**A “Swingable” straight-chain affinity molecule immobilized on a
semi-conductor electrode for photo-excited current-based molecular sensing**

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Abstract

The molecular affinities of biomolecules have found applications in the areas of clinical diagnostics, drug discovery, as well as allied fields of study. An affinity sensor is a unique *in situ* assay tool, which is valuable and convenient in practical situations. In this study, we designed a photo-excitabile molecular interface with an affinity domain and fabricated with a “swingable” straight-chain affinity molecule immobilized on a semi-conductor electrode (SCE). The straight-chain affinity molecule possessed a photo-excitabile dye at one end and was bound to the SCE surface at the other by the EC tag method, which was developed previously. A straight-chain molecule is too long to transfer electrons from the photo-excited dye to the conduction band of the SCE. However, the straight-chain molecule was designed with a “swing” structure, which made the transfer of electrons possible. The central region of the chain molecule has an affinity to the activated estrogen receptor (ER). When the activated ER bound to the affinity region, the molecular lost its swingable function, the electron transfer from the photo-excited dye to the SCE was consequently suppressed. Based on the unique swingable molecular interface, the affinity sensor can be used to determine the *in situ* concentration of endocrine disrupter ESTROGEN (17 β -estradiol) at concentrations ranging from 2 to 10 nM with very good reproducibility. The superior assay

1 reproducibility is responsible for the success of the EC tag method, which is a
2 quantitative method for immobilizing molecules on SCE.

3 **Keywords:** photo-excited current, estrogen receptor, affinity sensor,

4 **1. Introduction**

6 Affinity assay of biomolecules is important technique in fields such as clinical
7 diagnostics and drug discovery, because the loss of affinity of various biomolecules is
8 often an indicator of tumultuous diseases. In the pre-clinical drug discovery, lead
9 substances have been identified from large collections of compounds. Currently, the
10 affinity assay has been developed and applied in diagnostic and analytical tools. Affinity
11 assay methods are divided into two types: labeled or non-labeled affinity assay. The
12 binding assays using radioisotope [1] and fluorescence resonance energy transfer
13 (FRET) [2] are needed to label for receptors or ligands. On the other hand, the affinity
14 assays utilized enzyme-linked immunosorbent assay (ELISA) [3], electrochemical
15 luminescence (ECL) [4], surface plasmon resonance (SPR) [5] and Quartz crystal
16 microbalance (QCM) [6] are unnecessary to label for them. In this study, we focused
17 the detection methodology of non-labeled receptors and ligands using the photo-excited
18 current. Photo-excited current technology [7] has been recognized for promoting

1 photo-energy devices such as organic dye-sensitized solar cells [8-11].

2 In case of immobilization dye on the semi-conductor, the photo-excited current is
3 affected by the distance between the immobilized dye and semi-conductor because
4 photo-excited electrons are transferred from lowest unoccupied molecular orbital
5 (LUMO) of the organic pigment to the semi-conductor conductive band in the case of
6 adaption energy levels, respectively [8].

7 By a combination of the photo-excited current and our proposed concept of “swingable
8 molecular”, it is possible to actualize the novel affinity assay. We previously reported the
9 catalytic activity of an immobilized enzyme with this unique structure [12] and
10 successfully demonstrated that the swing-structured immobilized enzyme has a superior
11 catalytic activity compared to a conventional immobilized enzyme. We expect to
12 expand that the “swingable structure molecule” utilized affinity assay. Figure 1 were
13 illustrated the detection principle of the affinity assay. The assay can detect a target by
14 decrease photo-excited current detection form making a complex with the target
15 molecular and immobilized molecular including photo-excited dye.

16 In this work, the detection target is nuclear receptor which is activated by complex
17 formation with its agonist ligand. The activated nuclear receptor forms a specific
18 complex with its co-activator protein and binds to a specific DNA sequence [13-15].

1 Nuclear receptors are a transcription factors which regulate expression of
 2 hormone-responsive genes involved in embryonic development [16, 17]. Recently,
 3 nuclear receptor and their ligands have garnered attention as therapeutic targets [14,
 4 18-20]; apart from the 48 known types of nuclear receptors [21] genome analyses have
 5 revealed the presence of “orphan” receptors, the ligands of which are unknown.
 6 Moreover, the ligands of these orphan receptors could be potential candidates for novel
 7 drugs [22-24]. It is therefore important that these candidate ligands be identified even at
 8 low concentrations by screening methods. We propose a simple and quick principle of
 9 affinity assay for the nuclear receptor and its agonist ligand,
 10 However, it was difficult to fabricate the molecular interface by immobilization of the
 11 bio-affinity molecule, and therefore this approach was not feasible for the development
 12 of the affinity sensor. We previously developed the novel immobilization technique -EC
 13 tag method[25, 26] for electrodes including semi-conductors, which at the time was a
 14 technical break through. The EC tag method, in principle, is based on the
 15 electrochemical deposition of a coordinated metal ion with an acyclic peptide and can
 16 be used for the construction of a molecular interface by the design and synthesis of an
 17 acyclic peptide-tagged organic dye.
 18 We developed an affinity sensor based on a photo-excitable swingable straight-chain

1 affinity molecular layer immobilized on a semi-conductor electrode.

2 **2. Experimental Section**

3 **Chemicals and Reagents**

4 Copper chloride, potassium chloride, sodium chloride, HEPES, purified recombinant
5 human estrogen receptor (ER), and 17 β -estradiol were obtained from Wako Pure
6 Chemical Industries, LTD. (Osaka, Japan). Reagents for peptide synthesis such as the
7 amino acids were purchased from Watanabe Chemical Industries, LTD. (Hiroshima,
8 Japan) Tetra-n-propylammonium iodide was purchased by Tokyo Chemical Industry Co.,
9 LTD. (Tokyo, Japan)

10 **Design and synthesis of peptide molecules**

11 To immobilize an organic dye (Cy3) across a linker, which consists of 12 amino acids
12 (SRC1 peptide), we designed an immobilized molecule that could allow Cy3 to
13 approach the semi-conductor. The ER-activated agonist ligand enables the SRC1
14 peptide to recognize and form a complex. Peptide molecules (supplemental information,
15 Figure S1) were synthesized by a solid-phase method based on
16 fluorenylmethoxycarbonyl (Fmoc) chemistry. Detailed steps have been specified in a
17 previous study [27].

18 **Measurement of differential pulse voltammetry (DPV)**

1 In the preliminary preparation, the solution (100 μ M Cy3-PEG₂-His₆, 100 μ M CuSO₄ in
2 100 mM KCl, KNO₃) was prepared and adjusted to pH 5.0 using 100 mM KOH. The
3 working, counter, and reference electrodes were fluorine-doped tin oxide (FTO),
4 platinum wire, and Ag/AgCl in saturated KCl. DPV measurement was performed on
5 HZ3000 (HOKUTO DENKO Co., Tokyo, Japan) under conditions which pulse period,
6 pulse width, pulse height and ΔE were 60 ms, 40 ms, 30 mV, and 5 mV, respectively.

7 **Measurement of cyclic voltammetry (CV)**

8 After immobilization of 100 μ M ferrocene-PEG-His₆ (including 100 μ M CuSO₄) using
9 the EC tag method on FTO by -630 mV vs. Ag/AgCl in saturated KCl, CV was
10 measured as 20mV/sec under the condition that the counter and reference electrodes
11 were in 100 mM KCl KNO₃ platinum wire and Ag/AgCl in saturated KCl. In order to
12 calculate the electron transfer rate constant, the sweep rate of CV was changed from 50
13 to 150 mV/sec.

14 **Measurement of electrochemical impedance spectroscopy**

15 100 μ M ferrocene-PEG-His₆ (including 100 μ M CuSO₄) using the EC tag method was
16 immobilized to FTO as - 630 mV vs. Ag/AgCl in saturated KCl for 0, 2, 5, 10, 15, 20,
17 25, 30 min. The FTO, platinum wire, and Ag/AgCl in saturated KCl were used as the
18 working, counter, and reference electrodes, respectively, in 25 mM [Fe(CN)₆]^{3-/4-}.

Preparation sensor device and FTO adsorbed Cy3-SRC1

In the preliminary preparation, 15 μM SRC1 peptides, including Cy3 (Cy3-SRC1) and 15 μM CuSO_4 , were mixed, and the solution was adjusted to pH 5.0 using 100 mM KOH. To inhibit non-specific adsorption, 20 μM β -cyclodextrin was added to the solution[28]. For immobilization of Cy3-SRC1 to FTO by the EC tag method, FTO was applied at -630 mV vs. Ag/AgCl for 15 min in the solution. We defined the sensor device that was FTO immobilized Cy3-SRC1 by using the EC tag method. Molecular immobilization by using the EC tag method was confirmed by fluorescent observation using a fluorescent microscope (Nikon, Tokyo, Japan) (Figure S2).

Preparation of the formation complex between the immobilized peptide and ER

In the preliminary preparation, the ER solution was prepared by mixing agonist ligand (E2) of various concentrations in ER reaction buffer (10 mM HEPES (pH 7.4), 200 mM NaCl, 10% glycerol, 0.05% Tween 20) for 10 min. The ER concentration was adjusted to a constant 45 nM. The prepared solution was reacted with the sensor device for 10 min. Subsequently, the sensor device was washed three times using the ER reaction buffer.

Measurement of the photo-excited current

The cells of the photoexcited current was configured with a two-electrode system

consisting of FTO and platinum plate as the working and counter electrodes, respectively, as shown scheme 1 (a). Photoexcitation light which was 530 nm on 47 mW was irradiated to the sensor device, resulting in the photoexcited electron transfer as shown in scheme 1 (b).

3.Results and discussion

“Swingable function” effect to electron transfer

Immobilization proofs by EC tag method were indicated supplemental information as the immobilization potential and fluorescent image of immobilization molecular (Figure S2). Figure 2 (a) shows the cole-cole plot for the immobilization of the Ferrocene-PEG-His₆ on FTO in 25 mM [Fe(CN)₆]^{3-/4-}. The immobilization molecular inhibits the faradaic process of [Fe(CN)₆]^{3-/4-} redox[29]. Therefore, the immobilization coverage can be equivalent to the charge transfer resistance (R_{ct}) values. The R_{ct} of immobilized Ferrocene-PEG-His₆ on FTO increased with the applied immobilization potential in a time-dependent manner. We defined the immobilization coverage (θ) as:

$$\theta = 1 - R_{ct_{bare}} / R_{ct_{immobilization}}$$

R_{ct_{bare}} and R_{ct_{immobilization}} are charge transfer resistances of before and after Ferrocene-PEG-His₆ immobilization by the measurement of electrochemical impedance

spectroscopy.

Figure 2 (b) shows the relationship between immobilization time and coverage. The result indicated that the immobilization by the EC tag method was based on Langmuir isotherm, i.e. the monolayer. These results indicated that the amount of immobilization on the semi-conductor can be controlled using the EC tag method.

A cyclic voltammogram of ferrocene immobilized on the FTO electrode at a scan rate of 20 mV/sec revealed a reversible one electron redox reaction and the redox potential peaks were very close (Figure 3 (a)). Charge involved in the reaction (Q) was calculated by integrating the area under the cathode peak of the cyclic voltammogram and surface concentration of electro-active species (Γ) was determined by the following equation.

$$\Gamma = Q/nFA$$

Where n is the number of electrons as electron transfer, A is the electrode area (7.07 mm²) and F is Faraday constant. Figure 3 (b) shows the relationship between immobilization time and surface concentration of electro-active species. Γ is decreased after 15min of immobilization despite an increase in $R_{ct_{immobilization}}$. This result indicated that an increase in immobilization density inhibited of electron transfer between ferrocene to FTO.

1 Additionally, Figure 3 (b) shows the relationship between immobilization time and
2 electron transfer rate constant (k_s) that was determined by Laviron's approach[30]
3 (Figure S3) from the cyclic voltammogram as changing scan rate (20mV/sec ~ 150
4 V/sec). The k_s dramatically decreased after 15min of immobilization. Similarly, these
5 data indicated hindrance by high immobilization density. Thus, these data suggest that
6 the surface concentration electro-active species and k_s result in electron transfer by the
7 swingable function of immobilized molecules. This function mediates the easy transfer
8 of electrons from the FTO electrode; however, this transfer is hindered by increasing
9 immobilization density (Figure 4). We focused on this phenomenon and employed this
10 hindrance of electron transfer for the development of a nuclear receptor affinity sensor
11 and its agonist ligands.

12 **Definition of sensor response**

13 We prepared the sensor device which is FTO immobilized Cy3-SRC1 by EC tag method.
14 The dot line of Figure 5 is photo-excited current when non-immobilization FTO were
15 irradiated by phot-excited lay. In the case of Cy3-SRC1 immobilization on FTO by EC
16 tag method, the photo-excited current was obtained (Figure 5, black line) and decrease
17 of the current was observed from reaction of activated ER by E2 (Figure 5, gray line).
18 This suggests that the detection system of photo-excited current functioned as described

1 above (scheme 1 (b)). As expected, the ER activated by E2 forms a specific complex
2 with SRC1 immobilized on FTO. Therefore, the photo-excited electron transfer was
3 inhibited from the LUMO of Cy3 to the conductive band by steric hindrance of the
4 complex between the SRC1 peptide and activated ER by E2. We defined the sensor
5 response which is decrease rate of photo excited current at 120 second later of
6 photo-irradiation. The obtained response current had been calculated by the following
7 equation.

$$\text{Sensor response (\%)} = \{(A-C) - (B-C)\} / (B-C) * 100$$

8 A, B, C are photo-excited currents of after reaction, before reaction and non-reaction
9 detection target, respectively.

10 The time of photo-excited current stable is attributed to equilibrating electron cycle in
11 sensor circuit especially in the part of electro transfer by iodine (scheme 1 b).

12 **Specificity of Sensor response**

13 To indicate the specificity of sensor response, some molecules reacted with the sensor
14 device. Figure 6 (a) was observed high sensor response in the condition that Cy3-SRC1
15 was immobilized on FTO by EC tag method and mixed ER activated by E2 (in shown
16 the illustration under the graph). Contrastively, Figure 6 (b) was low sensor response in
17 spite of addition of ER activated by E2. Sensor surface of Figure 6 (b) was prepared by

1 non-specific adsorption of Cy3-SRC1. This result shows that the immobilization
2 method like the EC tag method is important for designing and constructing
3 molecule-immobilized affinity sensors. Moreover, Figure 6 (c) and (d) are few sensor
4 response in spite of preparation sensor surface by EC tag method. The additional
5 molecules of Figure 6 (c) and (d) are not make specific complex of SRC1 peptide.
6 These results insisted that the prepared sensor surface by EC tag method can detect and
7 evaluate the target molecule by observation of sensor response. The mixed molecular of
8 Figure 6 (c) and (d) are non-activated ER and bovine serum albumin (BSA),
9 respectively.

10 **Quantitative analysis of E2 by Sensor response**

11 Using the sensor device, we determined E2 concentrations based on the sensor response.
12 Figure 7 shows that the sensor response, at varying E2 concentrations and at a constant
13 ER concentration (45 nM), is linear E2 concentrations below 10 nM. However, at
14 concentrations higher than 20 nM, the sensor response reached a plateau. Moreover, the
15 sensor response values were linearly plotted against the E2 concentration could be used
16 to determine the E2 concentration reproducibly from 2.5 nM to 10 nM (Figure 7 (b)).
17 Limit of E2 detection are 2 nM because signal-noise (S/N) of sensor response was
18 within 3 %. Additionally, E2 containing BSA showed a similar sensor response of E2

1 without BSA (Figure S4). These result proved the reliability of sensor response
2 sufficiently because E2 concentration are determined by sensor response in spite of
3 including non-target molecular (BSA)..

4 **Stability of sensor response over a long time period**

5 We next evaluated the stability of the sensor device over a long period of time. For
6 commercialization of the sensor device, the storage stability is essential. The sensor
7 devices were preserved in 100 mM KCl, KNO₃ in a dark and cold place. The
8 photo-excited current of immobilized Cy3-SRC1 on FTO was defined as 100 % at the
9 time of strage (Figure S5). The detection of photo-excited current was maintained at a
10 100 % for about 50 days. It is therefore certainly possible to detect E2 for up to 50 days
11 using the sensor device.

12 **4.Conclusion**

13 In the present study, using the immobilized molecules capable of a swing function we
14 designed and constructed an affinity sensor. The EC tag method was used to immobilize
15 functional molecules on a semi-conductor, with the functional domain of the
16 immobilized molecule separated from the immobilization point. Moreover, the sensor
17 device detected E2, which is the agonist ligand of ER, and could quantify this ligand
18 even at low concentrations ranging from 2-10 nM. Notably, the sensor response at E2

1 concentrations below 10 nM indicated a good sensitivity and reproducibility. Moreover,
2 the sensor device can be preserved for up to 50 days under dark and cold conditions.
3 These findings potentiate the industrialization of this novel sensor device. The sensor
4 device and its detection mechanism can be applied for the high-throughput analysis of
5 novel nuclear receptor agonist ligands in clinical drug development. The method also
6 has the added advantage of the nuclear receptor and its ligand not requiring any labeling.
7 These facets of our novel sensor device should lay the foundation for development of
8 new analytical methods for studying nuclear receptor ligand in medicine. Furthermore,
9 this method would be useful for agonistic ligand screening of “orphan” nuclear
10 receptors. We expect that this method will contribute to the pharmaceutical development
11 of diagnostic tools for studying nuclear receptors.

List of abbreviation

14 Semi-conductor electrode (SCE), Estrogen receptor (ER), Fluorescence resonance
15 energy transfer (FRET), Enzyme linked immunosorbent assay (ELISA),
16 Electrochemical luminescence (ECL), Surface plasmon resonance (SPR), Quartz crystal
17 microbalance (QCM), lowest unoccupied molecular orbital (LUMO), Deoxyribonucleic
18 acid (DNA), Electrochemical tag (EC tag), Steroid receptor coactivator (SRC),

Fluorenylmethyloxycarbonyl (Fmoc), Differential pulse voltammetry (DPV), Polyethylene glycol (PEG), Cyclic voltammetry (CV), Fluorine-doped tin oxide (FTO), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Charge transfer resistance (R_{ct}), 17β -estradiol (E2), Bovine serum albumin (BSA), signal-noise (S/N).

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References

- [1] E.S. Delpassand, D.J. Yang, S. Wallace, A. Cherif, S.M. Quadri, J. Price, A. Joubert, T. Inoue, D.A. Podoloff, Synthesis, biodistribution, and estrogen receptor scintigraphy of indium-111-diethylenetriaminepentaacetic acid-tamoxifen analogue, Journal of pharmaceutical sciences, 85 (1996) 553-559.
- [2] M. Awais, M. Sato, K. Sasaki, Y. Umezawa, A genetically encoded fluorescent indicator capable of discriminating estrogen agonists from antagonists in living cells, Analytical chemistry, 76 (2004) 2181-2186.
- [3] A. Patton, M.C. Mullenix, S.J. Swanson, E. Koren, An acid dissociation bridging ELISA for detection of antibodies directed against therapeutic proteins in the presence of antigen, Journal of Immunological Methods, 304 (2005) 189-195.
- [4] M.D. Xue, T. Haruyama, E. Kobatake, M. Aizawa, Electrochemical luminescence immunosensor for alpha-fetoprotein, Sensors and Actuators B-Chemical, 36 (1996) 458-462.
- [5] D. Habauzit, J. Armengaud, B. Roig, J. Chopineau, Determination of estrogen presence in water by SPR using estrogen receptor dimerization, Analytical and Bioanalytical Chemistry, 390 (2008) 873-883.
- [6] I. Mannelli, M. Minunni, S. Tombelli, M. Mascini, Quartz crystal microbalance (QCM) affinity biosensor for genetically modified organisms (GMOs) detection, Biosensors &

- 1 Bioelectronics, 18 (2003) 129-140.
- 2 [7] Y. Matsuo, T. Ichiki, E. Nakamura, Molecular Photoelectric Switch Using a Mixed SAM
- 3 of Organic [60]Fullerene and [70]Fullerene Doped with a Single Iron Atom, Journal of the
- 4 American Chemical Society, 133 (2011) 9932-9937.
- 5 [8] L. Andrade, J. Sousa, H.A. Ribeiro, A. Mendes, Phenomenological modeling of
- 6 dye-sensitized solar cells under transient conditions, Solar Energy, 85 (2011) 781-793.
- 7 [9] M. Gratzel, Dye-sensitized solar cells, Journal of Photochemistry and Photobiology
- 8 C-Photochemistry Reviews, 4 (2003) 145-153.
- 9 [10] C. Magistris, S. Martiniani, N. Barbero, J. Park, C. Benzi, A. Anderson, C. Law, C.
- 10 Barolo, B. O'Regan, Near-infrared absorbing squaraine dye with extended pi conjugation for
- 11 dye-sensitized solar cells, Renewable Energy, 60 (2013) 672-678.
- 12 [11] X. Wang, L.J. Zhi, K. Mullen, Transparent, conductive graphene electrodes for
- 13 dye-sensitized solar cells, Nano Letters, 8 (2008) 323-327.
- 14 [12] Y. Takatsuji, R. Yamasaki, A. Iwanaga, M. Lienemann, M.B. Linder, T. Haruyama,
- 15 Solid-support immobilization of a "swing" fusion protein for enhanced glucose oxidase
- 16 catalytic activity, Colloids and Surfaces B-Biointerfaces, 112 (2013) 186-191.
- 17 [13] C. Carlberg, Ligand-mediated conformational changes of the VDR are required for gene
- 18 transactivation, Journal of Steroid Biochemistry and Molecular Biology, 89-90 (2004)
- 19 227-232.
- 20 [14] S. Christakos, P. Dhawan, Y. Liu, X.R. Peng, A. Porta, New insights into the
- 21 mechanisms of vitamin D action, Journal of Cellular Biochemistry, 88 (2003) 695-705.
- 22 [15] M.M. Gonzalez, P. Samenfild, M. Perakyla, C. Carlberg, Corepressor excess shifts the
- 23 two-side chain vitamin D analog gemini from an agonist to an inverse agonist of the vitamin
- 24 D receptor, Molecular Endocrinology, 17 (2003) 2028-2038.
- 25 [16] A. Aranda, A. Pascual, Nuclear hormone receptors and gene expression, Physiological
- 26 Reviews, 81 (2001) 1269-1304.
- 27 [17] K.S. Carmon, R.E. Baltus, L.A. Luck, A biosensor for estrogenic substances using the
- 28 quartz crystal microbalance, Analytical Biochemistry, 345 (2005) 277-283.
- 29 [18] J.L. Staudinger, K. Lichti, Cell signaling and nuclear receptors: new opportunities for
- 30 molecular pharmaceuticals in liver disease, Molecular pharmaceuticals, 5 (2008) 17-34.
- 31 [19] D. Panigrahy, A. Kaipainen, S. Huang, C.E. Butterfield, C.M. Barnes, M. Fannon, A.M.
- 32 Laforme, D.M. Chaponis, J. Folkman, M.W. Kieran, PPAR alpha agonist fenofibrate
- 33 suppresses tumor growth through direct and indirect angiogenesis inhibition, Proceedings of
- 34 the National Academy of Sciences of the United States of America, 105 (2008) 985-990.
- 35 [20] H. Yki-Jarvinen, Thiazolidinediones, The New England journal of medicine, 351 (2004)
- 36 1106-1118.

- [21] Z. Zhang, P.E. Burch, A.J. Cooney, R.B. Lanz, F.A. Pereira, J. Wu, R.A. Gibbs, G. Weinstock, D.A. Wheeler, Genomic analysis of the nuclear receptor family: new insights into structure, regulation, and evolution from the rat genome, *Genome research*, 14 (2004) 580-590.
- [22] H. Wang, W. Chu, S.K. Das, Z. Zheng, S.J. Hasstedt, S.C. Elbein, Molecular screening and association studies of retinoid-related orphan receptor gamma (RORC): a positional and functional candidate for type 2 diabetes, *Molecular genetics and metabolism*, 79 (2003) 176-182.
- [23] Y. Shi, Orphan nuclear receptors in drug discovery, *Drug discovery today*, 12 (2007) 440-445.
- [24] S. Safe, U.H. Jin, E. Hedrick, A. Reeder, S.O. Lee, Minireview: role of orphan nuclear receptors in cancer and potential as drug targets, *Molecular Endocrinology*, 28 (2014) 157-172.
- [25] T. Haruyama, Design and Fabrication of a Molecular Interface on an Electrode with Functional Protein Molecules for Bioelectronic Properties, *Electrochemistry*, 78 (2010) 888-895.
- [26] H. Sakamoto, T. Haruyama, Electrochemical preparation of junction between a molecule and solid surface through a metal coordinative peptidic tag, *Colloids and Surfaces B-Biointerfaces*, 79 (2010) 83-87.
- [27] Y. Takatsuji, S. Ikeno, T. Haruyama, Gold Nanoparticles Functionalized with Peptides for Specific Affinity Aggregation Assays of Estrogen Receptors and Their Agonists, *Sensors*, 12 (2012) 4952-4961.
- [28] R. Wakabayashi, T. Haruyama, Suppressive Method for Unspecific Adsorption on a Semiconductor Electrode in the Specific Molecular Immobilization Process Using EC tag Method, *Electrochemistry*, 80 (2012) 302-304.
- [29] J. Rickert, W. Gopel, W. Beck, G. Jung, P. Heiduschka, A 'mixed' self-assembled monolayer for an impedimetric immunosensor, *Biosensors & bioelectronics*, 11 (1996) 757-768.
- [30] E. Laviron, General Expression of the Linear Potential Sweep Voltammogram in the Case of Diffusionless Electrochemical Systems, *Journal of Electroanalytical Chemistry*, 101 (1979) 19-28.

Figure and Scheme captions

Figure 1. Illustration of the electron transfer inhibition by the binding of ER-activated E2 to Cy3-SRC1 immobilized on a semi-conductor.

Figure 2. Immobilization of ferrocene-PEG-His₆ by the EC tag method (a) Cole-cole plots of the immobilization of ferrocene-PEG-His₆ against time. (b) Coverage against immobilization time

Figure 3. Electron transfer of immobilized ferrocene (a) Cyclic voltammogram of immobilized ferrocene-PEG-His₆ on FTO, (b) relationship between immobilization time, surface concentration of electro-active species and electron transfer rate constant (ks).

Figure 4. Cartoon of electron transfer limit by steric hindrance of immobilization molecular.

Figure 5. Photo-excited current measurements: A (black line); sensor device, B (gray line); sensor device reacted ER activated E2, C (dot line); FTO

Figure 6. Measurements of sensor response: (a) sensor device reacted ER-activated E2,

1 (b) FTO adsorbed Cy3-SRC1 reacted ER activated E2, (c) sensor device-reacted ER, (d)
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7 sensor device-reacted BSA.

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12 4 Figure 7. Quantitative determination of E2 by sensor response: E2 concentration
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25 8 Scheme 1 (a) Illustration of cell configuration for affinity sensor using photo-excited
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Figure 1

Figure 2

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Figure 3

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(b)

Figure 4

Figure 5

Figure 6

Figure 7

(a)

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