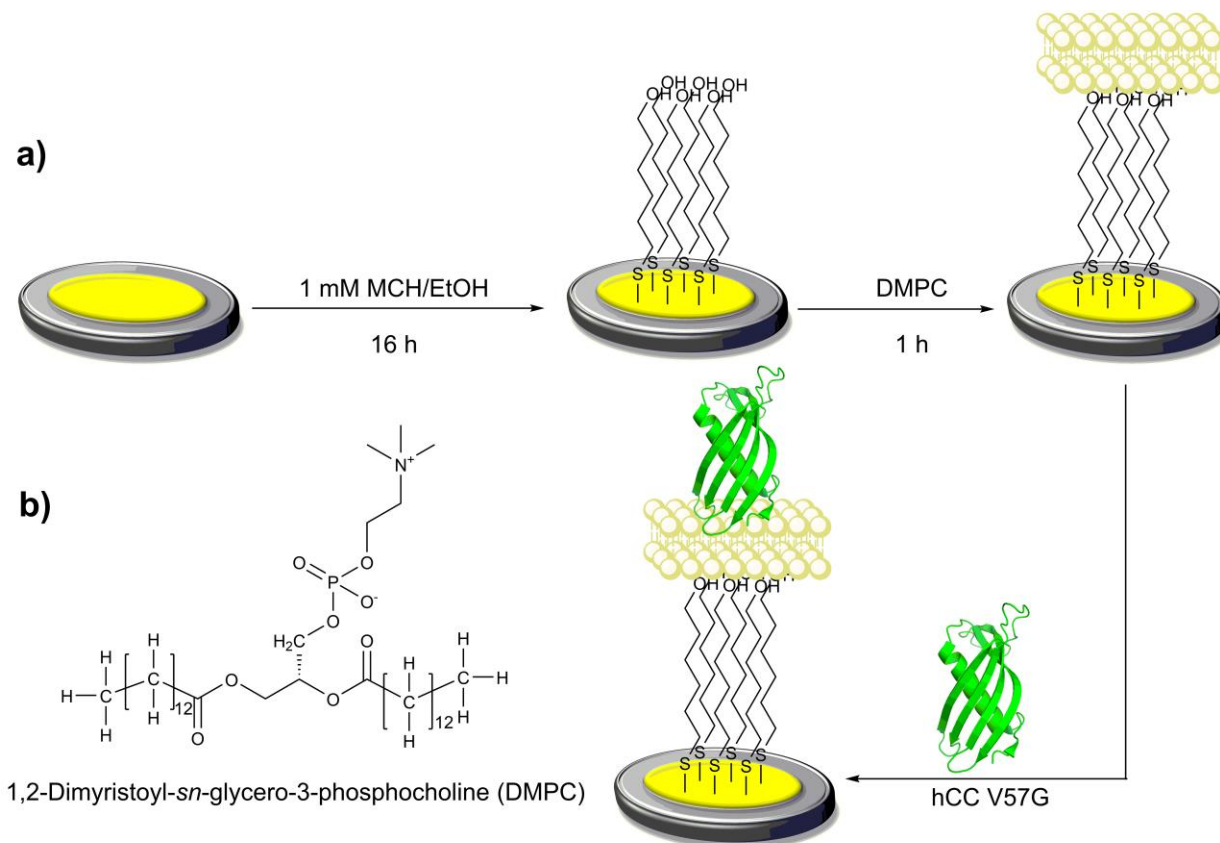
173 *2.7. Electrochemical impedance spectroscopy and cyclic voltammetry measurements*

174 The cyclic voltammetry (CV), and electrochemical impedance spectroscopy (EIS) measurements
175 were performed using M204 multichannel potentiostat (Autolab, Netherlands) equipped with FRA32M
176 electrochemical impedance spectroscopy module in a three-electrode cell. The unmodified and modified
177 gold electrodes served as working electrodes, Ag/AgCl (0.1 M KCl) was a reference electrode and platinum
178 wire was a counter electrode. All electrochemical measurements were performed in 0.01 M PBS solution
179 of pH 7.0 containing $\text{Fe}(\text{CN})_6^{3-/4-}$ as redox probes, containing 1 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$ and 1 mM $\text{K}_4[\text{Fe}(\text{CN})_6]$
180 (1 : 1). The cyclic voltammograms were obtained at scan rate of 0.1 V/s. The EIS spectra were obtained
181 with frequency range of 0.1 Hz to 10 kHz, at with perturbation amplitude of 10 mV, at the open circuit
182 potential (OCP). All the spectra were analyzed using ZSimpWin 3.21 impedance analysis software. All EIS
183 spectra were fitted using modified Randles equivalent circuit $R_s(Q(R_{ct}W))$, where R_s is electrolyte
184 resistance, Q_{dl} – constant phase element, R_{ct} – charge transfer resistance, and W – Warburg element.

185 **3. Results and discussion**

186 *3.1. The gold electrode modification procedure*

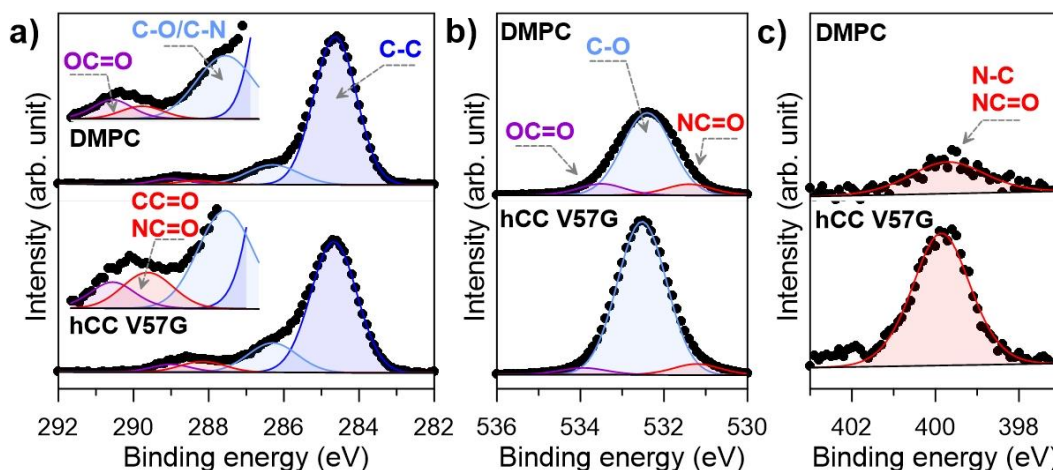
187 The gold electrodes used for detection of hCC V57G were modified according to the scheme shown
188 in Figure 1a. The crystallographic structure of hCC V57G presented in Figure 1 was previously reported in
189 [41]. In the first stage of the modification process MCH was applied to create a stable self-assembled
190 monolayers (SAMs) on the gold electrode surface due to the fact that, it forms an organized layer on the
191 electrode [42]. The SAMs formation is usually used in the first step of the process of electrode
192 functionalization for construction of electrochemical sensors and biosensors [43–45]. In this work 1 mM
193 ethanolic solution of MCH was used for electrode modification. The modification of gold electrodes with
194 MCH takes place not only in ethanolic solution [46], but also in an aqueous solutions [47] or buffer solutions
195 [48]. During the second stage the gold electrode was modified with DMPC by immersing electrode in 1
196 μ M DMPC solution in PBS for 1 h. The chemical structure of the DMPC membrane is shown in Figure 1b.
197 The electrode fabricated in this procedure was directly applied to measurements using CV and EIS methods.



198
199 **Figure 1.** (a) The modification of gold electrode with MCH and DMPC for the hCC V57G detection. (b)
200 The chemical structure of DMPC.

201 3.2. XPS Measurements

202 The XPS spectra were registered to analyze Au electrode surface chemistry and confirm the
203 successful anchoring of hCC V57G to the DMPC-functionalized surface. The results of XPS analysis for
204 primary film constituents are presented in Figure 2.



205
206 **Figure 2.** High-resolution XPS measurements of the electrode surface after DMPC and hCC
207 functionalization steps, studied in the core-level binding energy range of (a) C1s, six times enhanced in the
208 inset, (b) O1s, and (c) N1s with proposed deconvolution model.

209 The dominant component of the C1s spectra (Figure 2a) is C-C aliphatic bond (284.6 eV) within
210 the DMPC and hCC V57G. Furthermore, three additional components can be distinguished, representing
211 different oxidized forms of organic carbon. Their peak positions are characteristic for C-O and C-N
212 interactions (286.3 eV), aliphatic esters, carbonyls, amides, imides (288.3 eV), and carboxyl groups (289.0
213 eV). The share of all oxidized species in total [C] equals 18.0 % in case of DMPC and increases
214 significantly, reaching even 27.3 % after hCC V57G anchoring. Here, the share of the C=O/NC=O moieties
215 increases the most, from 2.8 up to 5.9 % of total [C]. Unfortunately, the signals representing single
216 phosphorus atoms from within DMPC structure or S atom present in the thiol groups were too low for the
217 threshold of the spectroscope and were not detected. The detailed deconvolution analysis is presented in

218 Table 1.

219
220
221
222

223 **Table 1.** Results of XPS data deconvolution (in at.%).

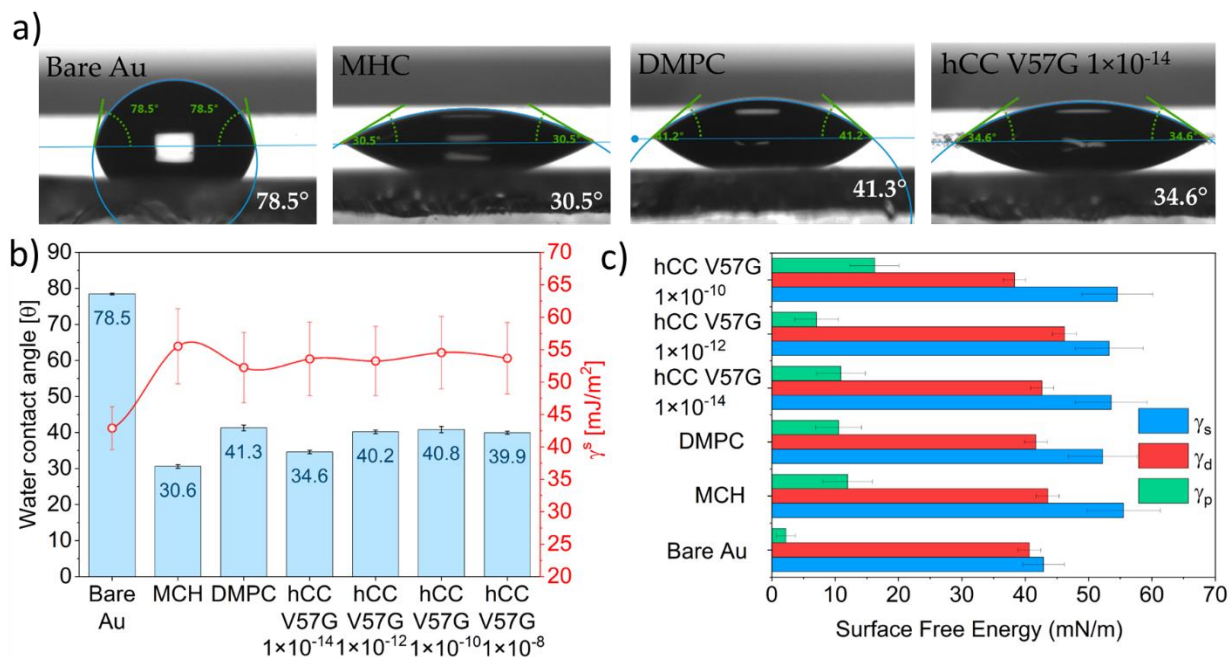
	C1s					O1s		N1s
	C-C	C-O	C=O/ NC=O	OC=O	NC=O	C-O	OC=O	N-C/ NC=O
BE (eV)	284.6	286.3	288.2	289.0	531.2	532.5	533.9	399.9
DMPC	46.1	7.5	1.6	1.1	33.9	4.6	4.5	0.7
hCC V57G	24.7	5.9	1.3	2.0	57.5	4.2	2.5	1.9

224

225 The above-mentioned observations are assisted by a tremendous rise in oxygen content, analyzed
 226 based on the O1s spectra (Figure 2b). The total share of all [O] species increases from 43.0 up to 64.21
 227 at.%, when $C_{580}H_{910}N_{170}O_{176}S_7$ (hCC V57G) is anchored to the modified electrode surface. The surface
 228 modification with hCC V57G particularly affects the C-O bonds (O1s at 532.5 eV), which corroborate with
 229 the findings observed from the C1s peak analysis. Moreover, over two-fold increase in the N1s share, from
 230 0.7 to 1.9 at.% was also observed. The N1s peak used for the deconvolution is located at 399.9 eV, a value
 231 characteristic for amide or imides but also amines.

232 3.3. Contact angle and surface free energy measurements

233 Wettability measurements at the modified gold electrode surface allowed to determine the influence
 234 of the modification process on the characteristics of the tested systems (Figure 3). The obtained results
 235 clearly indicate that the modification of the gold electrode with MCH causes a significant change in its
 236 properties. The decrease in the contact angle from 78.5° for the bare electrode to 30.6° for the 1-hexanethiol-
 237 modified surface is a significant change. The presence of MCH made the surface more hydrophilic, which
 238 is the result of the presence of -OH groups on the modified electrode surface. The introduction of the DMPC
 239 membrane into the system, in turn, causes an increase in hydrophobicity, which is manifested in an increase
 240 in the contact angle to 41.3° (Figure 3a). Further studies showed that the interaction with hCC V57G is
 241 visible for the concentration of 10^{-14} M. For electrodes incubated in such protein concentration, we observe
 242 a decrease in hydrophobicity by approx. 7° compared to electrodes with a DMPC membrane. In turn, the
 243 electrodes incubated in higher hCC V57G concentrations showed only slight changes in the contact angle
 244 (approx. $1-2^\circ$). This clearly indicates that the electrode is sensitive only below the concentration of 1×10^{-14}
 245 M.



246

247

248 **Figure 3.** (a) Wettability measurement photos, (b) water contact angle (blue bar) and surface free energy γ^s (red line), and (c) surface free energy γ^s diagram (blue bar) with disperse (red bar) and polar (green bar) parts, for each step of the modification of the gold electrode for hCC V57G sensing.

251 Surface free energy allows to determine the biocompatibility of a given system, as well as to track
 252 the interactions or physicochemical changes resulting from the interactions of the system with the analyte.
 253 The modification of the gold electrode with a thiol layer increases the surface free energy (around 15 mN/m)
 254 (Figure 3b,c). This is due to a significant increase in the share of the polar part (Figure 3c), resulting from
 255 the appearance of functional groups on the surface of the electrode, which were not present in the case of a
 256 bare electrode. Further anchoring of the DMPC membrane on the thiol surface reduces the free energy, but
 257 only by about 5 mN/m (Figure 3c). Such an effect may be caused by the arrangement of the lipid bilayer
 258 formed by DMPC. Changes in the contact angle and surface free energy (Figure 3b) indicate that the
 259 membrane surface has hydrophilic heads pointing outward from the layer. Hence, the hydrophilic character
 260 of the system is maintained, although it is weaker compared to the thiol layer rich in -OH groups. In the
 261 case of surface free energy parameters, we do not observe such a large variability in the presence of
 262 interaction with hCC V57G as in the case of the contact angle value. Nevertheless, the share of both
 263 dispersive and polar interactions (hydrogen bonding and dipole-dipole interactions) is visible.

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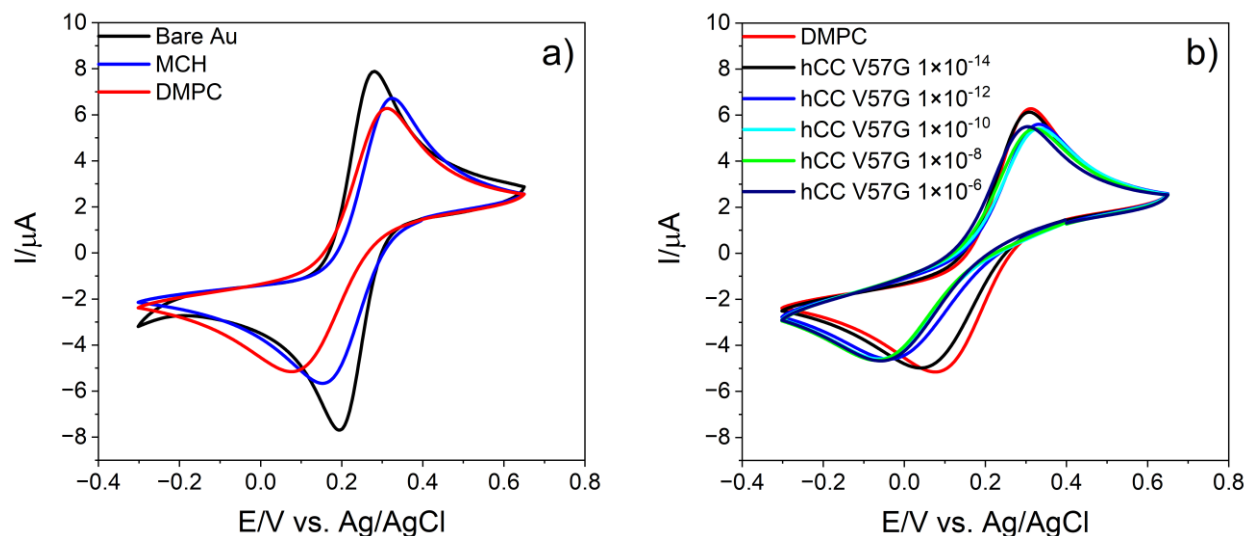
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266 3.4. Electrochemical measurements

267 3.4.1. Cyclic voltammetry (CV)

268 The cyclic voltammetry (CV) was used to characterize the response of an electrode during the
269 modification steps and in order to examine the electrode after incubation in hCC V57G solutions at
270 concentrations ranging from 1×10^{-14} M to 1×10^{-6} M. Figure 4a shows the cyclic voltammograms obtained
271 for bare gold electrode before and after modification with MCH and DMPC. All measurements were
272 performed in 0.01 M PBS solution (pH 7.0) containing 1 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ used as redox probe. The two
273 reversible peaks are observed on cyclic voltammograms with peak-to-peak separation (ΔE) of 76 mV. The
274 intensity current of peaks decrease after modification with MCH with the peak-to-peak separation
275 increasing to 159 mV, suggesting the SAMs formation on the electrode surface. Similar value of (ΔE) was
276 observed in the previous paper indicating the formation of monolayer on a gold electrode [49]. Further
277 functionalization of the electrode with DMPC leads to an even greater decrease in current intensity and an
278 increase in peak-to-peak separation to 214 mV. The above-mentioned changes indicate that electron transfer
279 to the surface of the electrode has been inhibited, indicating that its surface has been modified. The cyclic
280 voltammograms of the modified electrode after incubation in PBS solution containing different
281 concentrations of hCC V57G are shown in Figure 4b. The incubation in 1×10^{-14} M hCC V57G solution
282 causes a slight decrease in anodic peak and a shift in the position of the cathodic peak, with peak-to-peak
283 separation (ΔE) of 250 mV relative to the voltammogram observed for DMPC. The changes observed in
284 the cyclic voltammograms for hCC V57G at the concentration of 1×10^{-14} M are most significant. Further
285 incubation in a solution containing 1×10^{-12} M hCC V57G solution causes further decrease in the current
286 intensity and an increase of (ΔE) to 311 mV. At the same time, after incubation in hCC V57G solutions at
287 the concentration of 1×10^{-10} M and 1×10^{-8} M, almost identical voltammograms were obtained with ΔE
288 of 362 mV and 359 mV respectively. The incubation in 1×10^{-6} M hCC V57G solution also does not cause
289 changes in the peak heights, only a shift of the anode peak towards negative values is observable resulting
290 in ΔE decrease to 329 mV. The CV analysis indicates that the most significant changes are observed after
291 incubation of the electrode in 1×10^{-14} M hCC V57G solution.



292
 293 **Figure 4.** (a) Cyclic voltammograms of bare Au electrode and electrodes modified with MCH and DMPC.
 294 (b) Cyclic voltammograms of the DMPC-modified electrode after incubation in hCC V57G solutions at
 295 concentrations ranging from 1×10^{-14} M to 1×10^{-6} M recorded in 0.01 M PBS (pH 7.0) containing 1 mM
 296 $\text{Fe}(\text{CN})_6^{3-/4-}$, scan rate 100 mV/s.

297 3.4.2. Electrochemical Impedance Spectroscopy (EIS)

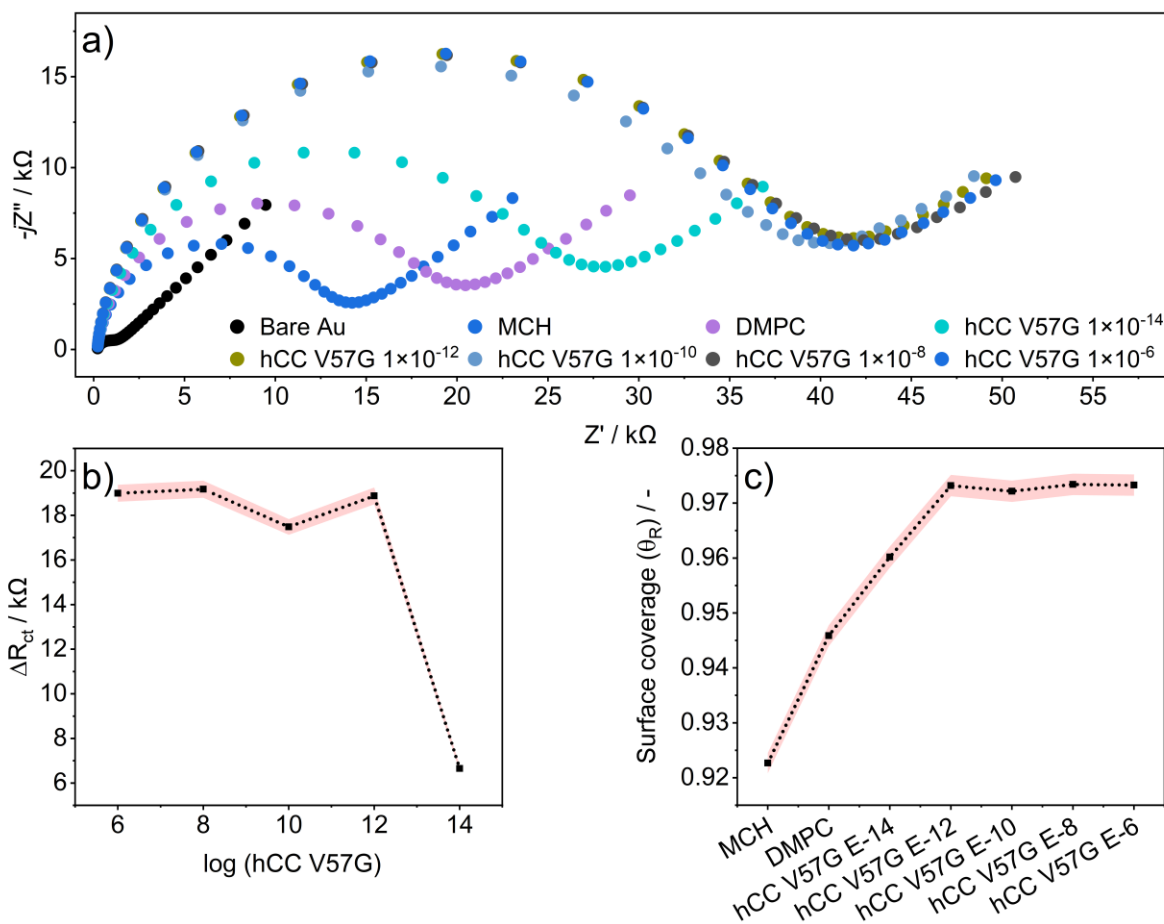
298 The EIS measurements were performed in 0.01 M PBS solution (pH 7.0) containing $\text{Fe}(\text{CN})_6^{3-/4-}$.
 299 Figure 5a shows the impedance spectra obtained for bare Au electrode, electrode modified with MCH,
 300 electrode modified with DMPC and the modified electrodes after incubation in hCC V57G solutions at
 301 concentrations ranging from 1×10^{-14} M to 1×10^{-6} M. All spectra were fitted using modified Randles
 302 equivalent circuit $R_s(Q_{dl}(R_{ct}W))$. The charge transfer resistance calculated for a bare electrode ($R_{ct} = 1.003$
 303 $\text{k}\Omega$) increases significantly after modification with MCH to ($R_{ct} = 12.970 \text{ k}\Omega$) with the decreases of constant
 304 phase element (Q_{dl}) from $1.426 \mu\text{F}$ for bare Au electrode to $0.191 \mu\text{F}$ after MCH modification. A similar
 305 phenomenon was also observed in previous work [49]. The Au electrode coated with MCH additionally
 306 modified with DMPC causes an increase of R_{ct} to $18.530 \text{ k}\Omega$ and increase of constant phase element (Q_{dl})
 307 to $0.212 \mu\text{F}$ (see Table S1 in Supplementary Information). Further incubation in hCC V57G solutions at
 308 the concentrations of 1×10^{-14} M cause an increase of the charge transfer resistance R_{ct} to $25.180 \text{ k}\Omega$. A
 309 subsequent incubation in hCC V57G solutions at higher concentrations causes an increase of R_{ct} to 37.400
 310 $\text{k}\Omega$ after incubation in 1×10^{-12} M hCC V57G and remains constant ($37.520 \text{ k}\Omega$) for 1×10^{-6} M hCC V57G
 311 solution. It's also worth noting that after DMPC modification the parameter n is constant regardless of any
 312 incubation in hCC V57G solution and is close to 1 [36]. This indicates that the surface roughness does not
 313 change significantly during subsequent stages of hCC V57G detection at different concentrations [50]. The
 314 understanding of the mechanism of interactions between the DMPC layer immobilized on the gold electrode

315 and hCC V57G at different concentrations requires additional research. Nevertheless, the previous EIS
316 studies on interaction between amyloid β monomers ($A\beta$ Ms) and the floating bilayer lipid membrane
317 (fBLM) do not lead to the formation of pores. Only the β oligomers ($A\beta$ Os) induced the pore formation in
318 examined membrane [36].

319 Figure 5b shows the ΔR_{ct} correlation, where ΔR_{ct} was calculated as difference between the R_{ct} after
320 electrode incubation in hCC V57G solutions of different concentration and R_{ct} of DMPC and the logarithm
321 of the hCC V57G concentration. The above relationship clearly shows that the calculated change in ΔR_{ct}
322 for the electrode after incubation in 1×10^{-14} M hCC V57G solution reaches a value of 6.650 k Ω , then
323 rapidly increases to 18.870 k Ω after incubation in 1×10^{-12} M hCC V57G solution. Subsequent incubations
324 in higher concentrations of hCC V57G result in only slight changes in ΔR_{ct} values, suggesting that the
325 resulting electrode is not so sensitive to the presence of hCC V57G at the higher concentrations ranging
326 from 1×10^{-12} M to 1×10^{-6} M.

327 Figure 5c shows the plot of surface coverage θ_R after each step of bare Au modification, calculated
328 according to the following equation $\theta_R = 1 - (R_{ct}(\text{Bare Au}) / R_{ct}(\text{Modified Au}))$ [51,52], where $R_{ct}(\text{Bare Au})$ and R_{ct}
329 (Modified Au) corresponds to the charge transfer resistance for the bare electrode and the appropriately modified
330 electrodes respectively. The surface coverage of gold electrode after SAMs formation was 0.923, while
331 after DMPC modification the surface coverage increased to 0.946, then after incubation in hCC V57G
332 solution at the concentration of 1×10^{-14} M the surface coverage was 0.960. The incubations of the modified
333 gold electrode in hCC V57G solution with concentrations ranging from 1×10^{-12} M to 1×10^{-6} M did not
334 change the electrode coverage. The calculated surface coverage value for each electrode was constant at
335 0.973. The 0.97 degree coverage of the electrode surface may indicate that pores can be present on the
336 bilayer of DMPC deposited on the electrode, which allows the redox probe to reach the electrode. This
337 unequivocally proves that the gold electrode used in this study shows the most significant changes after
338 incubation in the solution of hCC V57G at concentration of 1×10^{-14} M. The lack of significant changes
339 above the concentrations of 10^{-14} M of hCC V57G can be attributed to the saturation of binding sites on the
340 electrode surface. The previously published data shows that only an external random coil loop region of
341 hCC protein interacts with DMPC bilayer and the protein does not migrate into the bilayer, but interacts
342 with its surface [15].

343



344
 345 **Figure 5.** The electrochemical impedance spectra obtained for (a) bare Au electrode, electrodes modified
 346 with MCH and DMPC and modified electrodes after incubation in hCC V57G solutions at concentrations
 347 ranging from 1×10^{-14} M to 1×10^{-6} M. (b) Correlation between ΔR_{ct} and the logarithm of the hCC V57G
 348 concentration. (c) The electrode surface coverage after each step of modification. The red lines indicate the
 349 error bars.

350 CV and EIS technique have not been applied before for studies on interactions between hCC and
 351 membrane surface or protein-membrane interactions with the use of gold electrodes. The presented studies
 352 proves that the CV and EIS methods can be applied for the monitoring of changes occurring on the surface
 353 of an electrode modified with the DMPC bilayer during incubation in the V57G solution at different
 354 concentrations. EIS measurements also indicate that the hCC V57G can be detected at the concentrations
 355 ranging from 1×10^{-14} M to 1×10^{-6} M, nevertheless the most significant changes were observed for the
 356 hCC V57G at the concentration of 1×10^{-14} M.

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 358

359 **4. Conclusion**

360 The presented research indicates that the gold electrode modified with MCH and DMPC bilayer,
361 allows for monitoring the interactions between DMPC and V57G mutant of human cystatin C. The
362 interactions between the modified electrode and the hCC V57G protein were monitored at concentrations
363 ranging from 1×10^{-14} M to 1×10^{-6} M. Both the CV and EIS measurements indicate that the most significant
364 changes were observed for the protein detection at the concentration of 1×10^{-14} M. The incubation in hCC
365 V57G solutions at higher concentration did not cause any changes relevant in the experimental conditions.
366 Furthermore, the EIS measurements indicate that incubation of the modified electrode in hCC V57G
367 solutions, at concentrations ranging from 1×10^{-12} M to 1×10^{-6} M, did not change the electrode coverage.
368 The electrochemical data obtained in this study were also confirmed with water contact angle and surface
369 free energy measurements. The decrease in contact angle upon MCH modification highlights the
370 enhancement of surface hydrophilicity, while the subsequent increase in contact angle after the addition of
371 the DMPC membrane signifies the increase of hydrophobicity of the modified surface. The sensitivity of
372 the electrode in relation to the interaction with hCC V57G at different concentrations was reflected in the
373 contact angle measurements. The most noticeable decrease in hydrophobicity (by approximately 7°) was
374 observed for electrodes incubated with hCC V57G at the concentration of 10^{-14} M. Obtained results confirm
375 that the presented method of electrode functionalization allows to monitor the interactions between the
376 electrode surface and hCC V57G protein.

377

378 **Declaration of interests**

379 The authors declare that they have no known competing financial interests or personal relationships that
380 could have appeared to influence the work reported in this paper.

381

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