Unusual long-term stability of a Bilirubin Oxidase biocathode in organic solvents

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Organic solvent tolerant enzymes are of tremendous importance for the pharmaceutical industry in search of high added-value chemicals. However, enzymes exhibit usually weak long-term activity and stability in various media. Here, we report a general strategy allowing stabilizing and keeping enzymes active in organic solvents upon immobilization on an electrode surface. It consists in the combination of an engineered solvent-tolerant enzyme, combined with fine-tuned osmium-based redox polymers where the concentration of osmium complex has been specifically tuned to minimize its deswelling associated with the use of porous gold electrodes. This approach is validated with bilirubin oxidase as a model system. This copper enzyme is able to oxidize a wide

range of substrates, combined with the reduction of O_2 to water. While all other enzymatic systems irreversibly lose their activity and stability in the presence of 7.5 M methanol or below, this optimized enzymatic system stays functional in 12.5 M methanol with no loss in current density. It exhibits a half-life of more than 8 days, which is unprecedented in the literature. We show that this electrode can also operate in DMSO, dioxane, acetonitrile and acetone, thus opening up a very wide variety of applications in the field of bioelectrocatalysis and bioelectrosynthesis.

Introduction

Enzymatic bioelectrocatalysis in mixed aqueous/organic solvents is recently gaining a lot of attention. This field of research is fueled by the pharmaceutical industry, in quest of enantiopure and high added-value chemicals.[1] However, various substrates of industrial interest are insoluble in water and many enzymes are deactivated in pure organic solvents. The choice of the organic solvent is critical to benefit from the advantages of enzymes (selectivity, activity, specificity). As a result, a compromise is needed between the solubility of substrates and the denaturation of enzymes in the organic medium.[2, 3]

The decline of enzymatic activity in organic solvents is due to conformational changes and reduced flexibility of enzymes. It is explained by the lack of water molecules acting as “lubricant” within the protein. Enzyme molecules become more rigid, thus they are less active.[3-6] It was hypothesized that the denaturation effect of solvents would scale with their hydrophobicity, characterized by log P (partition coefficient between n-octanol and water). The lower this value, the lower the hydrophobicity, the higher the ability of the organic solvent to remove water from enzymes. Many attempts have been made to rationalize solvent effects on enzyme activity. However, a direct correlation between loss of activity and the nature of the organic solvent remains

elusive as it depends on many interrelated parameters (dielectric constant, polarity, dipolar moment, nature of the substrate, ..).[3, 7]

Laccases are copper-based metalloenzymes that are expected to find applications in numerous fields, including, but not limited to, wood, food, beverage, paper, textile, cosmetics and pharmaceutical industries, as well as in biosensors/biofuel cells.[8] They can oxidize a wide range of substrates while reducing O_2 to water.[9-13] This duality allows them to be used either for substrate oxidation or oxygen reduction.[14, 15] Nevertheless, their poor stability/activity in organic solvents restricts their use to low volumetric content of organic solvents. For example, Rodakiewicz-Nowak et al. reported that in acetone/, acetonitrile/, methanol/, ethanol/ and DMSO/water mixtures, various laccase species were inhibited by the organic solvent.[16, 17]

The effect of ethanol and DMSO has been studied electrochemically for the oxidation of catechol and O_2 reduction for laccase/Nafion composite electrodes.[18] The authors showed that once immobilized, the enzyme was inactivated at higher concentrations of organic solvent than their counterpart free in solution. In 2005, Barton et al. envisioned mediated laccase electrodes in a direct methanol fuel cell (DMFC).[19] They hypothesized that the high selectivity of an enzymatic O_2 cathode could solve methanol cross over issues encountered in DMFC, improving fuel efficiency and recovery.[20, 21] By combining a laccase from *Trametes versicolor* and an osmium based redox polymer,[22, 23] they elaborated a biocathode implemented in a membrane electrode assembly.[19] In the presence of 10 M methanol and at 40 °C in a pH 4 citrate buffer, the current density of their cathode decreased by 46 % compared to methanol free conditions. In the 0-5 M range, they attributed the loss in current to the deswelling of the highly charged redox polymer leading to a decrease of the apparent electron diffusion.[24] Above 5 M, methanol affected the enzyme kinetics and the enzyme deactivation was irreversible.[25] Other authors investigated the

effect of methanol on laccase based cathodes.[26-30] For example, Armstrong et al.[31] reported the immobilization of laccase from *Pycnoporous cinnabarinus* on graphite edge electrodes modified with chrysene-2-diazonium. In the presence of 0.5 M methanol. They observed an increase in current density, decreasing reversibly by 20 % in the presence of 5 M methanol. The loss of current was irreversible at 9.7 M methanol.

In recent years, our work has been focused on the identification, production and use of Bilirubin oxidases (BOD) for bioelectrochemical applications.[32] BODs also belong to the Multi Copper Oxidases family, but, unlike laccases, are more stable in the presence of chloride and in a broader range of pH, which significantly increases their range of applications.[15] The main purpose of this work is to investigate whether BODs would be more stable than laccases in co-organic solvents and would offer new perspectives and opportunities in bioelectrochemistry in organic solvents.

Results and Discussion

To overcome the above mentioned issues, we combined a molecularly modified solvent tolerant enzyme and a redox polymer specifically tuned to prevent polymer deswelling, together with the use of porous electrodes. To validate our approach, we used bilirubin oxidase from *Bacillus pumilus* (BOD-*Bp*)[33] as a model candidate. Initially produced in BL 21 (DE3), we showed earlier that promoting the formation of disulfur bridges and co-expression of chaperone proteins could increase the BOD-*Bp* stability and enhances its activity by 83%.[34]

To examine the effect of methanol on the activities of the BODs produced in BL21 (DE3) and origami B (DE3), we performed steady-state experiments with 2,6-dimethoxyphenol (DMP) as a substrate at pH 7 (Figure 1). The BOD produced in origami (red dots) is unexpectedly more active in the presence of methanol. This may be attributed to the disulfur bridges and is currently under

investigation. The decrease of the relative activity observed upon increasing the concentration of methanol is not due to the enzyme deactivation. Circular dichroism in the presence or absence of 12.5 M methanol shows that the secondary structure remains unchanged (Figure S1). The loss of activity results from the competitive inhibition (Table S1)[35] between DMP and methanol, as reported for other multi copper enzymes.[36] The half-life of the BOD produced in origami B (DE3) was 4 days in the presence of 12.5 M methanol, and exhibits the highest stability reported so far for any multicopper oxidase in methanol.[37]

We then performed electrochemical experiments by immobilizing BOD-*Bp* within osmium redox polymers. In such a system, electron conduction occurs through collisions between reduced and oxidized osmium complexes tethered to the polymer backbone.[23] The rate of collisions increases with the mobility of the tethered redox centers. The greater their mobility, the faster the electrons diffuse. Decreasing the amount of Os complexes will minimize the polymer deswelling, resulting in an increase of the electron diffusion. However, if deswelling is too important or swelling is too high, the segmental mobility of the osmium complexes will be impacted.[38-40]

Thus, the challenge consisted in minimizing polymer deswelling without compromising the electron transfer rate. To reach this goal, we tuned the molar proportions of each monomer (vinylimidazole and acrylamide) to synthesize a redox polymer with a 9/1 Polyacrylamide (PAA)/Polyvinyl imidazole (PVI) ratio. Since the osmium complexes are grafted onto the imidazole moieties, increasing the amount of acrylamide in the copolymer led to a decrease in the number of immobilized osmium complexes and therefore to a decrease of the global ionic charge of the redox polymer. The electroactive osmium content was evaluated by square wave voltammetry and was 20 mM. Glassy carbon electrodes were then modified with 62.57 wt% of PAA_9-PVI_1-$[Os(4,4'\text{-dichloro-}2,2'\text{-bipyridine})_2Cl]^{+/2+}$, 30 wt% of purified BOD-*Bp* and 7.43 wt

% of crosslinker for a total loading of 108 µg.cm^{-2}. Cyclic voltammograms (CVs) were recorded at 50 mV.s^{-1} and 500 rpm (Figure 1A) in the absence (blue curve) or in the presence of various methanol concentrations; 2.5 M (black), 5 M (red), 8.75 M (purple), 10 M (green), 12.5 M (yellow).

The CV in the absence of methanol is characteristic of a polymer-bound osmium complex with an apparent redox potential of +0.35 V vs. Ag/AgCl. CVs of the redox polymer in the presence or absence of methanol are nearly identical in the 0-8.75 M range, suggesting that electron transport in the hydrogel is not altered by methanol. This is confirmed by the identical values of the apparent electron diffusion coefficient (D_{app}), determined by the Randles-Sevcik equation as reported earlier[41] (Table S2). In the 10-12.5 M methanol range, a shift of 30 mV of the oxidation peak is observed while the active osmium decreases slightly. The observations that D_{app}, the oxidation peak height and the active osmium complex content do not change in the presence of methanol, within experimental errors, suggests that polymer deswelling is not relevant in this range of concentration.[24]

Figure 2B shows the resulting catalysis measured under 1 atm O_2 for various methanol concentrations. For laccase-based systems, it has been reported that the current drops upon increasing the methanol concentration.[19, 31] This was irreversible once a threshold was reached (commonly for > 7.5 M). As seen in Figure 2B, this is not the case here. There is a 20 % rise in current density when the methanol concentration reaches 8.75 M and this is most likely due to an increase of oxygen solubility.[42, 43] The current then slightly decreases in the presence of 10 M and 12.5 M methanol, but remains comparable to values obtained in the absence of methanol. A drop of the electron transfer driving force, dictated by the potential difference between the Os centers

and the redox potential of the T1 Cu (~ +0.36 V vs. Ag/AgCl), may explain this slight decay at high methanol concentrations. This potential difference is 35 mV in the absence of methanol, but only 10 mV in the presence of 12.5 M. However, it cannot be excluded that the presence of methanol could alter the redox potential of the enzyme which may influence the driving force. Non catalytic signal of BOD in DET mode were not visible on those electrodes and redox titration measurement were perturbed by the presence of methanol. Therefore, to rule out this hypothesis, we performed Direct Electron Transfer (DET) experiments on carbon cryogel electrodes.[44] As seen in Figure S2, the onset potential of O_2 reduction is identical in the presence or absence of methanol. This a good indication that the T1 redox potential may be unaffected by methanol. The more resistive shape of the CV may be ascribed to the drastic change of the dielectric constant of the buffer going down to 55 in the presence of 12.5M methanol.

To assess the reversibility of the system, after running the CVs in the presence of methanol, new CVs were recorded in a N_2 and methanol free buffer to test the effects associated with methanol on the reversibility of the redox polymer, and then in an O_2 saturated buffer to evaluate the effect of methanol on the enzymatic activity. For each methanol condition, CVs recorded after the experiments are almost identical to the first CVs obtained in a methanol-free buffer, within 10 % experimental errors. For example, as can be seen in Figure S3 (red curves), the surface coverage and current density only declined by 15 % and 12 % respectively after the experiments with 12.5 M methanol. This reveals that such a methanol concentration has only a minimal effect on the redox polymer and on the enzyme, unlike earlier systems.

Chronoamperometric experiments at +0.1 V vs. Ag/AgCl were performed to test the stability of the biocathode for various methanol concentrations and the half-lives are summarized in Table S2.

Interestingly, the stability is identical whatever the methanol concentration used, but lower than the one measured for the enzyme present in solution. The divergence between those values lies in the measurement method. While in electrochemistry the measurement is continuous, it is not the case in homogeneous solutions, where the enzyme is incubated in the desired buffer and the activity measured from time to time.

To investigate the reason for the decay in current for the immobilized enzyme, CVs were recorded before and after long-term experiments. As observed in Figure S3 (green curves), after 21 h of continuous experiments, 56 % of the redox polymer was lost while the enzyme activity declined by 80 %. In a control experiment for which the electrodes were left without any imposed potential for 21 h in a buffer containing 12.5 M methanol, CVs showed a decrease of 43 % for the surface coverage and of 53 % for the current density. It illustrates that most of the decline in current is due to polymer leaching and not to BOD-*Bp* inactivation, protected by the redox polymer.

To reinforce this hypothesis, we synthesized on purpose a new redox polymer to increase its deswelling by raising its global ionic charge. The (PAA)/(PVI) ratio was fixed to 4/1 and the concentration of electroactive osmium was 86 mM. The current density was 40 % lower compared to the current obtained with the PAA_9-PVI_1 redox polymer (Figure S4). In the presence of 12.5 M methanol, D_{app} was almost an order of magnitude lower (5 ×10-10 $cm^{-2}.s^{-1}$) and decreases upon increasing the methanol concentration, in agreement with the earlier report of Calabrese Barton.(Figure S5) [24] The decrease in Dapp is correlated with the deswelling of the redox polymer upon introduction of a solvent with reduced dielectric constant, such as methanol.[24]

The long-term stability was critically altered. While the half-life in the presence of 12.5 M methanol for the PAA_9-PVI_1 redox polymer was 4.2 hours, it was only 22 minutes for the PAA_4-

PVI_1. (Figure S6) This result, combined with the data gathered earlier, supports the theory that increasing the charge of the redox polymer enhances the deswelling and the diffusion of methanol, which deactivates the enzyme (Scheme 1). On the contrary, a less charged redox polymer prevents methanol diffusion and serves as a protective membrane keeping the BOD in a hydrated environment. This is in agreement with the recent finding of Davari et al that showed that the degree of the hydration shell of an enzyme determines its resistance to organic cosolvents.[45] The more hydrated the more resistant.

To show the versatility of our system, we then performed experiments with 4 additional solvents based on the Chastrette's classification[46, 47] including acetone and acetonitrile (aprotic dipolar with a low polarity), DMSO (aprotic highly dipolar) and dioxan (electron-pair donor). The results are summarized in Table S1 and S2 and illustrate that BOD-*Bp* is merely affected by acetone, acetonitrile, and dioxane. Figure 3 shows the resulting electrochemical experiments performed for the highest concentration of organic solvent under Ar (Figure 3A) and O_2 (Figure 3B). The current density is in the same range for all solvents except for DMSO, which can be interpreted by a lower value of D_{app} and BOD-*Bp* activity.(Table S2)

No correlation could be established between the activity/stability of the modified electrodes and the nature of the organic solvent. It may result from an intricate combination of various interrelated parameters specific to the enzyme, the redox polymer and the method of immobilization.

Finally, electrochemical half-live values of a couple of hours would severely impact the value of such electrodes for any applications. If properly engineered, the use of 3 dimensional porous electrodes permits to solve this issue.[48, 49] It promotes the stabilization of the enzymes within the pores, while increasing the current density, resulting from an increase of the active surface area.

However, the thickness, geometry and pore size of the electrodes needs to be well chosen and specifically designed to precisely accommodate the enzyme within the entire porosity, without impeding fast mass transport of substrates. Our group has pioneered the use of miniaturized 3-dimensional and hierarchical cylindrical porous electrodes and their uses, maximizing mass transport of substrates.[50, 51] We developed unsteady mathematical models at the electrode scale to predict, in the direct electron transfer mode, the optimal electrode thickness and porosity, allowing to immobilize BOD-*Bp* for efficient oxygen reduction. Those models showed that numerous interrelated parameters have to be taken into account to reach an optimized geometry and perfect geometry highly depends on the enzyme kinetics. When operating at 0V *vs.* Ag/AgCl, the optimal thickness is about 12 half-layers.[52, 53] While we have not yet developed a mathematical model to predict the optimized geometry for a MET system, we selected porous gold electrodes with eleven half-layers of pores (1 μm pore size) for the proof-of-concept experiments.

Figure 4A shows an example of long term stability experiments in 12.5 M methanol made with a mediated bilirubin oxidase biocathode immobilized either on a control bare gold electrode (black curve - scheme B and C) or a porous gold electrode (red curve - scheme D and E). The stability was dramatically enhanced 50 times with the use of the porous electrodes, compared to experiments carried out with bare gold electrodes. A half-life of more than 8 days in the presence of 12.5 M methanol was reached, while the highest reported stability to date, for a mediated laccase cathode, was 1.6 days in 10 M methanol.[19] After 23 days of continuous operation, the modified electrode still exhibits 30% of its original current. Interestingly, using this approach, such an enhancement has also been observed with other solvents. The stability increased ten times in 4.7 M acetone, reaching a half-life of 25 h (Figure S7); five times in 7.8 M acetonitrile reaching a half

live of 6h (Figure S8); almost 30 times in 4.7 M dioxane reaching a half live of 11h (Figure S9) and twenty times in 7 M DMSO reaching a half live of 3.8 days. (Figure S10)

Conclusion

To summarize, we report a rational strategy allowing to mitigate the effect of organic solvents on enzymes. It consists in the combination of an engineered solvent-tolerant enzyme, combined with fine-tuned redox polymers in combination with the use of porous gold electrodes. Applied to bilirubin oxidase from *Bacillus pumilus*, the resulting synergy allowed the operation of this O_2 reducing cathode in various organic solvents for extremely long periods of time, never reached so far. The modified electrodes could even operate in 12.5 M methanol for more than 8 days, while most of the other reported systems only function at low methanol concentration for a couple of hours. Such a stabilizing effect has also been observed for other solvents.

This straightforward approach could be generalized to other redox enzymes, making it a very promising strategy not only for bioelectrochemical organic synthesis, but also for other applications involving enzymes and organic solvents.

Materials and Methods

Chemicals. The cross-linker poly(ethylene glycol) 400 diglycidyl ether (PEGDGE) was purchased from Polysciences Europe GmbH (Hirschberg, Deutschland). All other chemicals were of analytical grade or higher and purchased from Merck (Saint-Quentin Fallavier, France).

Enzyme and redox polymer. Bilirubin oxidase from Bacillus Pumilus (BOD-*Bp*) with an activity of 873 ± 27 U/mg was produced and purified as already described.[34] The redox polymers PAA-

PVI-[Os(4,4'-dichloro-2,2'-bipyridine)$_2$Cl]$^{+/2+}$ were synthesized as previously reported by tuning the ratio of vinylimidazole and acrylamide.[39]

Enzymatic assay and kinetics measurements. The specific activity was determined spectrophotometrically at 37°C in an oxygenated 100 mM phosphate buffer pH 7 on a Cary 100 system from Varian, Inc. (Palo Alto, CA), equipped with a Peltier thermostable multicell holder, using a 10 mm path length glass cell. 2,6-Dimethoxyphenol (DMP) at 8 mM was used to measure the enzyme activity at 468 nm (ε468 = 14.8 mM^{-1}.cm^{-1}). One unit was defined as the amount of enzyme that oxidizes 1 µmol of substrate per minute. The enzyme concentration was chosen to be in initial velocity and was between 0.4 nM and 4.3 nM while the concentration of DMP was varied between 1 mM and 8 mM. The influence of methanol on the DMP oxidation by BOD was performed in a 100 mM sodium phosphate buffer pH 7 with 0, 20, 50% v/v of methanol. The enzyme stability in methanol was performed by incubating the BOD at 37°C with different methanol concentrations in 100 mM sodium phosphate buffer pH 7. The activity of the enzyme was then measured at different times in 100 mM sodium phosphate buffer pH 7. Each experiment was performed in triplicate. Data were analyzed using different inhibition models and kinetic constants were determined by fitting the Michaelis-Menten equation with Origin software.

Circular dichroism. Measurements were performed at room temperature on a MOS-450 spectrometer (BioLogic, France) with increments of 1 nm, an acquisition time of 0.5 s and 10 consecutive scans. CD spectra were recorded between 190 and 300 nm with a protein concentration of 1.3 mg/mL. The path length for the quartz cell was 0.1 mm. All measurements were performed in triplicate. The data were baseline corrected using the CD spectra for the buffer alone with or without methanol. Data were processed using Excel 2007

Determination of D_{app}. Electroactive redox mediator loading was measured using square wave voltammetry with a pulse height of 25 mV and a pulse width of 50 ms between 0V and +0.6V *vs.* Ag/AgCl in a O_2 free PBS buffer.[54] D_{app} was measured as described by Gallaway & al.[41] using glassy carbon electrode.

Preparation of electrodes. Glassy Carbon Electrodes (GCE, diameter: 5 mm) were polished on a felt with alumina (0.05 µm), rinsed, polished once more with deionized water and then cleaned by sonication in deionized water for 2 min. Electrode surfaces are exposed to a 150 m Torr O_2 plasma for 5 min before coating. Macroporous Gold Electrodes (MGE, length: 2 cm) were synthesized as described earlier.[55] Experiments were performed with 11 half layer electrodes. Before coating, the MGE were immersed in a Piranha solution for 45 minutes, rinsed with H_2O milliQ, characterized by cyclic voltammetry (40 scans, 100 mV/s) in deaerated 0.5M H_2SO_4 solution and then rinsed again with H_2O milliQ before modification. Carbon cryogel electrodes were prepared as reported earlier. [56]

Modification of electrodes. PAA-PVI-[Os(4,4'-dichloro-2,2'-bipyridine)$_2$Cl] $^{+/2+}$ (10 mg/ml in water, 1.66 µl for GCE, 3.21 µl for MGE), additional milli-Q water (1.66 µl for GCE, 3.21 µl for MGE), purified BOD-Bp (5 mg/ml in water, 3.18 µl for GCE, 6.16 µl for MGE) and PEGDGE (2 mg/ml in water, 1.97 µl for GCE, 3.81 µl for MGE) were mixed in a microtube. Then 3.39 µl (for GCE) and 16.38 µl (for MGE) of this mixture were deposited by drop-casting onto each electrode. The electrodes were thus modified with 62.57 wt % polymer, 30 wt % enzyme and 7.43 wt % cross-linker for a total loading of 108 µg.cm^{-2} or 600 µg.cm^{-2} on porous and non-porous gold electrodes and then dried in a humidity-controlled atmosphere (99 %) for 18 h at 4 °C.

Electrochemical measurements. All experiments were performed using CH Instruments potentiostats (CHI760C, 842B) in a three-electrode cell configuration. An Ag/AgCl and a platinum

wire were used as reference and counter electrodes, the working electrode was mounted on a rotating Pine motor. All experiments were performed at 37 °C in a thermostated glass cell and at 500 rpm. To maintain a fixed volume of solution in the cell, the bubbled gases were presaturated with water by passage through a bubbler, which contained water. Sodium phosphate buffer (0.1 M) adjusted at pH 7.2 was used as a supporting electrolyte. Since the pH value in mixed solvent/water solutions differs from the starting pH value of the aqueous buffer, each prepared mixture was adjusted to pH 7.2 after mixing. Experiments were performed with electrolytes containing at maximum a concentration of organic solvent for which precipitation of phosphate starts. The long term experiments were performed at +0.1 V *vs.* Ag/AgCl and the data were recorded every 30 seconds. To circumvent evaporation, a small volume of the respective buffer with adjusted ionic strength was added when necessary to maintain the volume and the ionic strength in the cell. This resulted in the steps visible in some graphs. For those reasons, all long term experiments were performed four times and half-lives values were evaluated from those sets of 4 long terms experiments. Raw data are presented on purpose.

Figure Captions

Figure 1. Relative activity of BODs produced in BL21 (black dots) and origami B (red dots) upon increasing the methanol concentration. Measured at pH 7 as detailed in the experimental section.

Figure 2. Cyclic voltammograms of the mediated bilirubin oxidase biocathode in the absence or presence of methanol. A) under Ar at pH 7.2, 500 rpm and 50 $mV.s^{-1}$ B) under 1 atm O_2 at 5 $mV.s^{-1}$ and 500 rpm. The color code is identical for both Figures.

Figure 3. Cyclic voltammograms of the mediated bilirubin oxidase biocathode in the presence of different cosolvents. A) under Ar at pH 7.2, 500 rpm and 50 mV.s-1 B) under 1 atm O_2 at 5 $mV.s^{-1}$ and 500 rpm. The color code is identical for both Figures.

Figure 4. Example of a long-term stability experiment for a mediated bilirubin oxidase biocathode in 12.5 M methanol. A) Black curve: immobilized on a bare gold electrode; red curve: immobilized on a 11/2 layers porous gold electrode. Experiments performed at +0.1V *vs.* Ag/AgCl. The total loading was fixed at 600 $\mu g.cm^{-2}$ for both experiments. SEM images of a bare (B) and porous gold electrode (D). Anticipated schematic illustration of a section of a modified bare (C) and porous gold electrodes (E). The blue color represents the enzyme, the green color the osmium based redox polymer and the yellow color the gold electrode.

Scheme 1. Schematic representation of the deswelling effect. Left) with the PAA_9-PVI_1 redox polymer. Right) with the PAA_4-PVI_1 redox polymer. The blue color represents the enzyme and the red color the osmium based redox polymers.

ASSOCIATED CONTENT

Supporting Information. Inhibition values of the enzyme by the organic solvents, kinetic parameters measured electrochemically and spectrophotometrically for all organic solvents, CVs of DET experiments, carbon cryogel experiments, circular dichroism experiments, long term experiments, CVs before and after stability experiments. The following files are available free of charge.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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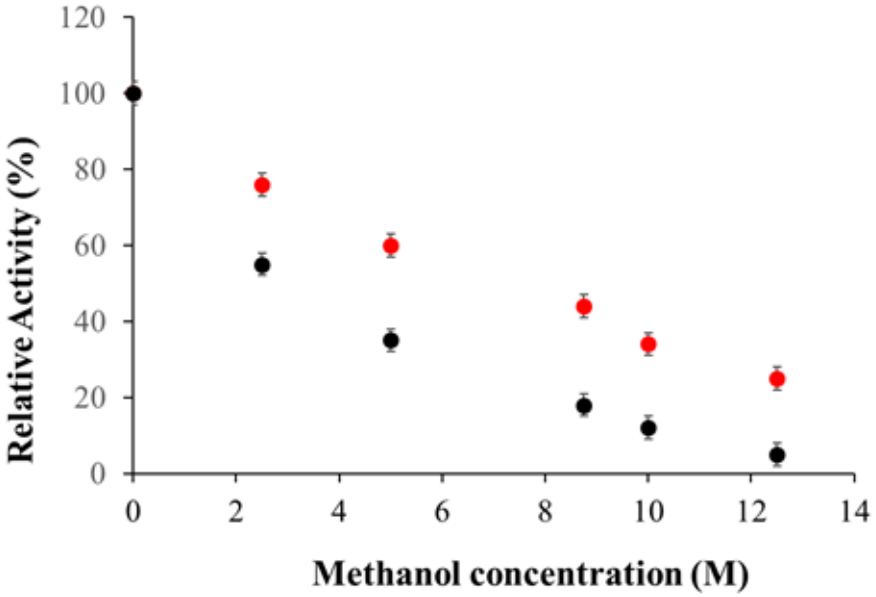

120
100
80
60
40
20
0
Relative Activity (%)
0
2
4
6
8
10
12
14
Methanol concentration (M)

Figure 1

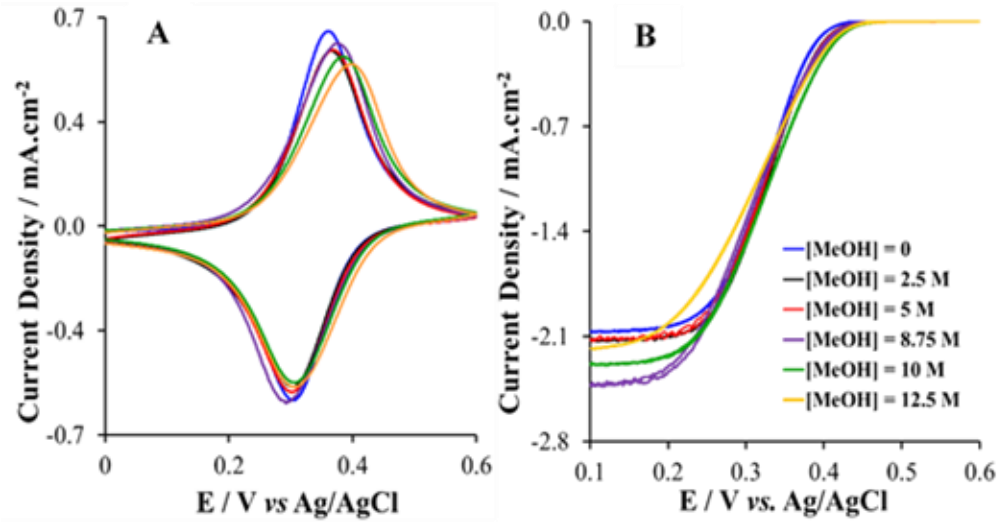

A
B
Current Density / mA.cm-2
E / V vs Ag/AgCl
E / V vs. Ag/AgCl
[MeOH] = 0
[MeOH] = 2.5 M
[MeOH] = 5 M
[MeOH] = 8.75 M
[MeOH] = 10 M
[MeOH] = 12.5 M

Figure 2

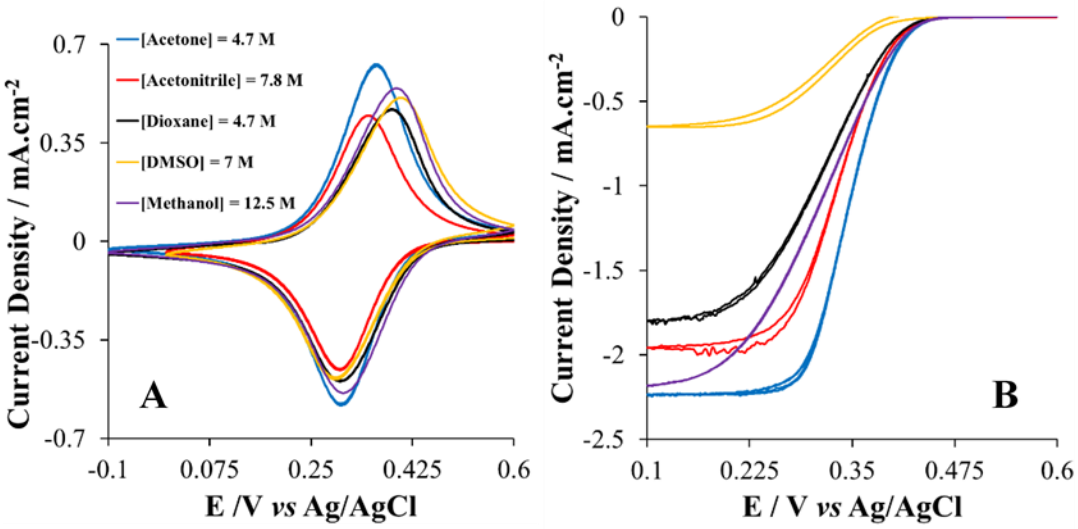
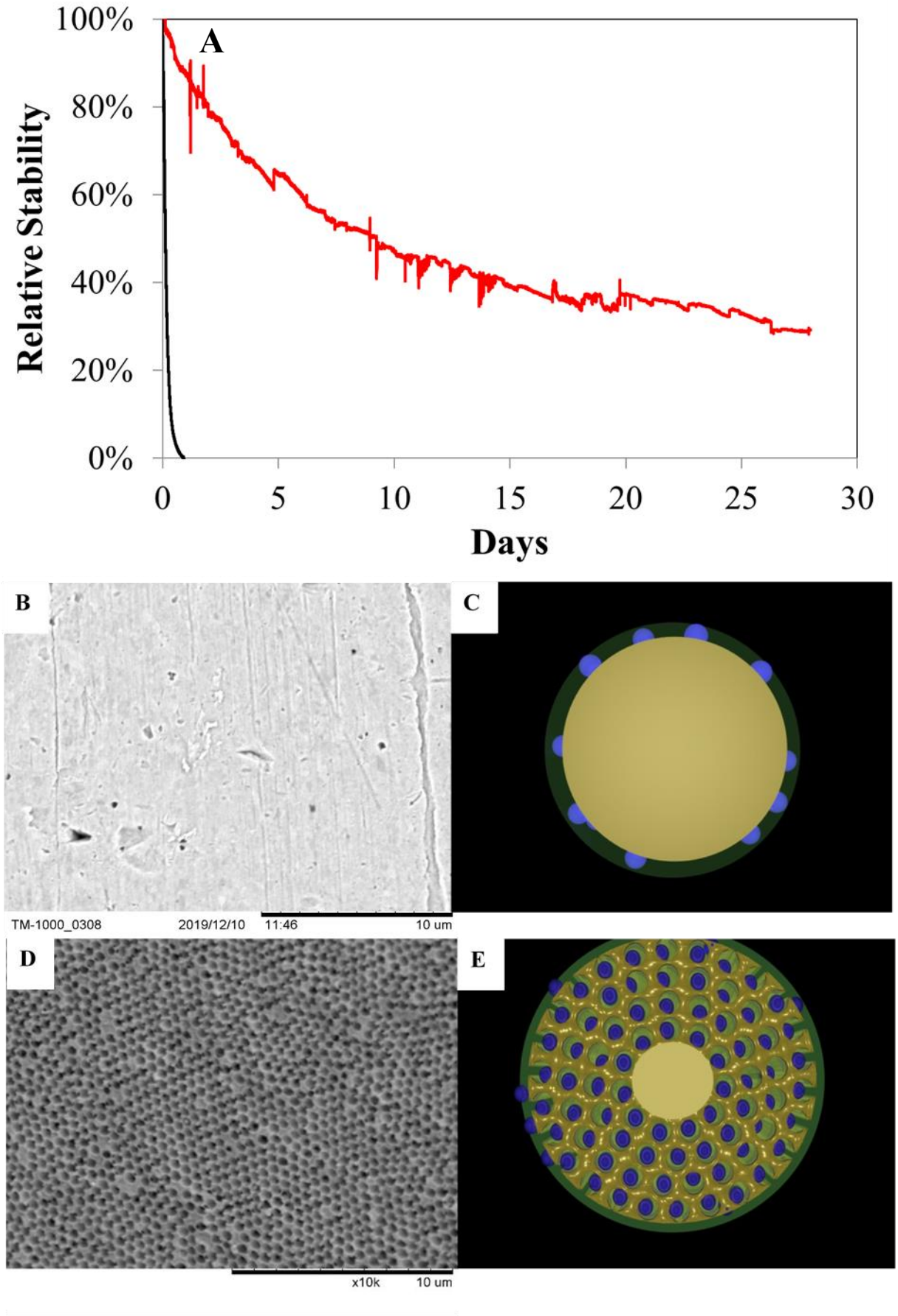

[Acetone] = 4.7 M
[Acetonitrile] = 7.8 M
[Dioxane] = 4.7 M
[DMSO] = 7 M
[Methanol] = 12.5 M
Current Density / mA.cm-2
E /V vs Ag/AgCl
A
B
Current Density / mA.cm-2
E / V vs Ag/AgCl

Figure 3


A
100%
80%
60%
40%
20%
0%
Relative Stability
0
5
10
15
20
25
30
Days
B
TM-1000_0308
2019/12/10
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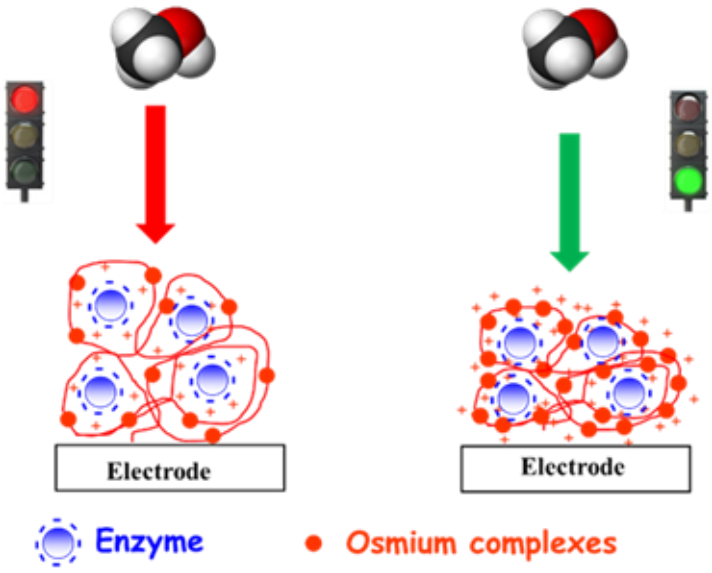
10 um
C
D
x10k
10 um
E

Figure 4



Scheme 1

Table of content