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Improved Stability of Continuous Operation of Lactate Oxidase-hydrogel Electrodes Using Os(bipyridine)$_2$Cl-pendant Poly(vinyl Imidazole)

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Hydrogel-modified redox electrodes are fabricated using lactate oxidase and a poly(vinyl imidazole) polymer containing an Os(bipyridine)$_2$Cl complex to facilitate the improved continuous measurement of lactate concentration. The stability of a MgO-templated carbon modified with a hydrogel was improved by quaternizing 10 mol% of the imidazole content of poly(vinyl imidazole) with bromoethylamine. Stability was further improved by cross-linking the polymer using ethylene glycol diglycidyl ether and sorbitol polyglycidyl ether, which increased the density of the hydrogel structure.

1. Introduction

High demand for statistics regarding lactate production from the healthcare, sport, and food industries necessitates the accurate, preferably continuous measurement of lactate concentration.[1,2] Although invasive lactate sensors are available, frequent or continuous measurements of lactate concentration remain difficult. Therefore, minimally invasive or noninvasive strategies for the continuous monitoring of lactate concentration have attracted considerable attention.[3–8]

Lactate oxidase (LOx) is used as a catalyst for the selective oxidation of lactate in electrochemical lactate sensors.[3,4,6,8,9] Disposable sensors typically contain ferricyanide as a redox mediator, which shuttles electrons from the LOx active center to the electrode.[1,10] Many continuous sensors measure oxygen reduction activity or the amount of hydrogen peroxide generated by the oxidation of lactate by LOx to determine the lactate concentration.[1,11–14] The generated hydrogen peroxide is typically reduced by a Pt or Prussian blue electrode, whereas the hydrogen peroxide reduction current is used to monitor the lactate concentration. However, the measurable dynamic range of lactate concentration is limited by the oxygen concentration supplied and the damage caused to the enzyme by the accumulation of hydrogen peroxide.

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Long-term, stable measurements can be achieved over a wide range of concentrations using an alternative system involving a mediator.[6] Sode and colleagues have recently modified the surfaces of LOx with amine-reactive mediators.[15] The mediator must be added to an appropriate position on the enzyme to enable the transport of electrons to the electrode. Additionally, the stability of the mediator is critical to ensure the long-term continuous operation of the sensor. Heller pioneered the use of hydrogel technology, which uses a redox polymer containing an Os complex, in electrochemical sensors.[16–19] With the use of a cross-linker, a hydrogel matrix is formed between redox enzymes and poly(vinyl imidazole) containing an Os(bipyridine)$_2$Cl complex (PVI-Os) on the electrode surface. The Os complex exhibits a redox potential that can be tuned to the desired potential for sensor applications, and both its oxidized and reduced forms exhibit exceptionally high stability.

The LOx–hydrogel can be stably immobilized; however, immobilization halves the electrode response within a few hours. Durability must therefore be improved, particularly for implantable/wearable sensors, to expand the application of this hydrogel and enable the long-term continuous measurement of lactate concentration. The lactate electrode is less stable than the glucose oxidation electrode with the same Os-polymer (PVI-Os), suggesting that the long-term stability of the electrode is determined either by the durability of the enzyme structure itself, by the elution of the enzyme or polymer from the electrode surface, or by the stability of the enzyme–polymer complex. The cross-linking of the enzyme with the polymer forms a hydrogel structure on the electrode. If the degree of cross-linking is insufficient, the enzyme and hydrogel detach from the electrode.

We previously reported that the use of porous carbon can improve the stability of glucose dehydrogenase hydrogel-modified electrodes.[20–22] In this study, the effect of the structure of the PVI-Os and the cross-linking agent on the stability of the hydrogel electrode response was investigated using the porous carbon electrode. The reaction of poly(vinyl imidazole) with alkyl halides containing various functional groups was employed to alter the properties of the polymer.[16,23–25] The cross-linking of enzymes and redox polymers was previously achieved using poly(ethylene glycol) diglycidyl ether (PEGDGE). In this work, ethylene glycol diglycidyl ether (EDGDE) and sorbitol polyglycidyl ether (SPGE) were employed as cross-linkers.

2. Materials and Methods

2.1 Materials

PVI-Os[17] and LOx (from *Enterococcus faecium*)[6,26] were prepared according to previously reported methods. MgO-templated porous carbon (MgOC, CNovel®, 150 nm pore size) was purchased from Toyo Carbon (Japan).[21] Polyvinylidene difluoride [PVdF, #9305, 5% in N-methylpyrrolidone (NMP)] was purchased from Kureha (Japan). EDGDE, NMP, and 2-propanol were purchased from Fujifilm Wako Pure Chemical Corp. (Japan). SPGE was kindly donated by Nagase Chemtex (Denacol 614B, Japan).

MgOC (40 mg) and PVdF (200 μL) were added to NMP (400 μL) and mixed for 2 min using an ultrasonic homogenizer (UH-50, S.M.T. Co., Ltd.). MgOC ink (2 μL) was applied to the

surface of a clean GC disc electrode (3 mm diameter), and the electrode was then dried in an oven at 60 °C for 16 h to obtain the MgOC electrode.

LOx (20.0 mg/mL), PVI-Os (9.5 mg/mL), and PEGDGE (8.0 mg/mL) were mixed in a microtube at a weight ratio of LOx:Os-polymer:cross-linker of 20:70:10 to form the hydrogel solution, which was subsequently deposited onto the plasma-treated MgOC electrode. The amount of hydrogel modified was calculated exclusively on the basis of the weight of the solutes. The electrodes were then dried at 25 °C for 16 h in a dry box (McDry, ERC Co., Ltd., Japan) with a humidity level of 1%. The electrodes were immersed in a pH 7.0 phosphate buffer solution for 30 min before measurements to clean the electrode surface. The electrodes were evaluated by cyclic voltammetry and chronoamperometry (CA) using a three-electrode system with counter and reference electrodes consisting of a Pt wire and a Ag/AgCl electrode, respectively. An air-saturated phosphate buffer solution (pH 7.0, 0.1 M) containing lactate (0.1 M) was used as an electrolyte solution. CA measurements were taken using a potentiostat (HA-151B, Hokuto Denko Co., Ltd., Japan) with a voltage of 0.6 V [vs Ag|AgCl|KCl (sat.)] applied to the working electrode.

2.2 Quaternization reaction of imidazole with alkyl halides

PVI-Os (1.0 mL, 9.5 mg/mL), methanol (0.4 mL), and a quaternization reagent [either 2-bromoethylamine (1.15 mg), bromoethane (0.61 mg), iodomethane (0.80 mg), or bromoacetic acid (0.78 mg)] were mixed in a sealed glass bottle and heated in an oven at 45 °C for 24 h. The solvents were then volatilized at 70 °C using a rotary evaporator before distilled water (1.0 mL) was added to dissolve the precipitate. The quaternization ratio of 2-bromoethylamine was changed to 10, 25, 50, and 75% by modifying the mass of 2-bromoethylamine to 1.15, 2.88, 5.76, and 8.64 mg, respectively.

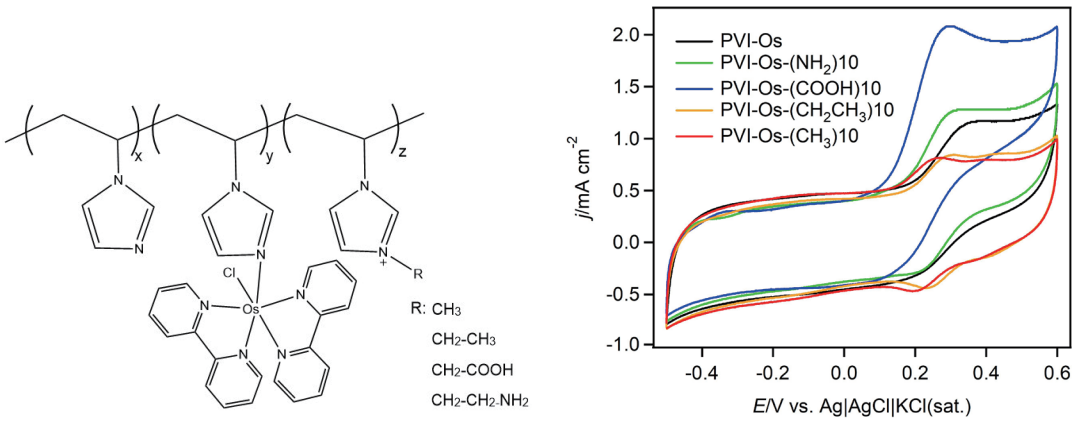
2.3 Cross-linking reagent

The enzyme, redox polymer, and cross-linker were mixed in a microcentrifuge tube at a LOx:Os-polymer:cross-linker weight ratio of 20:70:10. PEGDGE, EGDGE, and SPGE in aqueous solutions (8.0 mg/mL) were used as cross-linkers, and the mixed solution was then dropped onto the plasma-treated MgOC electrode. The amount of hydrogel added to the electrode was calculated on the basis of the weight of the solvent, excluding that of water. The electrodes were then dried at 25 °C for 16 h in a dry box with a humidity level of 1%.

3. Results and Discussion

3.1 Quaternization reaction of imidazole with alkyl halides

2-Bromoethylamine, bromoethane, iodomethane, and bromoacetic acid were added to nitrogen at the imidazole 3-position in poly(vinyl imidazole) and quaternized to introduce amino, ethyl, methyl, and carboxy groups, respectively. Figure 1(a) shows the structure of PVI-



(a) (b)

Fig. 1. (Color online) (a) Structure of quaternized PVI-Os. (b) CVs of the MgOC electrodes modified with quaternized polymers bearing different functional groups. LOx, PVI-Os, and PEGDGE in a ratio of 20:70:10 were deposited on the MgOC electrode at 600 μg cm^{-2}. Measurement conditions: 10 mV s^{-1}, pH 7.0, phosphate buffer solution (0.1 M) containing lactate (0.1 M), and 25 °C.

Os and quaternized PVI-Os. The quaternization percentage was defined as the percentage of imidazole quaternized without osmium [$= z/(x + z) \times 100$, in Fig. 1(a)]. Herein, the quaternization percentage of each of the four different polymers was set to 10%, and the resultant polymers were named PVI-Os-(NH_2)10, PVI-Os-(CH_3)10, PVI-Os-(CH_2CH_3)10, and PVI-Os-(COOH)10. Each of the quaternized polymers was mixed with LOx and PEGDGE at a LOx:PVI-Os:PEGDGE ratio of 20:70:10, and the MgOC electrode was modified using 600 μg cm^{-2} of the hydrogel. Figure 1(b) shows the cyclic voltammograms (CVs) obtained during lactate oxidation on the LOx–hydrogel MgOC electrode modified with the above-mentioned polymers. The current density on the PVI-Os-(NH_2)10-hydrogel electrode during lactate oxidation was higher than that on the PVI-Os electrode. The current on the PVI-Os-(COOH)10 electrode was larger than those on other electrodes. However, modification with PVI-Os-(CH_3)10 and PVI-Os-(CH_2CH_3)10 resulted in a significant reduction in lactate oxidation current. The redox signal of the Os complex was clearly observed in the PVI-Os-(CH_3)10 and PVI-Os-(CH_2CH_3)10-based electrodes, indicating that electron transfer between LOx and the Os complex tethered to the polymer backbone was significantly inhibited. This may be explained by the phase separation between the polymer and LOx during mixing. The chronoamperogram of the PVI-Os-(NH_2)10-based electrode (Fig. 2) shows that after measurement, its residual current density was approximately 80%, which was higher than that of the PVI-Os-based electrode (60%). The PVI-Os-(COOH)10-based electrode exhibited a comparatively high initial current density, but the residual current after the measurement was only approximately 30%. The introduction of ethyl and methyl groups resulted in a significant reduction in current density, with virtually no current being observed. These results demonstrate that the quaternization of PVI-Os with 2-bromoethylamine and the introduction of amino groups improve current density and electrode stability.

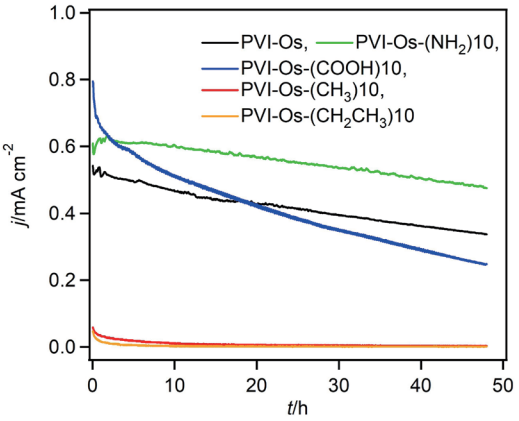


Fig. 2. (Color online) CAs of the MgOC electrode modified with quaternized polymers bearing different functional groups. Measurement conditions: 0.6 V, pH 7.0, phosphate buffer solution (0.1 M) containing lactate (0.1 M), and 25 °C.

3.2 Optimization of quaternization ratio of PVI-Os

The quaternization ratio of PVI-Os was optimized by performing the quaternization reaction with 10, 25, 50, and 75% 2-bromoethylamine. The resulting polymers were named PVI-Os-(NH_2)10, PVI-Os-(NH_2)25, PVI-Os-(NH_2)50, and PVI-Os-(NH_2)75. A mixture of LOx, PVI-Os-(NH_2), and PEGDGE (in a 20:70:10 weight ratio) was deposited on the MgOC electrode at 600 μg cm^{-2} and evaluated by CA. Figure 3 shows the 48 h chronoamperograms of the LOx–hydrogel electrodes using PVI-Os, PVI-Os-(NH_2)10, PVI-Os-(NH_2)25, PVI-Os-(NH_2)50, and PVI-Os-(NH_2)75 as redox polymers during lactate oxidation. The initial and residual currents decreased with an increasing quaternization ratio. PVI-Os–(NH_2)10 exhibited the highest response current and stability, retaining 80% of the residual current after 48 h. PVI-Os-(NH_2)25, PVI-Os-(NH_2)50, and PVI-Os-(NH_2)75 exhibited response currents at 70, 60, and 45% of the initial currents, respectively. These trends in current response and stability arise from the introduction of positive charges into the PVI-Os by quaternization. These charges enable the formation of an electrostatic adduct with the negatively charged LOx, thus forming a highly dense hydrogel structure on the electrode. However, the residual current decreased as the quaternization ratio increased above 10%, reaching a minimum of approximately 50% residual current after 48 h at a quaternization ratio of 75%. One of the possible reasons is that the increased hydrophilicity of the polymer may promote the desorption of the hydrogel from the carbon surface. Another reason is that the introduction of excess amino groups and positive charges into the polymer may reduce its redox activity. The electrons received from the enzyme are transferred to the electrode by hopping along the redox centers of the PVI-Os, and the high number of positively charged imidazole groups may cause electrostatic repulsion within the polymer, thereby inhibiting electron hopping. To investigate the effect of the introduction of a positive charge on the electron transfer, the CVs of PVI-Os-(NH_2)10-and PVI-Os-(NH_2)75-based hydrogel electrodes were measured in phosphate buffer solution without lactate. The CV of the PVI-Os-(NH_2)75-based electrode showed a smaller peak corresponding to the Os complex

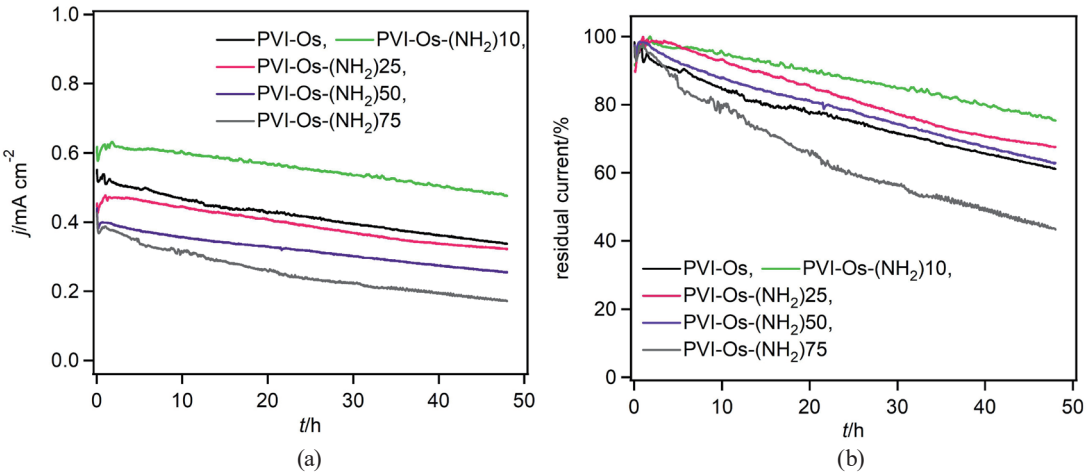


Fig. 3. (Color online) (a) Chronoamperograms of LOx–hydrogel-modified MgOC electrodes using PVI-Os-(NH_2) at various quaternization ratios. LOx, PVI-Os, and PEGDGE in a ratio of 20:70:10 were deposited on the MgOC electrode at 600 μg cm^{-2}. Measurement conditions: 0.6 V, pH 7.0, phosphate buffer solution (0.1 M) containing lactate (0.1 M), and 25 °C. (b) Residual current vs time.

than did the CV of the PVI-Os-(NH_2)10 electrode (data not shown), suggesting that an increase in quaternization ratio reduces the number of electrochemically active Os complexes. The excess amino groups may promote cross-linking between redox polymers and reduce the effective cross-linking between the polymer and the enzyme.

3.3 Effect of cross-linking reagent

The effects of the molecular weight and structure of the cross-linker on the durability of the hydrogel were investigated. PEGDGE is a widely used cross-linking reagent. In this study, PEGDGE was used alongside alternative reagents EGDGE and SPGE. The molecular weights of PEGDGE, EGDGE, and SPGE are 500, 174, and 275 g mol^{-1}, respectively. SPGE contains four glycidyl groups and has the smallest epoxy equivalent of 68 g eq^{-1}. A mixture of LOx, PVI-Os-(NH_2)10, and PEGDGE/EGDGE/SPGE (in a 20:70:10 weight ratio) was deposited onto the MgOC electrode at 600 μg cm^{-2} and evaluated by CA over 48 h.

Figure 4 shows the 48 h chronoamperograms of the LOx–hydrogel formed by different cross-linking agents. The SPGE-based electrode shows the highest lactate oxidation current response. The molecular weight of SPGE is larger than that of EGDGE; moreover, in contrast to the linear structures of PEGDGE and EGDGE, SPGE has a branched structure with each branch containing an epoxy group. This structure enables SPGE to form a finer network structure and thus achieve a higher cross-link density. A residual current of 84 ± 1% of the initial was observed in the SPGE-based electrode after 48 h; this percentage was higher than that measured for the electrode using the hydrogel formed with the PEGDGE cross-linker. With EGDGE, the initial and residual currents after 48 h were similar to that exhibited by the SPGE-based electrode. The high residual current density observed is likely due to the high cross-linking density and fine network structure of the hydrogels, as well as the suppression of enzyme desorption from the electrode surface.

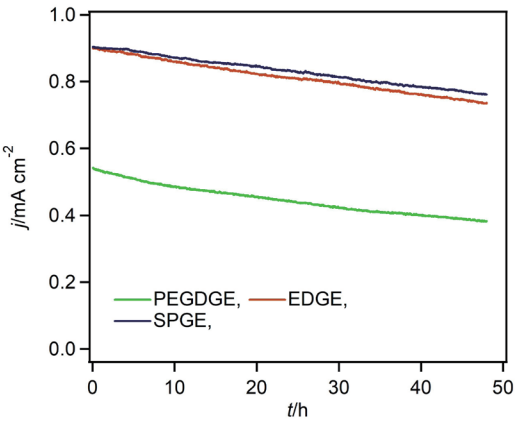


Fig. 4. (Color online) Chronoamperograms of LOx–hydrogel-modified MgOC electrodes with different cross-linkers. LOx, PVI-Os-(NH_2)10, and PEGDGE/EGDGE/SPGE (in a 20:70:10 weight ratio) were deposited on the MgOC electrode at 600 μg cm^{-2}. Measurement conditions: 0.6 V, pH 7.0, phosphate buffer solution (0.1 M) containing lactate (0.1 M), and 25 °C.

4. Conclusions

The long-term continuous operational durability of hydrogel-modified redox electrodes based on LOx and a poly(vinyl imidazole) polymer containing an Os(bipyridine)$_2$Cl complex was evaluated. The structures of the polymer and cross-linking agent were adopted to form a hydrogel film that is tightly packed with the enzyme. The quaternization of 10% of the imidazole in the side chain of the polymer backbone with ethylamine resulted in optimum LOx activity. Additionally, a cross-linker with a small epoxy equivalent improves the current density and stability. This new polymer modification strategy and cross-linker optimization is expected to be applicable to other hydrogel-based electrodes.

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References

1 L. Rassaei, W. Olthuis, S. Tsujimura, E. J. R. Sudhölter, and A. van den Berg: Anal. Bioanal. Chem. **406** (2014) 123. https://doi.org/10.1007/s00216-013-7307-1
2 I. Shitanda and S. Tsujimura: J. Phys. Energy **3** (2021) 032002. https://doi.org/10.1088/2515-7655/abebcb
3 W. Jia, A. J. Bandodkar, G. Valdés-Ramírez, J. R. Windmiller, Z. Yang, J. Ramírez, G. Chan, and J. Wang: Anal. Chem. **85** (2013) 6553.https://doi.org/10.1021/ac401573r
4 W. Gao, S. Emaminejad, H. Y. Y. Nyein, S. Challa, K. Chen, A. Peck, H. M. Fahad, H. Ota, H. Shiraki, D. Kiriya, D.-H. Lien, G. A. Brooks, R. W. Davis, and A. Javey: Nature **529** (2016) 509. https://doi.org/10.1038/nature16521
5 S. Anastasova, B. Crewther, P. Bembnowicz, V. Curto, H. M. D. Ip, B. Rosa, and G.-Z. Yang: Biosens. Bioelectron. **93** (2017) 139. https://doi.org/10.1016/j.bios.2016.09.038
6 I. Shitanda, M. Mitsumoto, N. Loew, Y. Yoshihara, H. Watanabe, T. Mikawa, S. Tsujimura, M. Itagaki, and M. Motosuke: Electrochim. Acta **368** (2021) 137620. https://doi.org/10.1016/j.electacta.2020.137620

7 Q. Zhang, D. Jiang, C. Xu, Y. Ge, X. Liu, Q. Wei, L. Huang, X. Ren, C. Wang, and Y. Wang: Sens. Actuators, B **320** (2020) 128325. https://doi.org/10.1016/j.snb.2020.128325
8 L. J. Currano, F. C. Sage, M. Hagedon, L. Hamilton, J. Patrone, and K. Gerasopoulos: Sci. Rep. **8** (2018) 15890. https://doi.org/10.1038/s41598-018-33565-x
9 K. Rathee, V. Dhull, R. Dhull, and S. Singh: Biochem. Biophys. Rep. **5** (2016) 35. https://doi.org/10.1016/j.bbrep.2015.11.010
10 H. Uenoyama, H. Okuda, and M. Aizawa: Sens. Actuators, B **14** (1993) 657. https://doi.org/10.1016/0925-4005(93)85132-T
11 K. Nagamine, T. Mano, A. Nomura, Y. Ichimura, R. Izawa, H. Furusawa, H. Matsui, D. Kumaki, and S. Tokito: Sci. Rep. **9** (2019) 10102. https://doi.org/10.1038/s41598-019-46611-z
12 N. A. Hirst, L. D. Hazelwood, D. G. Jayne, and P. A. Millner: Sens. Actuators, B **186** (2013) 674. https://doi.org/10.1016/j.snb.2013.06.090
13 P. J. Lamas-Ardisana, O. A. Loaiza, L. Añorga, E. Jubete, M. Borghei, V. Ruiz, E. Ochoteco, G. Cabañero, and H. J. Grande: Biosens. Bioelectron. **56** (2014) 345. https://doi.org/10.1016/j.bios.2014.01.047
14 R. Garjonyte, Y. Yigzaw, R. Meskys, A. Malinauskas, and L. Gorton: Sens. Actuators, B **79** (2001) 33. https://doi.org/10.1016/S0925-4005(01)00845-0
15 K. Hiraka, K. Kojima, W. Tsugawa, R. Asano, K. Ikebukuro, and K. Sode: Biosens. Bioelectron. **151** (2020) 111974. https://doi.org/10.1016/j.bios.2019.111974
16 B. A. Gregg and A. Heller: Anal. Chem. **62** (1990) 258. https://doi.org/10.1021/ac00202a007
17 T. J. Ohara, R. Rajagopalan, and A. Heller: Anal. Chem. **65** (1993) 3512. https://doi.org/10.1021/ac00071a031
18 T. J. Ohara, R. Rajagopalan, and A. Heller: Anal. Chem. **66** (1994) 2451. https://doi.org/10.1021/ac00087a008
19 A. Heller and B. Feldman: Chem. Rev. **108** (2008) 2482. https://doi.org/10.1021/cr068069y
20 A. Suzuki and S. Tsujimura: Chem. Lett. **45** (2016) 484. https://doi.org/10.1246/cl.160053
21 S. Tsujimura and S. Takeuchi: Electrochim. Acta **343** (2020) 136110. https://doi.org/10.1016/j.electacta.2020.136110
22 S. Tsujimura, K. Murata, and W. Akatsuka: J. Am. Chem. Soc. **136** (2014) 14432. https://doi.org/10.1021/ja5053736
23 A. Aoki, R. Rajagopalan, and A. Heller: J. Phys. Chem. **99** (1995) 5102. https://doi.org/10.1021/j100014a034
24 R. Rajagopalan, A. Aoki, and A. Heller: J. Phys. Chem. **100** (1996) 3719. https://doi.org/10.1021/jp952160g
25 H. I. Unal, O. Erol, and O. Y. Gumus: Colloids Surf., A **442** (2014) 132. https://doi.org/10.1016/j.colsurfa.2013.04.054
26 I. Shitanda, K. Takamatsu, A. Niiyama, T. Mikawa, Y. Hoshi, M. Itagaki, and S. Tsujimura: J. Power Sour. **436** (2019) 226844. https://doi.org/10.1016/j.jpowsour.2019.226844