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Protein Engineering

Complete Oxidation of Methanol in Biobattery Devices Using a Hydrogel Created from Three Modified Dehydrogenases**

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Protein engineering involves the manipulation of amino acids to improve the properties of proteins. Breakthroughs are still being reported in the design and improvement of enzymes as well as efforts to improve structural proteins for biomaterials applications. Various protein and peptide domains have been engineered to create new functional materials for a variety of applications.[1] Here we report an advancement of this approach where we create a new catalytic biomaterial by engineering of three dehydrogenase enzymes for self-assembly. When combined, the resulting new catalytic biomaterial is able to fully oxidize methanol to carbon dioxide and we demonstrate the application of this material as an anode modification in two types of enzymatic biobattery devices.[2]

Hydrogels can be created from proteins and peptides by outfitting them with cross-linking domains. Pioneering work by Tirrell and co-workers demonstrated that alpha-helical leucine zipper domains could be used to create peptides that self-assemble into hydrogels through coiled-coil interactions,[3] and we have expanded on this line of research by demonstrating that these domains can be appended to globular proteins.[4] These hydrogel constructs are cross-linked through both the coiled-coil motifs formed by the appended leucine zipper domains and through additional protein/protein interactions because of the quaternary structure of the proteins. So far, we have described the addition of helical appendages to fluorescent proteins,[4a] a thermostable alcohol dehydrogenase,[4b] an organophosphate hydrolase enzyme,[4c] and a small laccase enzyme.[4d] When the latter enzyme was combined with osmium-modified peptides, a bioelectrocatalytic hydrogel was formed that could reduce oxygen to water and could function as a cathode modification for a biobattery or enzymatic biofuel cell.[4d] In almost every case, the addition of the helical appendages has had a minimal impact on the catalytic activity of the enzymes, and robust hydrogels have been demonstrated.

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Here we extend this approach to create an enzymatic hydrogel that supports a functional synthetic metabolic pathway. Three NAD(H)-dependent dehydrogenase enzymes from different sources were modified for self-assembly. The first enzyme was a tetrameric alcohol dehydrogenase (ADH) from *Bacillus stearothermophilus* which oxidizes methanol to formaldehyde.[5] The second enzyme was a tetrameric human aldehyde dehydrogenase (ALDH2) which oxidizes formaldehyde to formate.[6] The final enzyme, a dimeric formate dehydrogenase (FDH1) from *Saccharomyces cerevisiae,* oxidizes formate to CO_2.[7] When combined these enzymes produce a synthetic metabolic pathway capable of the complete oxidation of methanol.[8] A schematic diagram of this reaction is as shown in Figure 1 a.

An alpha-helical leucine zipper domain (H) and randomly structured soluble peptide domain (S) were genetically appended to the N-termini of each of the three dehydrogenase genes. The three new bifunctional enzyme constructs (HSADH, HSALDH2, and HSFDH1) were overexpressed in *E. coli* and purified as described in the Supporting Information. HSADH and HSFDH1 were readily expressed and purified, while the HSALDH2 enzyme required the addition of the maltose binding protein (MBP) to enable functional expression. An intein domain was added between the MBP and HSALDH2 such that it spontaneously cleaved after expression within the cells and thus the HSALDH2 protein could be purified as though no fusion protein had been included.[9]

The kinetics of the purified bifunctionalized enzymes, all of which follow the ordered bi-bi kinetic mechanism, were measured in dilute solution to determine the impact of modifications on the kinetic parameters (Table 1 and Figure S3 in the Supporting Information). The kinetic parameters of the HSALDH2 enzyme were similar to those reported in the literature, while unexpectedly the kinetic parameters of both the HSADH and HSFDH1 enzymes were both found to be improved by the addition of the helical appendages. Both modified enzymes showed significant increases in catalytic efficiency (k_{cat}/K_m) as compared to the published values for the wild-type enzymes. The Michaelis constant (K_m) for the substrate of HSADH was three orders of magnitude smaller than reported for the unmodified enzyme while the k_{cat} value was found to increase by 120-fold. As a result, the catalytic efficiency (k_{cat}/K_m) of HSADH was increased six orders of magnitude. The catalytic efficiency of HSFDH1 was found to be increased by two orders of magnitude compared to literature values. The change in K_m for the substrate was not significantly different, but the k_{cat} value was two orders of magnitude higher than for the unmodified enzyme. We have previously observed that the addition of the helical appen-

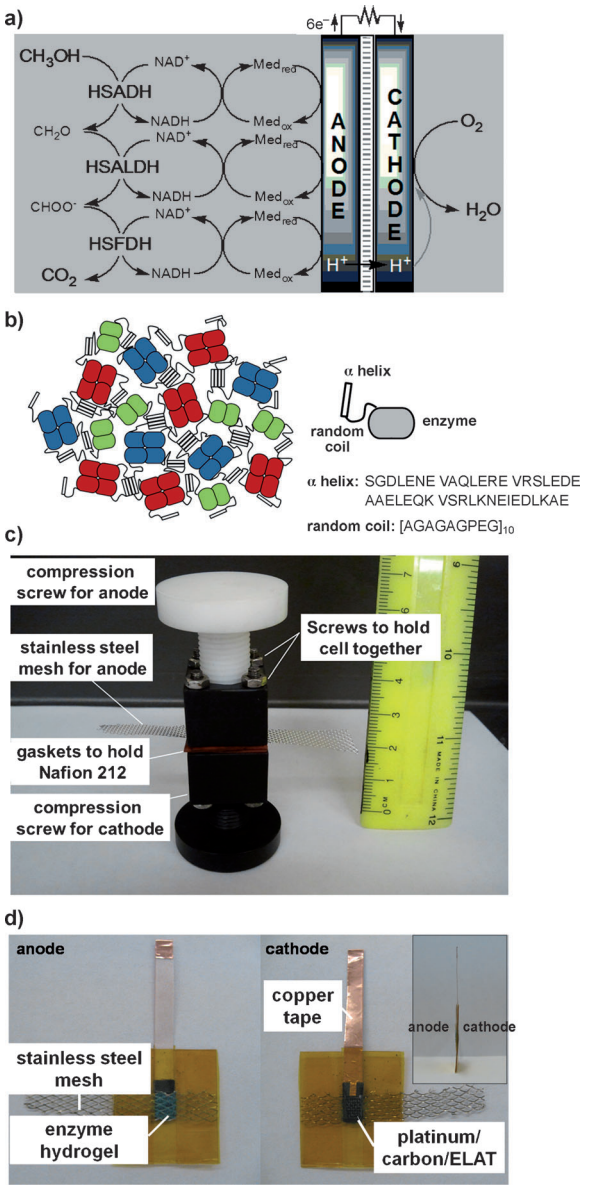

Figure 1. a) Schematic diagram of the methanol/O_2 enzymatic biobattery. b) Left: Schematic representation of the physically cross-linked enzyme hydrogel (red: ADH, purple: ALDH2, and green: FDH1). Right: Schematic representation of the protein modification where an α helix and random coil are added to the N-terminus of each monomer. c) The methanol/Prussian blue biobattery. d) The methanol/O_2 enzymatic biobattery developed to use the self-assembled protein hydrogel on the anode.

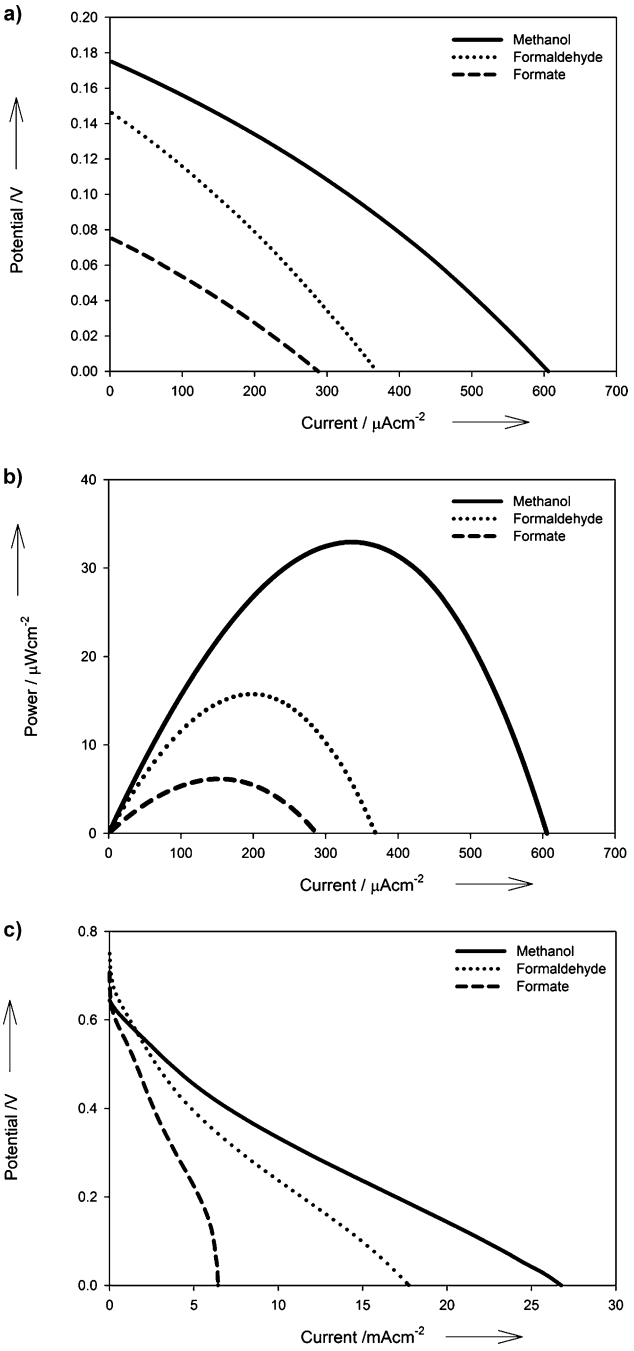

Figure 2. Representative polarization (a) and power curves (b) for the type 1 biobattery and polarization curve (c) for the type 2 biobattery when different fuels are added to the anode. Anode and cathode electrode areas for the type 1 biobattery were 0.785 cm^2 and the scan rate was 1 mVs^{-1}. Anode and cathode areas for the type 2 biobattery were 0.125 cm^2 and the scan rate was 100 mVs^{-1}.

dages to both ends of a different alcohol dehydrogenase enzyme resulted in almost no impact on the kinetic parameters.[4b] We have also observed that the addition of an N-terminal helical appendage can dramatically improve the functional expression of an organophosphate hydrolase enzyme.[4c] A similar increase in specific activity could partially explain the improvements in the k_{cat} values observed, as this modification could result in an increase in the amount of active enzymes that are purified. However, further experiments will be needed to prove this hypothesis.

Each of the newly bifunctionalized enzymes was purified, concentrated, (Figure S5 in the Supporting Information) and shown to be able to form a hydrogel (3.4 mM protein in 100 mM phosphate buffer pH 7.0) as assessed by an inversion test as well as by the ability of the material to retain its shape (Figure S5 in the Supporting Information). The hydrogels formed by the three enzymes as well as a hydrogels formed by a mixture of the three enzymes were very similar to the hydrogels formed by all other globular proteins we have previously investigated. Since all three enzymes are multi-

Table 1: Kinetic parameters of hydrogel-forming enzymes for the oxidation of methanol (HSADH), formaldehyde (HSALDH), and formate (HSFDH).

	k_{cat} [s^{-1}]	$K_{m,S}$ [mM]	K_{m,NAD^+} [mM]	k_{i,NAD^+} [mM]
HSADH[a]	7.2 ± 0.5	$(12 \pm 10) \times 10^{-3}$	$(7.6 \pm 2.6) \times 10^{-3}$	$(69.5 \pm 72.8) \times 10^{-3}$
ADH[b]	0.06	20	–	–
HSALDH2[a]	58.3 ± 19.8	21.7 ± 12.1	0.14 ± 0.18	0.21 ± 0.14
ALDH2[c]	67.5 ± 8.3	0.32 ± 0.08	–	–
ALDH2[d]	21.8	0.42 ± 0.08	–	–
HSFDH1[a]	1670 ± 80	7.2 ± 1.5	0.8 ± 0.1	1.7 ± 0.6
FDH1[e]	6.5 ± 0.4	5.5 ± 0.3	0.036 ± 0.005	–

[a] $T = 37$ °C, pH 8.5. [b] $T = 37$ °C, pH 7.0.[5] [c] $T = 25$ °C, pH 9.5.[6a] [d] T = ambient temperature, pH 9.5.[6b] [e] $T = 30$ °C, pH 7.0.[7]

mers, the hydrogels are cross-linked through protein/protein interactions in addition to the coiled-coil interactions added by the helical appendages (Figure 1b).

To create the metabolic pathway, a mixture of the three enzymes was made with equal activity of each enzyme (1 U each, where 1 U = 1 μmol min^{-1}) and this mixture was used to create anodes for use in two different enzymatic biobattery configurations. The first type of biobattery (type 1) is based on a previously published design[10] where the enzyme mixture in a dilute, non-hydrogel-forming concentration (2.5 wt%) is mixed with methylene blue, NAD^+, Na_2SO_4, carbon black, and carbon fiber and applied to a stainless-steel mesh, and this anode is combined with a Prussian blue cathode in a battery test cell with a Nafion proton exchange membrane (PEM; Figure 1c). The type 2 device was newly developed to take advantage of the hydrogel-forming ability of the enzymes. A small hydrogel (25 wt%) was formed by dissolving the lyophilized enzymes along with NAD^+, methylene blue, and Na_2SO_4. This material was spread on a stainless-steel mesh anode. A commercially prepared air-breathing cathode was assembled with a Nafion PEM and pressed to the anode (Figure 1d). Methylene blue is used to lower the overpotential required for the electrochemical oxidation of NADH at the electrode (Figure 1a).[11]

The three enzyme metabolic pathway was designed to fully oxidize methanol to CO_2 through formaldehyde and formate intermediates with a concomitant generation of 6 electrons per molecule of methanol. By operating the complete biobatteries with the two intermediates as fuels, the functionality of all three enzymes in the pathway can be investigated.

Biobattery type 1 used the enzymes in a dilute solution (2.5 wt%), which reduces transport issues, and allows for larger volumes of fuels to be used. The polarization curves for the type 1 biobattery show that the current increases as the reduction state of the fuel is increased from formate up to methanol, indicating that all three enzymes are functioning in the anodic pathway (Figure 2ab and Table 2). The maximum power density (shown in Figure 2b) also increases as the number of enzymes involved in the reaction increases. As expected, the highest power density (32.7 ± 0.2 μW cm^{-2}) and current density (577 ± 26 μA cm^{-2}) were observed when methanol was used in the biobattery.

The type 2 biobattery was constructed to take advantage of the hydrogel-forming ability of the engineered proteins (Table 3). To maintain the hydrogel state of the proteins (25 wt% protein), only a small amount of liquid fuel was added to the hydrogel, and a fast scan rate (100 mV s^{-1}) was required as the high loading of the enzymes in the anode caused the fuel to be consumed rapidly. As was seen in the type 1 biobattery design, the polarization curves (Figure 2c) increased with the different fuels, and the maximum current density (26.4 ± 1.8 mA cm^{-2}) was obtained with methanol fuel. There is a shape change between the polarization curves of the two biobatteries, as well as a difference in the open circuit potentials. The open circuit potential of the type 2 biobattery is considerably higher, because the oxygen cathode has a higher standard reduction potential than the Prussian blue cathode. The differences in shapes between the polarization curves are interesting. There is a kinetic polarization in the type 2 biobattery associated with the poor kinetics of oxygen reduction on platinum, but this is not observed in the Prussian blue cathode. On the other hand, the type 1 biobattery has higher resistance resulting in lower current densities.

Table 2: Average values for the maximum power density, current density, and open circuit voltage measured with three fuels in biobattery type 1 (2.5 wt% protein, Prussian Blue cathode, 1 mV s^{-1} scan rate).

	Maximum power density [μW cm^{-2}]	Maximum current density [μA cm^{-2}]	Open circuit potential [V]
methanol	32.7 ± 0.2	577 ± 26	0.195 ± 0.015
formaldehyde	15.9 ± 0.1	379 ± 16	0.146 ± 0.002
formate	6.7 ± 0.1	317 ± 24	0.075 ± 0.007

Table 3: Average values for the maximum current density measured with three fuels in biobattery type 2 (25 wt% hydrogel, air breathing platinum cathode, 100 mV s^{-1} scan rate).

	Maximum current density [mA cm^{-2}]
methanol	26.4 ± 1.8
formaldehyde	16.5 ± 3.8
formate	4.82 ± 1.44

In this work we have used a protein engineering approach to make a new catalytic biomaterial that supports a synthetic metabolic pathway and can be used as an anode modification for an enzymatic biobattery. The protein engineering strategy enabled the enzymes to self-assemble while either retaining or enhancing the catalytic performance of the enzymes. The biobatteries resulted in power and current densities comparable to those that have been previously reported in the

literature for similar systems. Palmore et al. reported a methanol/O_2 enzymatic biofuel cell using with the same cascade of three enzyme with a maximum power density of 0.68 mW cm^{-2}.[8a] In later work, Akers et al. constructed a methanol/oxygen enzymatic biofuel cell and Addo et al. made methanol/oxygen (air) enzymatic biofuel cells with maximum power densities of 1.55 mW cm^{-2}[8b] and (261 ± 7.6) μW cm^{-2}[8c], respectively. Higher power can certainly be obtained by further optimizing this system.

On the large scale, enzymes can be produced inexpensively, and these hydrogels may be a competitive alternative to other chemical catalysts for methanol oxidation. This general approach can also be applied to other enzymes so that new biocatalytic biomaterials could be used for additional bio-electrocatalysis applications as well as other technologies involving heterogeneous biocatalysis.

Experimental Section

The cloning, expression, and purification of the three modified dehydrogenases are described in the Supporting Information. The enzymatic activity of the hydrogel-forming proteins in dilute solution were measured with varying NAD^+ concentrations and varying the three different substrate concentrations (methanol, formaldehyde, and formate). The reaction rates of all three dehydrogenases were determined by following the change of absorbance from the produced NADH at 340 nm ($\varepsilon = 6.22$ mM^{-1} cm^{-1}) using SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA). All assays were performed in 96-well UV-transparent microplate at 37 °C. The kinetic parameters were determined by fitting the reaction rates obtained with varied substrate and cofactor concentration combinations to the ordered bi-bi kinetic rate equation [Eq. (1)] using the Enzyme Kinetics Module of SigmaPlot (Systat Software Inc., San Jose, CA). All measurements were repeated at least three times.

$$\text{rate} = \frac{[\text{E}]^{\text{total}} k_{\text{cat}} [\text{NAD}^+][S]}{k_{\text{i,NAD}^+} K_{\text{M,S}} + K_{\text{M,S}}[\text{NAD}^+] + K_{\text{M,NAD}^+}[\text{S}] + [\text{NAD}^+][\text{S}]} \quad (1)$$

Mixed hydrogels were prepared by combining equal units of each enzyme and the mixture was lyophilized. The enzyme unit was determined by following the change of absorbance at 340 nm using a UV/Vis spectrophotometer when 1 mM NAD^+ and 10 mM substrate were used, and the unit was defined as 1 μmol min^{-1} of activity.

In the type 1 biobattery, a 2.5 wt% protein solution was made with 1 mM methylene blue, 6 mM NAD^+, 200 mM Trizma (pH 8.5), 2 M Na_2SO_4, carbon black, carbon fiber, and 1-butyl-3-methylimidazolium chloride (BMIMCl) ionic liquid to form a conductive bioanode. A Prussian blue cathode made with 100 mg of iron(III) chloride, 100 mg of potassium ferricyanide, and 200 μL of BMIMCl was used. The anode and the cathode were separated by Nafion 212 and allowed to equilibrate for 3 h prior to the introduction of 100 mM fuel. Polarization curves were obtained by using linear sweep voltammetry scanned (1 mV s^{-1}) from open circuit voltage (OCV) to 0 V with a Digi-Ivy DY2100 potentiostat.

For the type 2 biobattery, a 25 wt% hydrogel was kneaded onto a 0.125 cm^2 stainless-steel mesh surface until the gel was equally distributed across the surface. The full biobatteries were constructed using hydrogel-coated anodes along with commercially available cathodes which consisted of a Nafion 112 assembled gas-permeable ELAT electrode with 0.5 mg platinum per 1 cm^2 carbon (Fuel Cell Store, San Diego, CA). Both the anode and cathode were exposed to air. 100 mM of fuel (methanol, formaldehyde, or formate) was added to the anode in a total final volume of 5 μL. Electroanalytical measurements were carried out using an Autolab PGSTAT 302 potentio/galvanostat. Polarization curves were obtained by using linear sweep voltammetry scanned (100 mV s^{-1}) from open circuit voltage (OCV) to 0 V.

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[1] a) S. Banta, I. R. Wheeldon, M. A. Blenner, *Annu. Rev. Biomed. Eng.* **2010**, *12*, 167–186; b) S. Banta, Z. Megeed, M. Casali, K. Rege, M. L. Yarmush, *J. Nanosci. Nanotechnol.* **2007**, *7*, 387–401; c) D. N. Woolfson, M. G. Ryadnov, *Curr. Opin. Chem. Biol.* **2006**, *10*, 559–567; d) W. F. Daamen, J. H. Veerkamp, J. C. van Hest, T. H. van Kuppevelt, *Biomaterials* **2007**, *28*, 4378–4398; e) S. A. Maskarinec, D. A. Tirrell, *Curr. Opin. Biotechnol.* **2005**, *16*, 422–426; f) F. G. Omenetto, D. L. Kaplan, *Science* **2010**, *329*, 528–531.

[2] a) S. C. Barton, J. Gallaway, P. Atanassov, *Chem. Rev.* **2004**, *104*, 4867–4886; b) S. D. Minteer, B. Y. Liaw, M. J. Cooney, *Curr. Opin. Biotechnol.* **2007**, *18*, 228–234; c) P. Atanassov, C. Apblett, S. Banta, S. Brozik, S. C. Barton, M. Cooney, B. Y. Liaw, S. Mukerjee, S. D. Minteer, *Interface* **2007**, *16,* 28–31.

[3] a) W. A. Petka, J. L. Harden, K. P. McGrath, D. Wirtz, D. A. Tirrell, *Science* **1998**, *281*, 389–392; b) W. Shen, R. G. H. Lammertink, J. K. Sakata, J. A. Kornfield, D. A. Tirrell, *Macromolecules* **2005**, *38*, 3909–3916; c) W. Shen, K. Zhang, J. A. Kornfield, D. A. Tirrell, *Nat. Mater.* **2006**, *5*, 153–158; d) H. R. Marsden, A. Kros, *Angew. Chem.* **2010**, *122*, 3050–3068; *Angew. Chem. Int. Ed.* **2010**, *49*, 2988–3005.

[4] a) I. R. Wheeldon, S. C. Barton, S. Banta, *Biomacromolecules* **2007**, *8*, 2990–2994; b) I. R. Wheeldon, E. Campbell, S. Banta, *J. Mol. Biol.* **2009**, *392*, 129–142; c) H. D. Lu, I. R. Wheeldon, S. Banta, *Protein Eng. Des. Sel.* **2010**, *23*, 559–566; d) I. R. Wheeldon, J. W. Gallaway, S. C. Barton, S. Banta, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 15275–15280.

[5] M. C. Sheehan, C. J. Bailey, B. C. Dowds, D. J. McConnell, *Biochem. J.* **1988**, *252*, 661–666.

[6] a) A. A. Klyosov, *Biochemistry* **1996**, *35*, 4457–4467; b) H. Glatt, K. Rost, H. Frank, A. Seidel, R. Kollock, *Arch. Biochem. Biophys.* **2008**, *477*, 196–205.

[7] A. E. Serov, A. S. Popova, V. V. Fedorchuk, V. I. Tishkov, *Biochem. J.* **2002**, *367*, 841–847.

[8] a) G. T. R. Palmore, H. Bertschy, S. H. Bergens, G. M. Whitesides, *J. Electroanal. Chem.* **1998**, *443*, 155–161; b) N. L. Akers, C. M. Moore, S. D. Minteer, *Electrochim. Acta* **2005**, *50*, 2521–2525; c) P. K. Addo, R. L. Arechederra, S. D. Minteer, *Electroanalysis* **2010**, *22*, 807–812.

[9] a) K. V. Mills, H. Paulus, *Homing Endonucleases and Inteins*, *Vol. 16* (Eds.: M. Belfort, D. W. Wood, B. L. Stoddard, V. Derbyshire), Springer, Berlin, **2005**, pp. 233–255; b) M.-Q. Chong, Xu, *Homing Endonucleases and Inteins*, *Vol. 16* (Eds.: M. Belfort, D. W. Wood, B. L. Stoddard, V. Derbyshire), Springer, Berlin, **2005**, pp. 273–292; c) K. Shingledecker, S. Jiang, H. Paulus, *Arch. Biochem. Biophys.* **2000**, *375*, 138–144; d) W. Y. Wu, C. Mee, F. Califano, R. Banki, D. W. Wood, *Nat. Protoc.* **2006**, *1*, 2257–2262.

[10] P. K. Addo, R. L. Arechederra, S. D. Minteer, *J. Power Sources* **2011**, *196*, 3448–3451.

[11] A. A. Karyakin, E. E. Karyakina, W. Schuhmann, H.-L. Schmidt, S. D. Varfolomeyev, *Electroanalysis* **1994**, *6*, 821–829.

