ELECTRONIC SUPPLEMENTARY FILE

Nano-modified Screen-Printed Electrodes-based Electrochemical Immunosensors for Oral Cancer Biomarkers Detection in Undiluted Human Serum and Saliva Samples

*Payal Gulati[1], Avinash Kumar Singh[1], Amit K. Yadav[1], Kiran Pasbola[2], Prerna Pandey[2], Rinu Sharma[2], Alok Thakar[3], Pratima R. Solanki[1]**

[1]Nano-Bio Laboratory, Special Centre for Nanoscience, Jawaharlal Nehru University, New Delhi-110067, India

[2]University School of Biotechnology, Guru Gobind Singh Indraprastha University, India

[3]All India Institute of Medical Sciences, Ansari Nagar, New Delhi, 110029, India

@ Authors Contributed Equally to this work.

Corresponding Author: E-mail Id: partima@mail.jnu.ac.in; pratimarsolanki@gmail.com*

S1: Experimental conditions optimization

In acidic medium, acidic residues of proteinaceous biomolecules are uncharged; similarly, in basic pH, basic residues are uncharged. Thus, due to ionic interactions, these states' residues cannot contribute to the electrostatic stability required for the folded conformation of proteinaceous molecules [1]. Proteins, when exposed to a medium away from physiological pH, they are unstable. Figure S1(a) shows the bar graph of the pH study (range: 6.0- 8.0), which demarcates the highest peak current at pH=7.4, the physiological pH. Figure S1(b) shows bar plot of antibody concentration optimization (1 µg mL^{-1}, 5 µg mL^{-1}, 10 µg mL^{-1}). This graph clearly states that 10 µg mL^{-1} concentration electrode has the highest peak current; therefore, it is the most appropriate concentration for immune sensor fabrication.

Figure S1(c, d, and e) represents the cyclic voltammogram (CV) of ink-printed substrates including PET, Ivory sheet, and sandpaper for substrate standardization. The electrode-printed sandpaper substrate showed well-defined oxidation-reduction peaks compared to the other ink-printed substrates, suggesting it be the most suitable for biosensor construction.

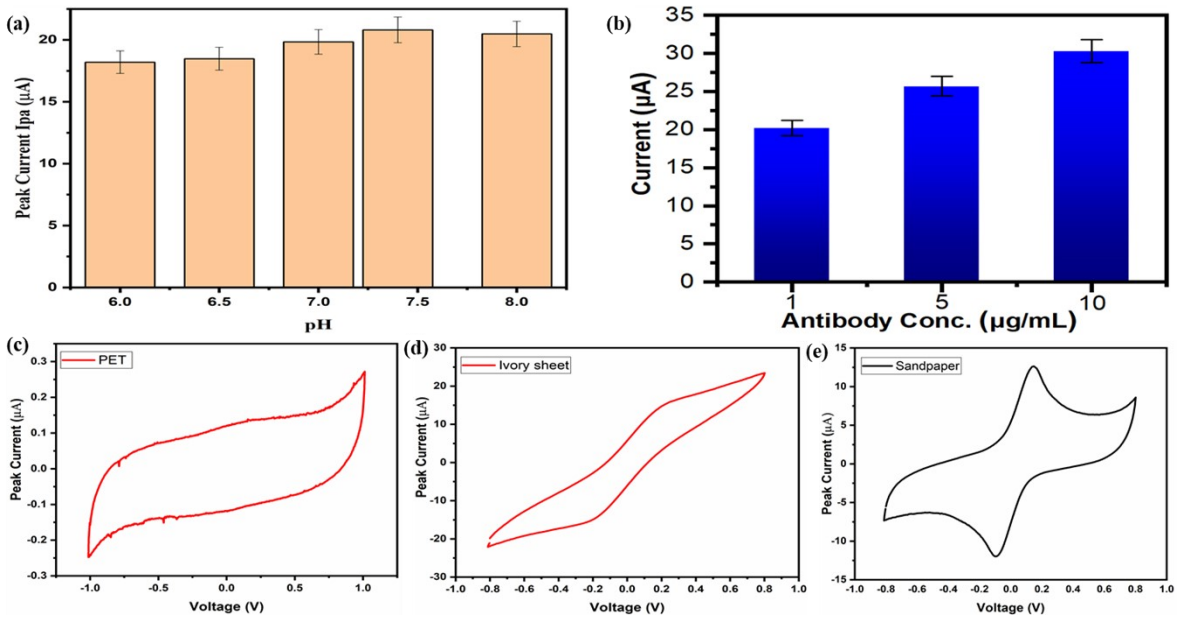

Fig S1: Standardization of some parameters of immunosensor: (a) Effect of pH on antibody; (b) optimization of Ab concentration; cyclic voltammetry plots of three different substrates (c) bare PET, (d) bare ivory sheet, and (e) bare sandpaper.

S2: Scan rate Study

The diffusion coefficient (D_f) was estimated using Randles–Sevcik equation, given below:

$$I_p = \left(2.69 \times 10^5\right) C\, n^{3/2} D_f^{1/2} \nu^{1/2} A \qquad \text{Eq. (i)}$$

representation of each symbol is as follows: I_p (I_{pa} or I_{pc}) - electrodes peak current, n - the number of electrons participating in the redox process and i.e., =1, A - the active area of the electrodes' surface, D - diffusion coefficient (cm^2s^{-1}), C - electrolyte redox species concentration = 5 mM, and υ - scan rate utilized in experimentation = 50 mVs^{-1} [23]. The bio-electrodes such as BSA/anti-CYFRA 21-1/SPE, BSA/anti-IL-8/SPE, and BSA/anti-TP-53/SPE demonstrated higher D value than bare SPE indicates superior electron transfer at the electrolyte/electrode interface of the former electrodes. The high D value demarcates the excellent analytical efficiency of this immunosensor, as the D value depends upon the surface area of electrodes and electrolyte concentration. D value for all the bio-electrodes and electrodes is mentioned in table S1.

In order to determine the number of maximum binding sites available at the surface of immune electrodes, the value of Ae was calculated by incorporating the D_f value (obtained from Randles–Sevcik equation) in Eq (ii). The relation is as follows:

$$A_e = \frac{S}{(2.69 \times 10^5) n^3 c D^{1/2}} \quad \text{Eq. (ii)}$$

Where S is the slope of the straight line obtained from the plot of Ipa v/s scan rate, and other symbols are the same as the above equation [23]. The electroactive area (Ae) value of BSA/anti-CYFRA 21-1/SPE, BSA/anti-IL-8/SPE, and BSA/anti-TP-53/SPE bio-electrodes are higher than bare SPE. Thus, showing the presence of more reactive sites per unit volume, after biomolecules (i.e., BSA and antibody) immobilization. This suggests them to be the most suitable platform for immunosensor fabrication instead of bare SPE. Ae values for all the electrodes are given in table S1.

Moreover, the surface concentration of ionic species (I* in mol.cm^{-2}) for the immune-electrodes were calculated using Brown-Anson equation:

$$I_p = \frac{n^2 F^2 I^* A V}{4 R T} \quad \text{Eq. (iii)}$$

Where, F represents the Faraday constant (96485 C mol-1), T is the temperature (300 K), R is the gas constant (8.314 J mol-1 K-1), and other symbols are same as mentioned in the previous equation[23]. The enhanced electro-catalytic behaviour of these bio-electrodes than bare SPE is due to presence of biomolecules including antibodies and BSA which implies it be successful bio-sensing platform. I* values for all the electrodes are given in Table S1.

The calculated D_f, A_e and I* for bare SPE, anti-CYFRA 21-1/SPE, BSA/anti-CYFRA 21-1/SPE, anti-IL-8/SPE, BSA/anti-IL-8/SPE, anti-TP-53/SPE and BSA/anti-TP-53/SPE are mentioned in the Table S1.

Table S1: Projected values of electrochemical factors for bare electrode and modified bio-electrodes.

S.N.	Electrode	D_f (cm^2s^{-1})	Ae (mm^2)	I* (mol cm^2)
1.	Bare SPE	7.524×10^{-14}	0.6	1.767×10^{-9}
2.	Anti-CYFRA 21-1/SPE	7.335×10^{-14}	1.8704	1.744×10^{-9}
3.	BSA/anti-CYFRA 21-1/SPE	9.663×10^{-14}	2.71	2.003×10^{-9}

4.	Anti-IL-8/ SPE	27.57×10^{-14}	2.099	3.383×10^{-9}
5.	BSA/anti-IL-8/SPE	39.31×10^{-14}	1.509	4.031×10^{-9}
6.	Anti-TP-53/SPE	20.96×10^{-14}	1.797	2.949×10^{-9}
7.	BSA/anti-TP-53/SPE	23.84×10^{-14}	1.717	3.145×10^{-9}

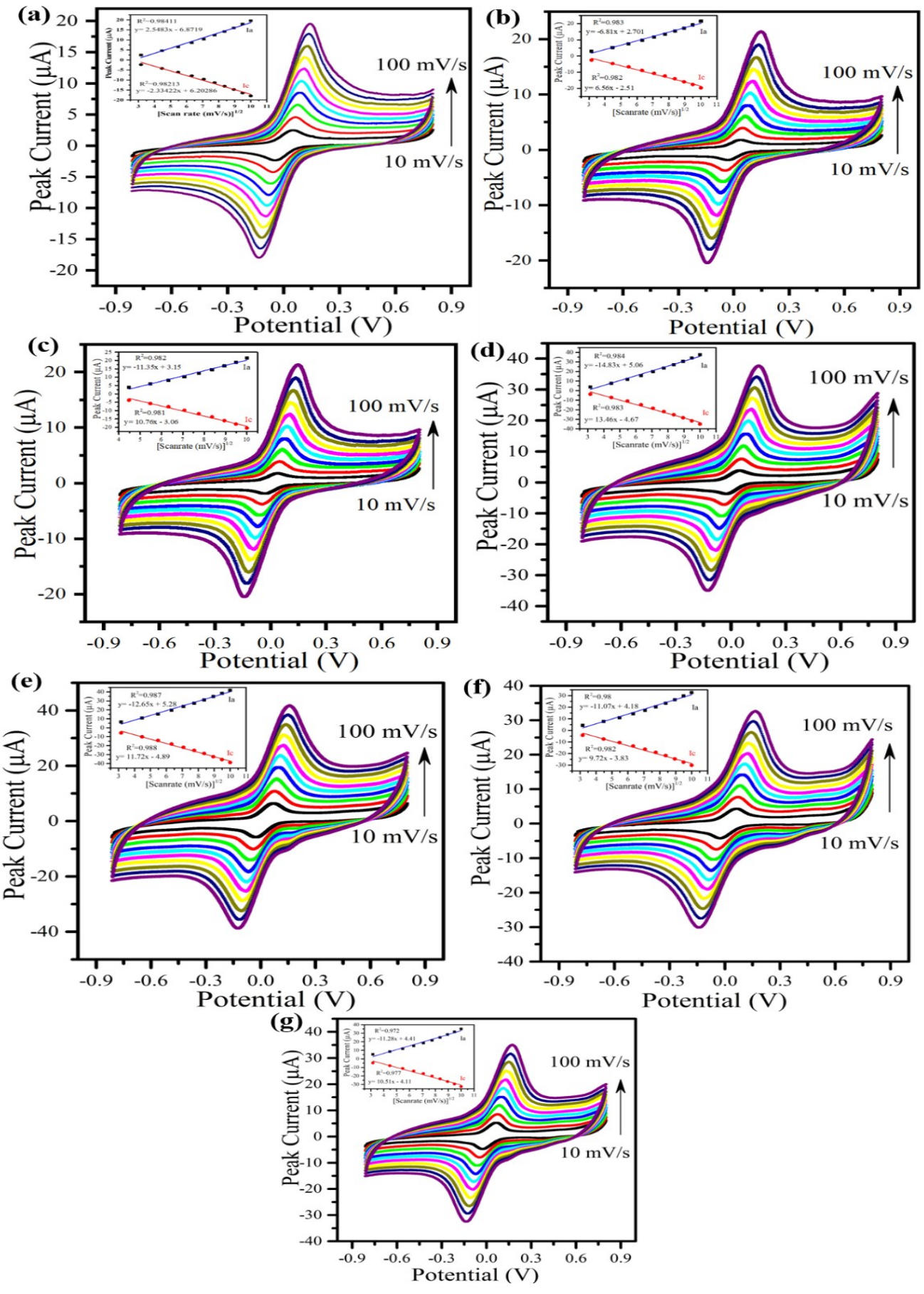
(a)
100 mV/s
10 mV/s
Peak Current (μA)
Potential (V)
(b)
100 mV/s
10 mV/s
Peak Current (μA)
Potential (V)
(c)
100 mV/s
10 mV/s
Peak Current (μA)
Potential (V)
(d)
100 mV/s
10 mV/s
Peak Current (μA)
Potential (V)
(e)
100 mV/s
10 mV/s
Peak Current (μA)
Potential (V)
(f)
100 mV/s
10 mV/s
Peak Current (μA)
Potential (V)
(g)
100 mV/s
10 mV/s
Peak Current (μA)
Potential (V)

Fig S2: Cyclic voltammetry (CV) of SPEs and immune-electrodes recorded at different scan rates from 10 to 100 mV/s with reference to the Ag and inset represents the Peak current (Ipa and Ipc) vs square root of scan rate plot: (a) bare SPE, (b) Anti-CYFRA 21-1/SPE, (c) BSA/Anti-CYFRA 21-1/SPE, (d) Anti-IL-8/SPE, (e) BSA/Anti-IL-8/SPE, (f) Anti-TP-53/SPE, (g) BSA/Anti-TP-53/SPE.

S3: List of patient samples data

These tables discuss about, biomarkers [CYFRA 21-1 (in manuscript in Table 2 and 3), IL-8 and TP-53] expression in patient's serum and saliva sample. Based on their expression level the clinician will be able to detect oral cancer in different patients. Thus, clinicians can head towards accurate prognosis and status of the diseased condition. These tables also represent the ELISA concentration of all the three biomarkers and RSD (%) values calculated from CV plot (peak current value) of patient serum and saliva samples and standard biomarker antigen concentration. The standard peak current values were obtained by extrapolating the graph. The RSD values are less than 3% for every patient serum and saliva samples, which is an acceptable range. Biomarker's concentration was found higher in the case of saliva than serum because saliva is in direct contact with the cancer.

Table S2: Estimation of % RSD among peak current obtained for standard sample and cancer patient serum samples, and determination of IL-8 concentration by ELISA using BSA/anti-IL-8/SPE immunoelectrode.

S No.	Patient No.	IL-8 conc. (pg/ml) determined using ELISA	Peak Current (µA) obtained from std. IL-8 samples	Peak Current (µA) obtained with patient serum sample	% RSD
1.	OCSe 1	126.1	28.18	28.42	0.6
2.	OCSe 2	71.64	26.95	26.39	1.82
3.	OCSe 3	22.7	28.18	28.07	0.28
4.	OCSe 4	36.05	25.72	26.16	1.2
5.	OCSe 5	90.66	24.49	24.12	0.81
6.	OCSe 6	58.63	24.49	24.60	0.32
7.	OCSe 7	13.87	23.47	23.20	0.82

8.	OCSe 8	22.7	24.49	24.68	0.55
9.	OCSe 9	67.06	24.49	24.63	0.4
10.	OCSe 10	21.84	24.49	23.63	0.4
11.	OCSe 11	85.96	24.49	240.4	1.31
12.	OCSe 12	80	25.72	26.37	1.76
13.	OCSe 13	110.61	25.72	26.25	1.44
14.	OCSe 14	26.79	23.47	23.18	0.88
15.	OCSe 15	7.31	24.49	24.85	1.03
16.	OCSe 16	16.26	21.47	21.36	0.36
17.	OCSe 17	39.68	21.88	21.77	0.36
18.	OCSe 18	410.5	20.19	20.87	0.14
19.	OCSe 19	27.62	20.55	19.72	2.91
20.	OCSe 20	57.28	20.55	20.29	0.9
21.	OCSe 21	867.9	20.55	18.64	6.89
22.	OCSe 22	0.09	21.88	21.91	0.1
23.	OCSe 23	426.8	20.55	19.94	2.13
24.	OCSe 24	63.81	20.55	19.20	4.8
25.	OCSe 25	17.36	20.55	18.77	6.63
26.	OCSe 26	22.83	20.55	19.60	3.35
27.	OCSe 27	0.145	20.55	16.07	17.3
28.	OCSe 28	35.82	20.55	16.65	14.83

Table S3: Estimation of % RSD among peak current obtained for standard sample and cancer patient serum samples, and determination of TP-53 concentration by ELISA using BSA/anti-TP-53/SPE immunoelectrode.

S No.	Patient No.	TP-53 conc. (pg/mL) determined using ELISA	Peak Current (µA) obtained from std. P-53 samples	Peak Current (µA) obtained with patient serum sample	% RSD
1.	OCSe 1	1073.95	37.04	37.46	0.80
2.	OCSe 2	584.62	38.08	38.67	1.09
3.	OCSe 3	909.04	39.4	38.80	1.09
4.	OCSe 4	618.14	39.42	40.47	1.86
5.	OCSe 5	1071.59	40.23	40.18	0.09
6.	OCSe 6	754.55	37.04	37.48	0.84
7.	OCSe 7	572.91	37.04	37.59	1.04
8.	OCSe 8	486.84	36.7	36.08	1.2
9.	OCSe 9	1651.03	38.08	38.24	0.30
10.	OCSe 10	1696.08	39.4	38.97	0.78
11.	OCSe 11	1280.21	37.99	37.30	0.17
12.	OCSe 12	1232.6	44.07	44.23	0.26
13.	OCSe 13	499.11	35.27	32.57	5.63
14.	OCSe 14	1073.95	42.95	43.01	0.08
15.	OCSe 15	671.74	36.7	36.01	1.34
16.	OCSe 16	1476.66	38.08	38.05	0.06
17.	OCSe 17	3340.29	36.7	36.91	0.40
18.	OCSe 18	638.73	38.08	38.36	0.52
19.	OCSe 19	1065.44	36.7	36.28	0.81
20.	OCSe 20	821.56	39.42	39.96	0.96
21.	OCSe 21	593.45	35.27	33.49	3.66
22.	OCSe 22	327.55	37.04	37.05	0.02

23.	OCSe 23	1915.22	40.23	40.46	0.4
24.	OCSe 24	684.13	38.08	38.65	1.05
25.	OCSe 25	2723.5	35.3	35.00	0.6
26.	OCSe 26	2824.42	38.08	38.20	0.22
27.	OCSe 27	1669.03	35.27	34.79	0.97
28.	OCSe 28	417.35	35.27	34.35	1.87

Table S4: Estimation of % RSD among peak current obtained for standard sample and cancer patient saliva samples, and determination of IL-8 concentration by ELISA using BSA/anti-IL-8/SPE immunoelectrode.

S. No.	**Patient No.**	**IL-8 conc. (pg/ml) determined using ELISA**	**Peak Current (µA) obtained from std. IL-8 samples**	**Peak Current (µA) obtained with patient serum sample**	**% RSD**
1.	OCSa 1	17850	80.29	80.53	0.21
2.	OCSa 2	4553	42.76	42.46	0.50
3.	OCSa 3	10100	92.17	92.28	0.08
4.	OCSa 4	442.3	39.09	40.95	3.29
5.	OCSa 5	956	36.86	37.01	0.29
6.	OCSa 6	6980	42.76	42.73	0.05
7.	OCSa 7	411.7	39.09	40.82	3.06
8.	OCSa 8	18045	89.53	89.47	0.05
9.	OCSa 9	20154	84.25	84.56	0.26
10.	OCSa 10	17050	42.76	43.98	1.99
11.	OCSa 11	134.4	54.55	54.31	0.31
12.	OCSa 12	142	95.47	95.42	0.04
13.	OCSa 13	9000	95.47	95.67	0.15
14.	OCSa 14	579.4	87.55	87.18	0.30
15.	OCSa 15	3172	102.07	101.98	0.06
16.	OCSa 16	8189	51.25	51.02	0.32

Table S5: Estimation of % RSD among peak current obtained for standard sample and cancer patient saliva samples, and determination of TP-53 concentration by ELISA using BSA/anti-TP-53/SPE immunoelectrode.

S No.	Patient No.	TP-53 conc. (ng/mL) determined using ELISA	Peak Current (µA) obtained from std. P-53 samples	Peak Current (µA) obtained with patient sample	% RSD
1.	OCSa 1	18.123	28.2	28.92	1.78
2.	OCSa 2	7.319	28.2	28.37	0.42
3.	OCSa 3	19.893	30.76	29.74	2.38
4.	OCSa 4	16.408	30.76	30.18	1.35
5.	OCSa 5	7.534	31.62	31.47	0.34
6.	OCSa 6	10.273	41.08	41.06	0.10
7.	OCSa 7	10.471	41.94	42.24	0.50
8.	OCSa 8	54.105	44.52	44.56	0.06
9.	OCSa 9	67.464	40.22	39.90	0.56
10.	OCSa 10	17.775	45.38	45.12	0.41
11.	OCSa 11	19.998	39.36	39.31	0.09
12.	OCSa 12	21.942	46.24	46.05	0.29
13.	OCSa 13	12.441	49.68	49.59	0.13
14.	OCSa 14	77.301	66.02	66.16	0.11
15.	OCSa 15	15.302	54.84	54.84	0
16.	OCSa 16	42.397	50.54	50.93	0.54

References:

1 M. Hebditch, R. Kean and J. Warwicker, *Comput. Struct. Biotechnol. J.*, 2020, **18**, 897–905.

2 A. K. Singh, T. K. Dhiman, G. Lakshmi and P. R. Solanki, *Bioelectrochemistry*, 2021, **137**, 107684.

3 S. Tiwari, P. K. Gupta, Y. Bagbi, T. Sarkar and P. R. Solanki, *Biosens. Bioelectron.*, 2017, **89**, 1042–1052.