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► **To cite this version:**

C. Artner, B. Bohrer, Luca Pasquini, Ievgen Mazurenko, Nora Lahrach, et al.. Effects of interactions between SPEEK or Nafion ionomers and bilirubin oxidase on O₂ enzymatic reduction. *Electrochimica Acta*, 2022, 426, pp.140787. 10.1016/j.electacta.2022.140787 . hal-03738192

HAL Id: hal-03738192

<https://hal.science/hal-03738192v1>

Submitted on 25 Jul 2022

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Effects of interactions between SPEEK or Nafion ionomers and bilirubin oxidase on O₂ enzymatic reduction

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Ionomers such as Nafion are used combined to redox enzymes in bioelectrocatalysis in order to prevent enzyme leakage and to enhance proton diffusion. In the search for an alternative to this costly and non-ecofriendly material, sulfonated poly(ether ether ketone) SPEEK, another ionomer free of perfluorinated functions can be envisioned. Here, we investigate the effect of ionomer type and concentration on enzymatic oxygen reduction by the *Myrothecium verrucaria* bilirubin oxidase (*Mv* BOD). We show that both SPEEK and Nafion ionomers reduce the catalytic activity of *Mv* BOD in enzymatic assays in solution as well as in electrochemical experiments once the enzyme is co-adsorbed with the ionomer. A pluridisciplinary approach associating microscopy, UV-Vis spectroscopy, dynamic light scattering, SDS gels and electrochemistry allows to point out a molecular interaction between the polymer particles and the enzyme governed by different principles depending SPEEK or Nafion is considered. The differences in hydrophilicity and conformation of SPEEK compared to Nafion account for a more severe effect on enzyme efficiency.

KEYWORDS: *Nafion*; *SPEEK*; *Enzyme*; *Electrocatalysis*; *ORR*

1. INTRODUCTION

1 Redox enzymes are eco-friendly catalysts applicable for many bioelectrochemical devices such
2 as biosensors, bioreactors or biofuel cells [1-4]. Among them, some multicopper oxidases,
3 namely laccases and bilirubin oxidases, catalyze O₂ reduction into water with low over-
4 voltages, making them attractive catalysts for fuel cells. Despite great advances in their
5 electrical wiring, the poor stability of the enzyme and/or the enzyme-based electrode prevents
6 any large-scale development [5-8]. As an illustration, we showed that an optimized cathode
7 based on the thermostable *Bacillus pumilus* bilirubin oxidase (BOD) presented a half-life of
8 only one week [9], and we expect that using non-thermostable BODs should lead to even less
9 durability. Many strategies have been developed to enhance enzyme stability, and their choice
10 depends on the reasons why the catalytic activity decreases [10]. As a modification of enzyme
11 conformation is often involved in intrinsic enzyme activity changes, ways to rigidify the
12 structure have to be employed. The use of additives such as polyols is one relevant example.
13 However, in most cases enhanced stability is gained against activity, because activity imposes
14 some fluctuation in the structure. The decrease in electrocatalytic activity can also originate
15 from the progressive leakage of enzyme from the electrode. Although some studies clearly
16 demonstrated that this is not the main process in many enzyme-based bioelectrodes [8], cross-
17 linking procedures proved to be efficient for stabilizing the enzymatic signal. The addition of
18 polymeric moieties acting as protective films is an alternative strategy. In that sense Nafion, a
19 perfluorinated sulfonated ionomer classically used as a proton conductive separator membrane
20 in fuel cells, has been used in many bioelectrochemical studies [11-14]. This strategy may be
21 of particular interest because it combines a protective action of the polymer against enzyme
22 leakage and a possible enhancement of proton diffusion due to the cation exchange capacity.
23 This can prevent any local pH variation that would be detrimental for the enzyme. In that way
24 also, the so-built bioelectrode will resemble the catalytic layer of classical fuel cell gas-diffusion
25 electrodes, where Nafion surrounds platinum particle catalysts. However, although ionomer
26 membranes have been largely studied for fuel cell performance optimization, interaction of
27 liquid ionomer in the ink composed of carbon and platinum particles has been much less
28 investigated and is poorly understood [15, 16]. Turning to biotechnology, the interactions
29 between liquid ionomers and enzymes at the molecular scale are lacking.

30 Recently, the group of Crespilho *et al.* formed a biogel matrix by mixing Nafion, glutaraldehyde
31 and BOD, and showed an increased stability of the bioelectrode [17]. However, it must be noted
32 that a huge amount of enzyme from solutions in the mM range was used at the electrode, which
33 will limit any device development because of the induced cost. The aim of the current work is

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to study ionomer interaction with BOD using concentrations in the μM range, with the view of determining the main physico-chemical properties of the ionomer that influence both enzymatic activity and stability of BOD. Furthermore, Nafion contains non-ecofriendly perfluorinated functions and is a costly material. We thus consider sulfonated poly(ether ether ketone) SPEEK, another ionomer free of these functions (Scheme 1) [18]. The homogeneous activity of BOD in contact with ionomers is first examined using classical spectrophotometric assays as a function of the ionomer nature and concentration. We extend the study to bioelectrochemical assays, and evaluate the conditions at which the ionomers may act as a protective/facilitator layer for enzyme activity. In combination to dynamic light scattering, SDS gels and microscopy, spectroscopic assays and electroactivity measurements allow us to propose chemical and structural parameters accounting for specific interactions between Nafion and SPEEK and the enzyme.

2. Experimental

2.1. Materials

BOD from *Myrothecium verrucaria* (*Mv* BOD) was a gift from Amano Enzymes Inc. (Nagoya, Japan). 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) ($\geq 98\%$) and potassium hexacyanoferrate (FeCN) were purchased from Sigma. Carbon nanotubes modified with COOH-groups (CNT) ($>95\%$, (USA)) were from NanoLab Inc. and dispersions (1 mg/ml) were prepared in Milli-Q water by sonicating for 1 h. Their characterization, including surface chemistry and charge, was previously reported in [19]. Potassium phosphate buffer (PPB) was used at the concentration of 50 mM at pH 6 (unless otherwise specified) and was prepared with potassium phosphate monobasic (99.0%) and potassium phosphate dibasic (99.0%), both purchased from Fisher. All other chemicals were used as received.

Nafion perfluorinated ion-exchange polymer (equivalent weight EW: 1100 g/mol, ion exchange capacity IEC = 0.9 meq/g, 5 wt. %) was obtained from Aldrich Chemical Company Inc. in solution in a mixture of lower aliphatic alcohols and 10% water. The SPEEK polymer was prepared as in reference [20, 21] (EW: 362 g/mol, Degree of sulfonation: 0.92, IEC = 2.5 meq/g); the solution (5 wt%) was prepared accordingly in a mixture of 2-isopropanol and 10% water.

2.2. Preparation of the enzyme/ionomer hybrid

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Initially, a stock solution of *Mv* BOD was prepared by diluting *Mv* BOD in the phosphate buffer 50 mM, pH 6 to a final concentration of 1 mM. This mixture was divided into aliquots and then frozen at -20°C. One aliquot was daily thawed and further diluted to a concentration of 10 µM in phosphate buffer for experiments. The ionomer solutions were diluted in the 50 mM phosphate buffer pH 6 to various ratios ranging from 1:1 to 1:500 (v/v) immediately prior usage. Then the *Mv* BOD (10 µM) was mixed with the ionomer solution in a 1:1 (v/v) ratio. For the highest ionomer concentrations, experiments were additionally made by adding small amounts of NaOH in ionomer solution to obtain a final pH of the mixture close to pH 6. To obtain a SPEEK 1:1 solution at a pH close to 6 the ionomer stock solution was mixed with a 50 mM K₂HPO₄ solution.

18 2.3. Methods

19 *Spectroscopic assays*

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Enzymatic assays were performed at 25°C by measuring the absorption at 420 nm using spectrophotometry (Agilent Technologies: Cary Series UV-VIS Spectrophotometer). ABTS was used as the electron donor. Four replicates were performed for each condition. *Mv* BOD was incubated with ionomer for 15 min before the measurement. In the cuvette, the *Mv* BOD concentration was 50 nM and ABTS concentration was 1 mM in 50 mM phosphate buffer pH 6. For pH-dependent activity measurements, the Britton Robinson buffer was used in the pH range 2-9 by mixture of different ratios of 1 M phosphoric acid, acetic acid and boric acid [22]. The final pH was adjusted with NaOH. Temperature was regulated to 25°C.

37 *Visualization of proteins by Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)*

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30 µL of each sample were centrifuged for 30 min at 13000 rpm. The supernatant was separated from the pellets, which corresponds to soluble and aggregated proteins, respectively. 30 µL loading buffer (62.5 mM Tris pH 6.8, 10% glycerol, 2% SDS, 0.5 mg/ml Coomassie Brilliant Blue R 250, 0.715 M β-mercaptoethanol) were added to the supernatant samples. The pellet was resuspended in 30 µL of PPB and 30 µL loading buffer. To denature the proteins, the mixture was incubated at 95°C for 5 min. The visualization of proteins was performed by loading samples on 12% SDS-PAGE. The migration of the samples on the gel (EZBiolab WSHT Precast Gel) was performed by applying a voltage of 150 V for 45 min. The gel was stained with Instand Blue™.

58 *Electrochemistry*

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Cyclic voltammetry (CV) and chronoamperometry (CA) were performed in a standard 3-
electrode cell using a potentiostat from Autolab PGSTAT30 controlled by Nova software 1.1
(Eco Chemie). A Hg/Hg₂SO₄ (sat. K₂SO₄) and a Pt-wire were used as reference and auxiliary
electrodes, respectively. All potentials are quoted vs Ag/AgCl reference electrode by adding
430 mV to the measured potential. The working electrode was a pyrolytic graphite electrode
(PG, geometric surface area 0.07 cm²) from Biologic. Before use, it was polished on a wet emeri
paper (1200), sonicated in 30% ethanol solution, and dried prior to any further modification.
Oxygen or nitrogen were continuously bubbled through the electrochemical cell during the
experiments. 50 mM phosphate buffer pH 6 was used as the electrolyte. Different protocols
were used for electrode modification. In most cases (protocole A) a small aliquot (typically 2
μl) of the enzyme solution, mixed with the ionomer or with only buffer, was drop casted on the
PG electrode modified by a film of carbon nanotubes. For carbon nanotube deposition, 5 μL (1
mg/mL) were drop casted on the surface of the PG electrode, then dried at 60°C. The
electroactive surface was calculated from the capacitive current, and the CNTs film was shown
to induce around one hundred times higher surface. To study the influence of the ionomer on
the electroactive surface, FeCN and ABTS were used as redox probes. To get rid of any errors
induced by the CNT film, FeCN or ABTS redox signals were first recorded on the electrode
modified by the CNT layer. After smooth washing and drying, the electrode was further
modified by the ionomer at different dilutions, and CVs were run before and after FeCN or
ABTS addition.

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Adsorption of the enzyme, alone or with the ionomer, was made by incubating the electrode
with the enzyme solution for 15 min at 4°C. Then the bioelectrode was washed with Milli-Q
water to remove loosely bound enzyme, and transferred to the electrochemical cell for
electroenzymatic experiments. An alternative protocol was used (protocol B) where the enzyme
aliquot was first adsorbed on the PG electrode by dropcasting, then the ionomer was deposited
on the enzyme layer and left for 15 min before rinsing. After adsorption, the electrode was
rinsed to ensure removal of loosely bound material before transfer to the electrochemical cell.
Temperature of the electrochemical cell was regulated to 25°C.

In a typical experiment, a CV curve was recorded at a scan rate of 5 mV/s with a scanning
window from 0.65 V to 0 V vs Ag/AgCl. To evaluate bioelectrode stability, immediately
afterwards a CA experiment was carried out at an applied potential of 300 mV vs Ag/AgCl for
an hour, followed again by another CV measurement. CV limiting catalytic currents are
measured at a potential of 0 mV vs Ag/AgCl. They are normalized against the current obtained

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2 for Mv BOD alone, in order to get rid of any variation in enzyme activity with time or sampling
3 conditions. All the electrochemical experiments are at least made in triplicate.

4 *Dynamic light scattering (DLS) measurements*

5 DLS was performed with the ionomer dilutions (v/v) alone as well as in combination with 10
6 μM Mv BOD in a Zetasizer Nano ZS machine. Mv BOD was centrifuged at 13500 rpm for 30
7 min before performing DLS measurements, in order to remove any impurities that could lead
8 to a high background noise and thus disturb the measurement.

9 In order to perform DLS, a ZEN0040 measuring cell was used with a constant temperature at
10 25°C. A volume of 60 μl Mv BOD (10 μM) was analyzed with added increments of 7 μl of
11 SPEEK 1:10, or 4 μL Nafion 1:10 or buffer control. The equilibration time was set to 120 s and
12 3 measurement cycles were performed for each sample over approximately 11 runs each. The
13 determined viscosity was specified in the settings for each individual ionomer dilution. Size
14 determination was based on Dv50 size distribution which corresponds to the size point below
15 in which 50% of the material is contained.

16 To measure the viscosity of the ionomers, we needed the density beforehand. The densities and
17 viscosities of the ionomers were measured using the DMA4100M for the density and viscosity
18 with the added dispositive Lovis 2000M from Anton Paar (FRANCE). For the density
19 measurement, 1ml ionomer samples at different concentrations were injected into the
20 measurement cell using a 2 ml syringe. To verify if the cell was clean and dry before the
21 injection, the measurement cell density was measured at 0.0014 g/cm^3 , which corresponds to
22 the air density. The measurement of the ionomer density was performed at 25°C using the
23 DMA4100M standard method. The density value of the ionomer was used in the standard Lovis
24 method to measure the viscosity. 1ml of ionomer sample was loaded on to a capillary which
25 contained a 1.59 mm diameter steel ball. Both ends of the capillary were closed and the capillary
26 was inserted into the capillary block. The viscosity used measured at 25°C. Two angles were
27 used to perform the measurement a forward one at 70° and a back one at 70°.

28 *Microscopy*

29 A scanning electron microscope Zeiss GeminiSEM 500 (EHT = 5 kV, Mag. 31x, Signal: HE-
30 SE2) was used to observe the surface of a CNT-modified graphite rod on which ionomer
31 solutions were deposited. Electrode samples were prepared similarly to those for
32 electrochemistry. In order to fit the SEM chamber, PG electrodes were replaced by a polished
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graphite stick that can be cut at the desired height. A layer of CNT was first deposited, followed by a deposition of mixtures of Nafion or SPEEK ionomer solutions (prepared in 2.2).

3. Results and discussion

3.1. Influence of SPEEK or Nafion dilution on *Mv* BOD activity in solution

Mv BOD activity was first quantified in solution using classical spectroscopic assays with ABTS as a redox mediator. Different ionomer dilutions with phosphate buffer pH 6 were investigated from 1:1 to 1:500. The influence of SPEEK ionomer was compared to Nafion ionomer (Figures 1A and 1B, respectively). The addition of ionomer to the enzyme dilution, either SPEEK or Nafion, induces a decrease of the activity relative to *Mv* BOD activity alone in buffer. For both ionomers, the 1:1 dilution does not allow any activity to be measured. The activity remains only 23 and 50 % of *Mv* BOD activity for SPEEK dilutions of 1:10 and 1:50 respectively. With large SPEEK dilutions (1:100 and more), the activity of *Mv* BOD alone tends to be recovered. However, no Nafion dilutions could allow full enzyme activity recovering. It is still only 65% of the *Mv* BOD alone activity even with the 1:500 Nafion solutions. Interestingly, more than 97% of the enzyme activity is maintained for 1 hour whatever ionomer type and dilution, suggesting that the loss of activity compared to *Mv* BOD alone occurs as soon as the enzyme is mixed with the ionomer with few modifications afterwards.

Viscosities of the various solutions were first considered as potentially affecting the overall enzymatic activity. From the data in Table S1, it can be concluded that viscosity cannot be the main factor affecting the overall catalytic activity. Indeed, SPEEK 1:10 for example has a viscosity value very close to those measured at high dilutions, although the activity stays very low.

Sulfonated ionomers like SPEEK and Nafion are strong acids, presenting respectively pK_a of -1 and -6 [23, 24]. Otherwise, the ion exchange capacity was titrated at 2.5 and 0.9 meq/g for SPEEK and Nafion respectively [18, 23]. We measured the pH of the different *Mv* BOD/ionomer solutions obtained after dilution of each ionomer with the 50 mM phosphate buffer pH 6. For SPEEK, dilutions of 1:1, 1:10 and 1:50 yield pH of 2.6, 4 and 5.7 respectively. Higher dilutions allowed complete neutralization of the ionomer solution with pH values around 6. The pH of various Nafion dilutions were 3.5, 5.2 and 6 for 1:1, 1:10 and 1:50 or higher dilutions, respectively. Both 1:1 ionomer dilutions thus present a low pH that can be detrimental to the enzyme. The need for higher dilution of SPEEK vs Nafion to reach neutral pH relates to the 2.5 higher content of sulfonate groups in SPEEK compared to Nafion [23]. *Mv* BOD activity is known to be dependent on pH, describing a bell-shape behavior with a

1 maximum activity around pH 6. Since *Mv* BOD is not an acidophilic enzyme, it can be
2 irreversibly inactivated at the lowest pHs. *Mv* BOD activity measurement in solution as a
3 function of pH clearly underlines loss of activity at extreme pHs (i.e. pH 2 and pH 9) (Figure
4 1C). This activity can be almost recovered when the enzyme is exposed to pH 3 or pH 9, but
5 exposition to pH 2 does not allow any activity recovering at pH 6.
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8 As a first hypothesis, the absence of activity of *Mv* BOD in 1:1 ionomer dilutions may be
9 explained by the acidic pH values of both SPEEK and Nafion that lead to irreversible
10 inactivation of the enzyme. We neutralized the 1:1 and 1:10 SPEEK or Nafion ionomer
11 solutions (see experimental section) and measured again the BOD activity in solution in the
12 presence of neutralized ionomers. Results are reported in Figure 1D. As can be seen, the activity
13 in the case of the 1:1 dilution remains lower than 10% of the activity of BOD alone. For 1:10
14 dilutions, the activity stays in the range of 20% of the activity relative to BOD alone. This
15 means that the pH effect is not the single cause of the low activity obtained in the presence of
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27 3.2. Effect of the ionomer deposition on the properties of CNT-modified PG electrode

28 The next step is to evaluate the effect of ionomer on the electroreduction of O₂ by *Mv* BOD.
29 However, the films made upon deposit of ionomer-based enzyme solutions on the CNT-
30 modified PG electrode may impact the electrode available surface, the interfacial electron
31 transfer rate as well as O₂ diffusion. These three parameters were thus first investigated using
32 ionomer solutions alone.
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38 Ionomers consist of a hydrophobic backbone and ionic side chains. Molecular interactions
39 between the ionomer and the solvent used for dispersion will control the molecule
40 conformation, hence the sizes and distributions of ionomer aggregates [25]. Tarokh et al.
41 proposed an atomistic study of Nafion dispersion as a function of the nature of the solvent [26].
42 A cylindrical structure was shown, confirmed by SAXS measurements, for Nafion in
43 water/alcohol mixtures where Nafion forms aggregates with a central core of perfluorinated
44 backbone with the ionic groups on the outer surface [27]. The morphology of the aggregates
45 was shown to be highly dependent on the counter ion, i.e. H⁺ or Na⁺. Using DLS, Lee et al. and
46 Li et al. showed a microscale dispersion of Nafion particles with hydrodynamic radius in the
47 range of 1 μm resulting from aggregation between the ionomer hydrophobic backbone and
48 water [25, 27]. On the other hand, Sung et al. reported particles of SPEEK with mean size 10
49 nm when SPEEK was in N,N-dimethylacetamide as a result of dissolution of the ionomer [28].
50 Nafion and SPEEK also differ by their chemical properties, including differences in the acidity
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1 of the sulfonic acid functional group [23]. SPEEK backbone is otherwise much less
2 hydrophobic than Nafion backbone [28]. Such differences between both ionomers are expected
3 to have a great impact on the conformation assumed in solution and on their interaction with
4 the nanotube layer, and further with the enzyme.
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7 In this work, we first carried out DLS measurements for SPEEK and Nafion solutions. SPEEK
8 presents a narrow distribution of sizes with a small hydrodynamic diameter Dv_{50} of 4.15 ± 0.60
9 nm. DLS measurements with Nafion show on the contrary the formation of a heterogeneous
10 mix of aggregates with a major hydrodynamic diameter by volume Dv_{50} of 461 ± 183.85 nm.
11 This large difference in the particle size can be explained by the hydrophobicity of Nafion
12 backbone compared to the SPEEK backbone which induces a high dispersion in solution [28].
13 SPEEK being dispersed in solution SEM images of the films made by either SPEEK or Nafion
14 deposit on the CNT layer reflect this difference in conformation (Figure 2). Although EDX
15 analysis reveals the presence of SPEEK by the S band (Figure S1), a thick film is not formed
16 in the case of SPEEK 1:1 solution deposit (Figure 2B) as evidenced from the highest
17 magnification. A very different situation is observed with Nafion 1:1 solution deposit (Figure
18 2C) where a film appears covering the top of the CNTs. The hydrophilic character and small
19 size of SPEEK aggregates compared to Nafion properties may allow different interactions with
20 CNTs and in-depth penetration of SPEEK in the carbon layer instead of forming a thick ionomer
21 layer [9, 18]. This would be advantageous for electrocatalysis by modifying proton diffusion,
22 but could affect both interfacial electron transfer process and substrate diffusion.
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37 To evaluate the effect of ionomer on electron transfer rates, FeCN was used as a redox probe.
38 To get rid of any errors induced by the first CNT film (the surface developed by the underlayer
39 CNT film varies by around 10 % in the various assays), FeCN redox signal was first recorded
40 on the electrode modified by the CNT layer. After smooth washing and drying, the electrode
41 was further modified by the ionomer at different dilutions and CVs before and after FeCN
42 addition were recorded again. Differences in CV peak potentials ΔE_p were compared before or
43 after ionomer deposition (Figure S2). Compared to CNT-modified PG electrode, ΔE_p was
44 increased by 30 mV and 50 mV when using SPEEK or Nafion 1:1 dilutions respectively. It can
45 be concluded that the presence of both ionomers on the electrode does not prevent the interfacial
46 electron transfer, although it is slowed down. Otherwise, a decrease of more than 40% in the
47 redox peak signal can be observed with Nafion 1:1 solution, while it is around 10% in the case
48 of SPEEK 1:1 solution deposit. These data could suggest a decrease in the electroactive surface
49 area in the case of Nafion at high concentration which would not be not unexpected considering
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1 the film of Nafion imaged by SEM compared to SPEEK. However, a role of the electrostatic
2 repulsion between the surface sensitive innersphere FeCN and the ionomer cannot be ruled out.
3 ABTS redox behavior was thus examined as a valuable probe used for mediated electron
4 transfer processes in ORR biocatalysis. As can be seen in Figure S3, ABTS oxidative peak
5 current is only decreased by around 10 % in the presence of Nafion 1:1 or 1:10, and around
6 15% in the presence of SPEEK. These results compare well with capacitive currents compared
7 at CNT-modified PG electrode before and after ionomer deposits in the absence of redox
8 probes. Indeed, capacitance values indicate a decrease of 10% in the electroactive surface area
9 with Nafion 1:1 solution, and around 25% in the case of SPEEK 1:1 (Figure S4).

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17 To evaluate how the presence of ionomers could affect O₂ diffusion, currents in the absence of
18 enzyme were measured at a potential of -250 mV vs Ag/AgCl mV corresponding to abiotic O₂
19 reduction (Figure 3). The currents in the presence of ionomers are normalized to the O₂
20 reduction at the CNT-modified PG electrode. As can be seen, Nafion films do not prevent O₂
21 access to the electrode. In the case of SPEEK, the tendency is a decrease in O₂ reduction current
22 with the SPEEK concentration. This result is in agreement with data on gas permeability in
23 cross-linked SPEEK which is lower than in Nafion membrane [29]. However, O₂ access is never
24 completely blocked whatever the ionomer deposit in our experimental conditions. Furthermore,
25 the continuous bubbling of O₂ inside the electrochemical cell during enzymatic experiments
26 inducing oxygen concentration (1.2 mM) much higher than the K_M of BOD for O₂, is expected
27 to restrict any O₂ diffusion limitation.
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40 3.3. Influence of SPEEK or Nafion ionomers on *Mv* BOD electroactivity and bioelectrode 41 stability 42

43 Both stock solutions of SPEEK and Nafion are available in water/alcohol mixtures. It is known
44 that enzymes may present fair stability in non-aqueous solvents, although many enzymes have
45 been shown to maintain their activity in alcohols [10]. We thus preliminary checked the
46 electrocatalytic efficiency of *Mv* BOD diluted in either isopropanol or ethanol. We showed that
47 the presence of 90% alcohol in the buffer only slightly affects the electroactivity of *Mv* BOD.
48 The electrocatalytic current is even enhanced most probably because of a better incorporation
49 of the enzyme inside the CNT layer (Figure S5). The next step is to evaluate the influence of
50 ionomers on enzymatic O₂ electroreduction by *Mv* BOD once the mixture enzyme/ionomer is
51 immobilized at the electrode surface. This was first evaluated by CV measurements. In Figure
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4 are overlaid typical CV curves for the enzyme alone or in mixtures with SPEEK or Nafion at the dilution 1:100. In the three cases, a sigmoidal curve develops at an onset potential of 600 mV vs Ag/AgCl. These typical CV curves are characteristic of direct catalytic reduction of O₂ by *Mv* BOD where the Cu T1, the physiological first electron acceptor in this multicopper oxidase, is connected to the electrode. Direct electrical communication is expected due to the negative charge induced at the electrode by the CNT layer which accommodates the positive Cu T1 environment of *Mv* BOD [7]. The limiting currents are however notably different decreasing from 37 $\mu\text{A} \pm 3 \mu\text{A}$ for *Mv* BOD alone to 17.5 \pm 0.5 μA in the presence of Nafion down to 10 $\mu\text{A} \pm 0.1 \mu\text{A}$ in the presence of SPEEK.

The different ratios of ionomers in buffer were then examined, and the limiting currents for O₂ catalytic reduction were compared to the k_{cat} measured in solution by UV-Vis spectrometric assays (Figure 5). Four main features can be extracted from these data. First, when the ionomer solution presents an acidic pH (cases of Nafion 1:1 and SPEEK 1:1 and 1:10), the electroactivity remains very low compared to the catalytic current obtained with *Mv* BOD alone. It is negligible for SPEEK and 15% for Nafion. When the neutralized SPEEK and Nafion 1:1 solutions are used instead, the catalytic current remains at a very low intensity for SPEEK whereas it is enhanced to 30 % of the signal of enzyme alone for Nafion. Using the 1:10 neutralized dilution for SPEEK only slightly enhances the catalytic current that keeps a low value around 6% of the activity of BOD alone. As concluded with UV-Vis measurements, electroactivity thus confirms that not only the pH influences *Mv* BOD activity in the presence of ionomers. However, a marked difference between UV-Vis activity measurement in solution and electrocatalytic activity is the much lower catalytic currents obtained with SPEEK compared to Nafion, even with diluted ionomer solutions. Simple effect of the interfacial electron transfer rate and available electroactive surface cannot be at the origin of these observations as we showed that both parameters do not have a drastic impact on the electrochemical surface properties. The electroactivity with Nafion dilutions other than 1:1 stays in the range of 50-70% of the electroactivity of *Mv* BOD, a very similar trend than observed in solution.

Additionally, we ran chronoamperometry measurements at an applied potential of +300 mV vs Ag/AgCl to follow the stability of the catalytic current for O₂ reduction as a function of ionomer type and dilution (Figure S6). In this experimental condition, *Mv* BOD alone was able to keep 80% of the initial activity after 1 hour. Regardless of the SPEEK dilutions, a small stabilization effect was detected switching the signal stability to values close to 90%. The same behavior was observed for the highest Nafion dilutions. However, for Nafion dilution less than 1:50, an

1 increase in the current was recorded over the one hour of chronoamperometry, being
2 respectively 110, 130 and 140% of the initial current for 1:50, 1:10 and 1:1 Nafion dilutions.
3 This peculiar behavior may suggest some reorganization of the thick Nafion film. But the fact
4 that the current is stable once the mixture enzyme/ionomer has been deposited on the electrode
5 suggests again that the main detrimental effect of ionomer was induced as soon as both
6 components were mixed with few changes afterward.
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10 11 12 13 3.4. SPEEK and Nafion/enzyme interactions 14

15 To further explain the dependence of the *Mv* BOD electroactivity on ionomer dilutions and the
16 discrepancies between electrochemical measurements and UV-Vis assays, we need to
17 determine the interactions between the enzyme and the ionomer at the molecular level.
18 Electrochemical investigation of enzyme orientation and specific effect of ionomer deposit
19 protocol on the electroactivity are completed by SDS gels and DLS measurements to provide
20 understanding of the enzymatic activity in the presence of ionomers.
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27 *Enzyme orientation.* The presence of ionomer in mixture with the enzyme may affect the wiring
28 of the enzyme on the electrode. The interactions between the ionomer, both SPEEK and Nafion,
29 should tune the orientation of the enzyme depending on the strength of the interactions between
30 either the enzyme / electrode or the enzyme / ionomer or the ionomer / electrode. The study of
31 mediated electrochemical signal may bring a first answer on the enzyme molecules present and
32 active at the interface but not able to be directly wired because of a large distance between the
33 Cu T1 and the electrode. We compared a CNT-based electrode and CNT-based electrodes
34 further modified by 1:10 SPEEK and 1:1 Nafion solutions as relevant ratios to detect a potential
35 mediated ET. Whatever the electrochemical interface, CNT alone or in the presence of SPEEK
36 or Nafion, ABTS reversible redox signal was detected (inset in Figures 6), denoted as already
37 mentioned with FeCN the absence of electron transfer blocking. As seen in Figure 6A, only a
38 slight change can be recorded in the direct catalytic current obtained for O₂ reduction by *Mv*
39 BOD alone at the CNT-modified PG electrode when the redox mediator ABTS is added into
40 the electrolyte. This result agrees with previous reports and is linked to the narrow orientation
41 of *Mv* BOD via the Cu T1 on the negatively charged carbon nanotube network [7]. We then
42 considered O₂ reduction by *Mv* BOD in mixtures with either SPEEK (Figure 6B) or Nafion
43 (Figure 6C). In the case of SPEEK (Figure 6B), the signal shape is greatly modified in the
44 potential range 0.4-0.7 V after ABTS addition. However, this shape is linked to ABTS redox
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signal induced by mass transport inside the CNT layer under O₂ bubbling. Indeed, ABTS alone under O₂ bubbling (green curve, Figure 6B) superimposes with the current obtained in the presence of BOD (blue plain curve, Figure 6B). The same phenomenon occurs in the presence of Nafion after ABTS addition, with in addition a decrease in the catalytic current (Figure 6C). Overall, no mediated catalytic current can be detected in the presence of ABTS which suggests that either all the enzymes are connected to the electrode or that the redox mediator cannot access enzymes isolated by or inside the ionomer.

Effect of enzyme and ionomer deposition protocol. Catalytic currents were recorded according to protocol B (see experimental section), where the enzyme was adsorbed on the CNT-modified PG electrode prior the ionomer deposit. Non-neutralized ionomer solutions were used for comparison with protocol A. Protocol B allows to recover higher enzyme activity than the protocol A with catalytic currents tending to very similar values in the presence or absence of the ionomer, except for the highest ionomer concentration (Figure 7). To be especially noted, Nafion deposited according to protocol B allows full activity to be recovered. This result reinforces the key role of deleterious interactions of the ionomer on the enzyme in the mixture.

Effect of ionomer on enzyme aggregation. A potential aggregation of the enzyme with the ionomer can be in part responsible for the decrease in the enzymatic activity. This has been evaluated in this work by separating soluble and insoluble proteins after interaction with ionomers by centrifugation and visualization on SDS-PAGE. As shown in Figure 8, *Mv* BOD is found in the supernatant (main band below 66 kDa) for all SPEEK dilutions except SPEEK 1:1 where it is only found in the pellet. In this last dilution condition, aggregation of the protein can be accompanied by a protein degradation as a large smear can be seen in the bottom of the expected size of the protein. This means that the low pH of the SPEEK 1:1 dilution induces some aggregated inactive forms of *Mv* BOD. For all the other dilutions, no aggregated proteins can be seen. The situation with Nafion is markedly different. *Mv* BOD can be detected on the gel only in the case of the 1:500 Nafion dilution where it appears to be present in the supernatant. With all the other dilutions, no bands can be observed on the gel, except a coloration at the top of the gel, suggesting that in the presence of the Nafion ionomer prevents the migration of the enzyme on the gel. This suggests that the formation of large Nafion aggregates may encage the enzyme, decreasing ABTS access or decreasing enzyme wiring to the CNT-surface.

1 *Effect of ionomer on enzyme conformation and availability.* After having measured the
2 refractive indexes, densities and viscosities of the various samples, we carried out DLS
3 measurements for *Mv* BOD in mixture with SPEEK and Nafion 1:10 dilutions. The
4 hydrodynamic diameter of *Mv* BOD is reduced in the presence of SPEEK, going from a Dv50
5 of 7.86 ± 0.32 nm as expected for this 60 kDa protein [30, 31] to a Dv50 of 5.73 ± 1.26 nm.
6 Remember that SPEEK alone has a Dv50 of 4.15 ± 0.60 nm (Figure 9A). A band at 11 nm also
7 appeared in the BOD/ionomer mixture, in minority however. This shift in size of the enzyme
8 suggests that some interaction exists between both components that affects their hydrodynamic
9 radius or their geometry. SPEEK being more hydrophobic than the protein, its binding to the
10 enzyme would induce a decrease in solvent molecules on the enzyme surface. One hypothesis
11 may be that SPEEK binds in the environment of the Cu T1 which is positively charged,
12 explaining the decrease in activity because restrictive access to ABTS in solution, or preventing
13 the wiring to the CNTs. Results are markedly different using Nafion. Mixtures of Nafion and
14 *Mv* BOD result in the appearance of peaks for Nafion but no BOD can be detected (Figure 9B),
15 confirming that the enzyme is either bound or engaged into the large Nafion aggregates.
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31 **4. Conclusive model for the enzyme/ionomer interaction**

32 SPEEK and Nafion ionomers were evaluated in this work in view of their potential to act as a
33 protective layer against enzyme leaching while potentially favouring proton diffusion.
34 However, one of the main results obtained here is that both SPEEK and Nafion ionomers reduce
35 the catalytic activity of *Mv* BOD in enzymatic assays in solution as well as in electrochemical
36 experiments once the enzyme is co-adsorbed with the ionomer. The decrease in the catalytic
37 activity is not related to the pH change uniquely, but points out a molecular interaction between
38 the polymer particles and the enzyme governed by different principles depending SPEEK or
39 Nafion is considered.
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49 For Nafion, the decrease of the catalytic activity follows the same trend considering enzymatic
50 assays in solution or electroactivity. SEM, DLS and SDS gel data highlight large particles of
51 Nafion. Additionally, the stability of the activity with time, both in solution or when the enzyme
52 is co-immobilized with Nafion on the electrode, is similar to that of BOD alone. Such results
53 indicate that Nafion does not affect the activity of the active available enzymes in the mixture.
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presence of Nafion is due to a decrease in the available enzymes in the sample, presumably by engagement in large Nafion aggregates that prevent ABTS access or wiring to the CNTs. The tendency of catalytic current to increase with time at the highest Nafion concentration may suggest a reorganization of the aggregates at the electrode that can liberate some new enzyme molecules. In the presence of SPEEK ionomer, the catalytic current decrease is even more pronounced than in the presence of Nafion, to such an extent that SPEEK 1:1 and 1:10 mixtures only allow weak enzymatic catalysis. The thinner layer of SPEEK film observed by SEM, with potential diffusion of SPEEK in the inner CNT layers, and the formation of SPEEK/BOD particle suggested by DLS support the hypothesis that SPEEK binds to the protein surface, so that a proper orientation of BOD for direct electron transfer is hindered. This complex formation between BOD and SPEEK seems to affect the electrocatalytic activity even more than the homogeneous activity in solution, because of O₂ diffusion decrease and decrease of CNT available surface by SPEEK adsorption.

AUTHOR CONTRIBUTIONS

Conducting experiments, C. A. and B. B.; DLS supervising, D. B.; SDS gels, N. L.; Electrochemistry supervising, I. M. and E. L.; Review and editing, I. M., A. P., L. P.; Writing, editing and supervision, E. L. All authors have read and agreed to the published version of the manuscript.

ACKNOWLEDGMENTS

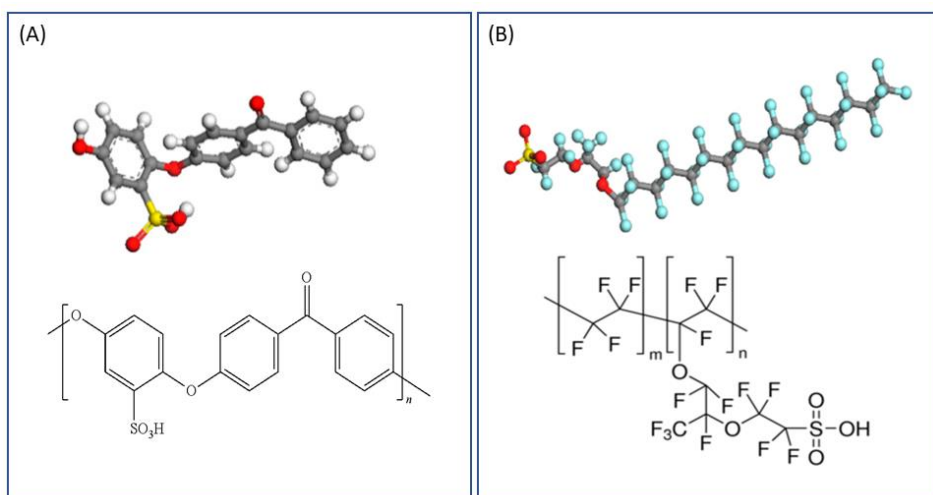
This work was supported by Excellence Initiative of Aix-Marseille Université-A*MIDEX (AMX-18-MED-002 ENZIM-FC) and by the Institute of Microbiology, Bioenergies and Biotechnology - IM2B (AMX-19-IET-006). The authors thank Prof. P. Knauth from MADIREL, CNRS, Aix-Marseille University and Dr M. Ilbert from BIP, IMM, CNRS, Marseille for fruitful discussion on ionomer properties and protein aggregation. They are also grateful to Prof. M. L. Di Vona from University of Rome Tor Vergata, Italy, for SPEEK synthesis, and Dr. A. Campos from the “Centre Pluridisciplinaire de Microscopie électronique et de Microanalyse (CP2M) at Aix Marseille University for the SEM images. *Mv* BOD was a gift from Amano incorporation, Japan, that the authors would like to greatly thank.

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Figures



Scheme 1. Comparative chemical structures of (A) SPEEK vs (B) Nafion.

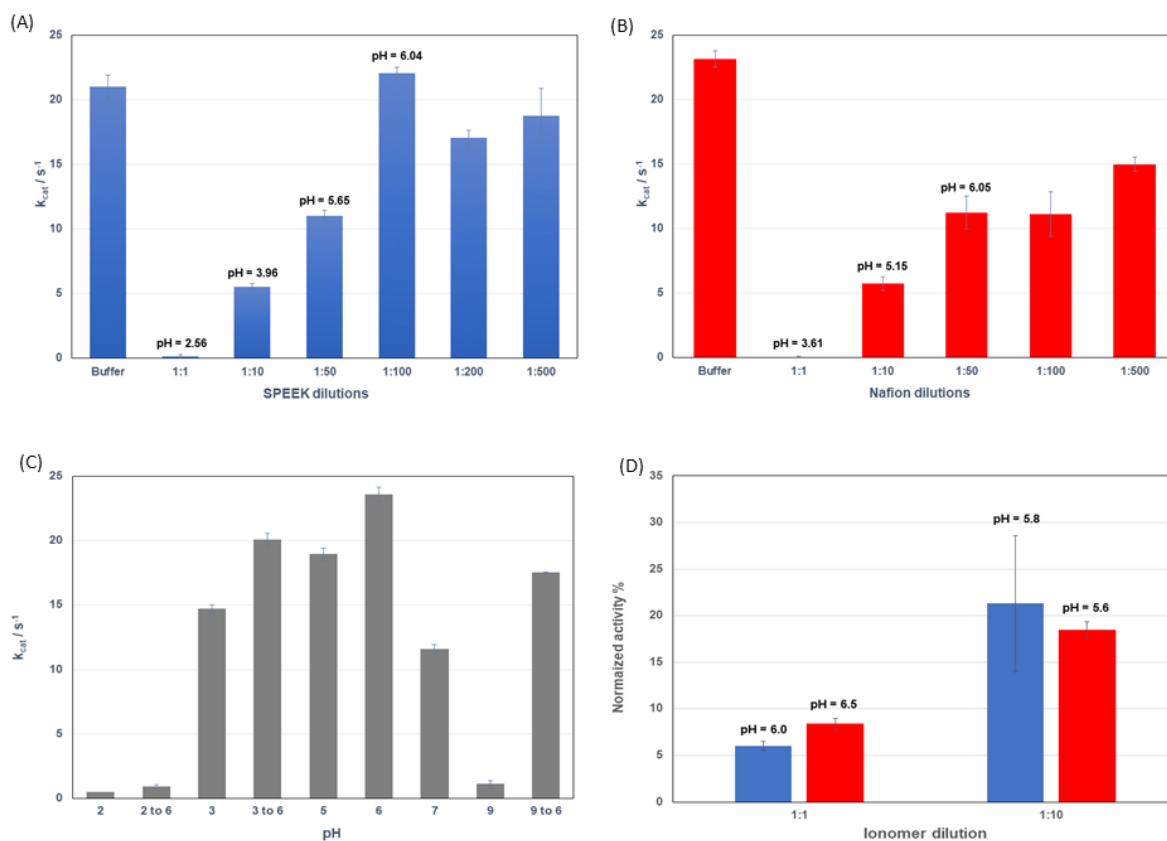


Figure 1. *Mv* BOD activity in solution as a function of ionomer dilution in the enzyme sample: (A) Speek, (B) Nafion. (C) activity of *Mv* BOD as a function of pH, (D) *Mv* BOD activity in the presence of neutralized SPEEK (blue columns) and Nafion (red columns) at high concentrations. “x to y” refers to transfer from pH x to pH y. The activity is normalized to the activity of *Mv* BOD alone. The respective pH of the enzyme/ionomer solutions is indicated over each column in (A), (B) and (D).

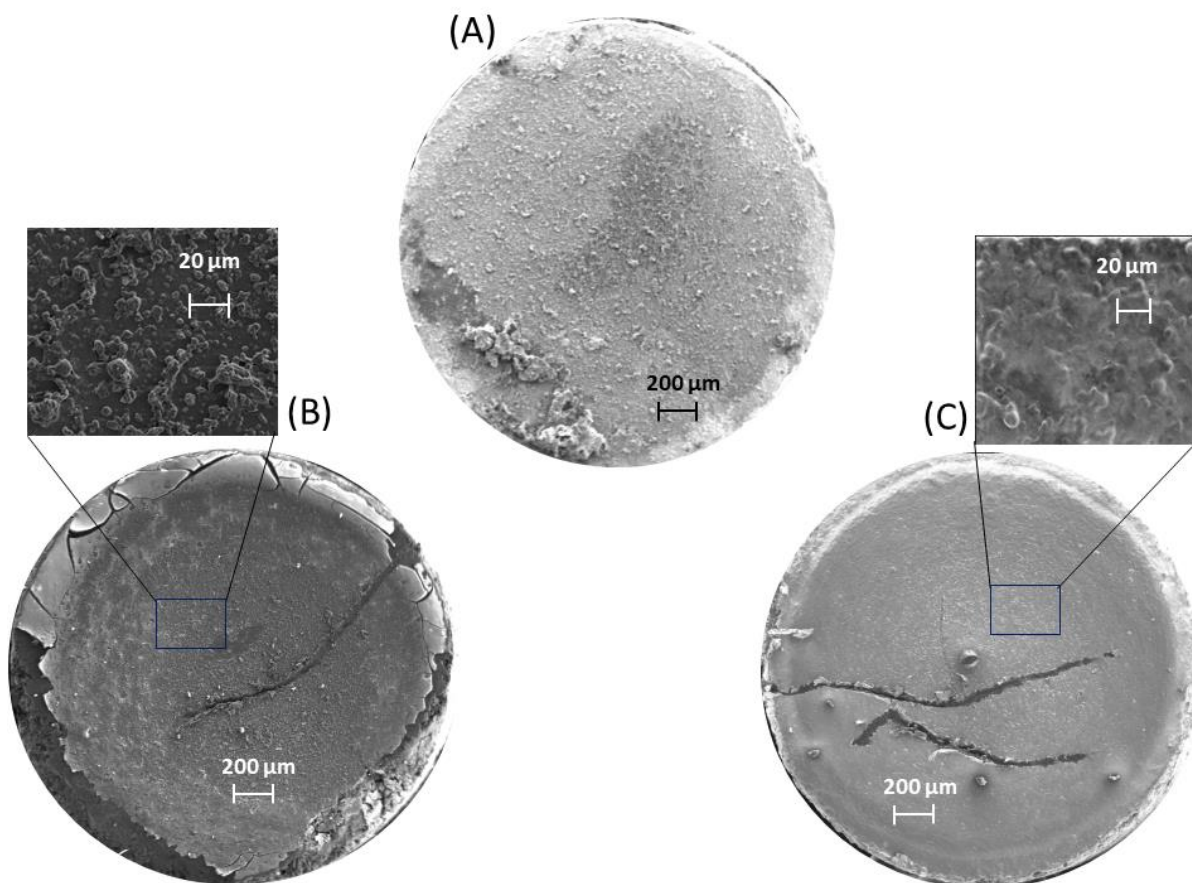


Figure 2. SEM images of the graphite electrode modified by CNTs (A), CNT layer on which a 1:1 SPEEK (B) or 1:1 Nafion (C) solutions have been deposited. Scratches made on the surface allows to visualize the film thickness. In (B) and (C) two different magnifications are provided.

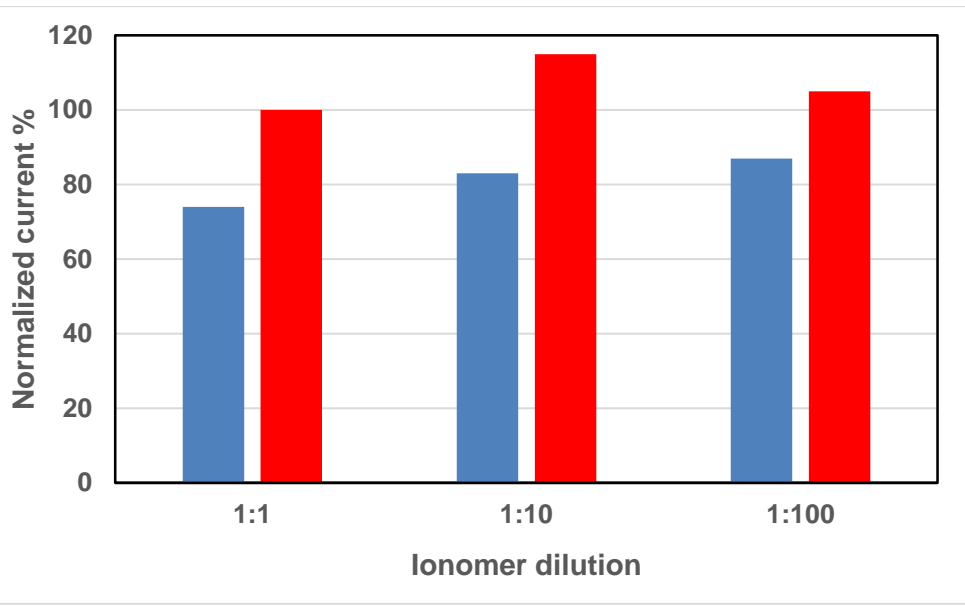


Figure 3. Normalized current for non catalytic O₂ reduction at the CNT-modified PG electrode in the presence of SPEEK (blue columns) or Nafion (red columns) films made from different ionomer dilution. 50 mM PPB buffer pH 6, $v = 5$ mV/s.

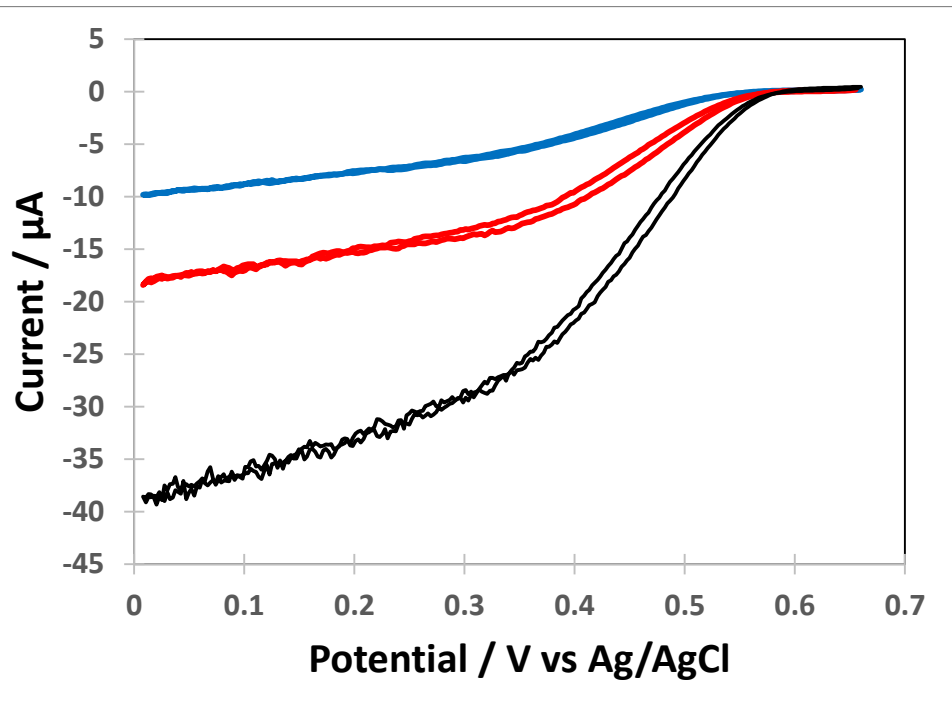


Figure 4. Typical CV curves for O₂ reduction with *Mv* BOD (black curve), *Mv* BOD/SPEEK 1:100 (blue curve) and *Mv* BOD/Nafion 1:100 (red curve) at the PG-modified CNT electrode. 50 mM PPB pH 6, $v = 5$ mV/s.

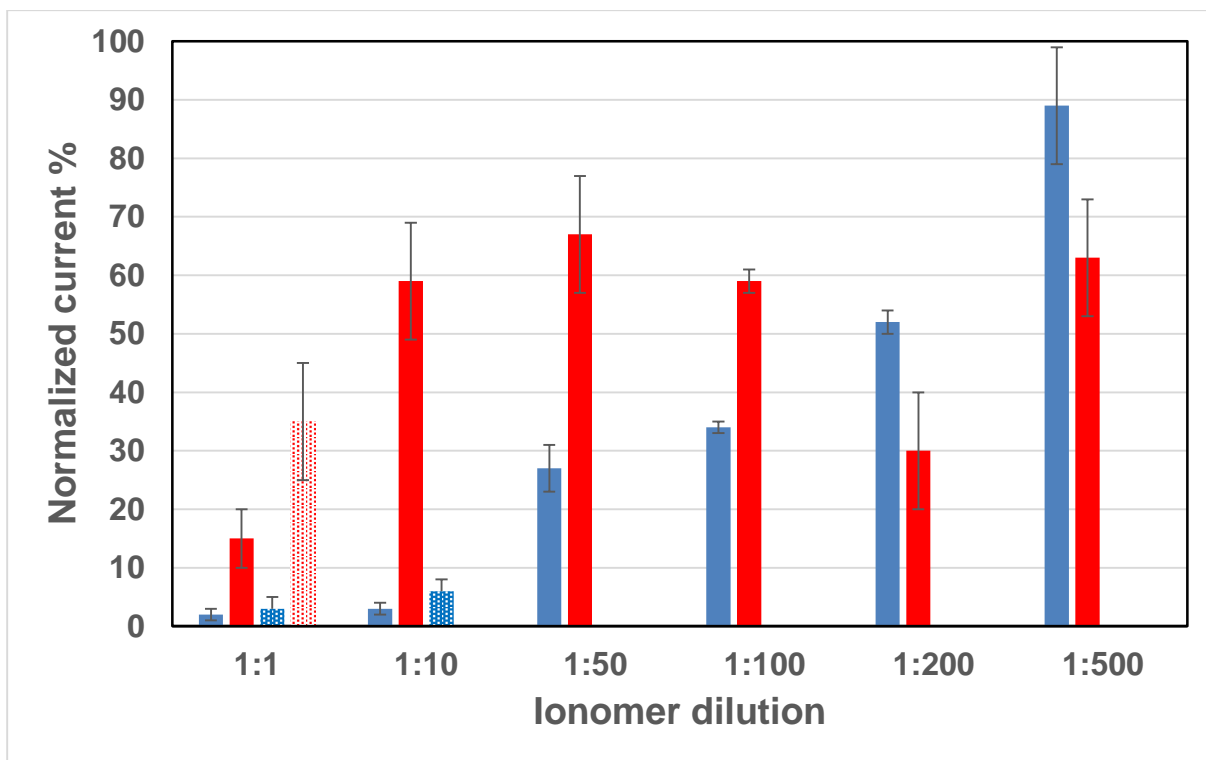


Figure 5. Normalized currents measured at 0 mV vs Ag/AgCl for O₂ reduction by *Mv* BOD in mixture with the different ratios of SPEEK (blue columns) or Nafion (red columns). Dotted colours are for neutralized ionomers. CNT-modified PG electrode, 50 mM PPB pH 6, $v = 5$ mV/s.

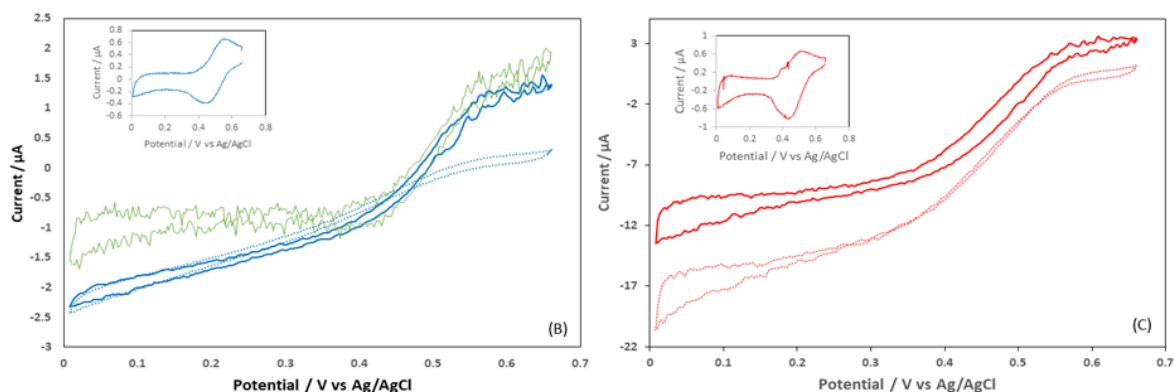
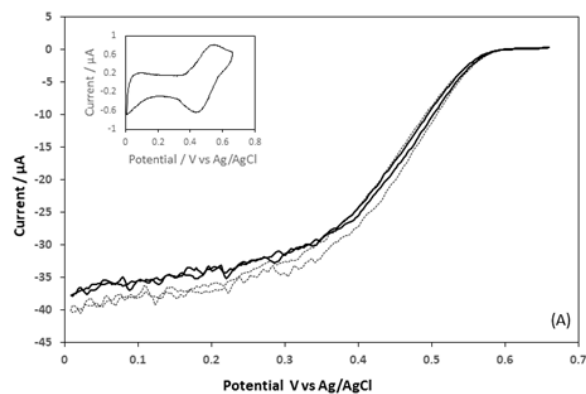


Figure 6. Mediated electrocatalytic reduction of O_2 on CNT modified PG electrode by Mv BOD (A), Mv BOD/SPEEK 1:10 (B) and Mv BOD/Nafion 1:1 (C). Dotted lines and plane lines are obtained before and after $200 \mu\text{M}$ ABTS addition respectively. SPEEK 1:10 and Nafion 1:1 are neutralized solutions. In (B), blue and green lines are obtained with and without Mv BOD, respectively. Inserts corresponds to the signal obtained under N_2 in the presence of ABTS. 50 mM PPB pH 6, $v = 5 \text{ mV/s}$.

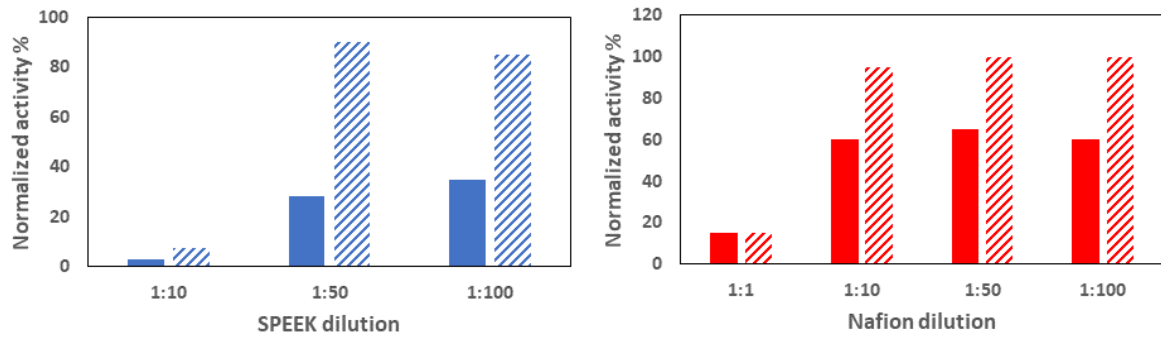


Figure 7. Comparison of normalized currents measured at 0 mV vs Ag/AgCl for O₂ reduction by *M_v* BOD as a function of protocole A (solid column) or B (hatched column). SPEEK (blue columns) or Nafion (red columns). CNT-modified PG electrode, 50 mM PPB pH 6, $\nu = 5$ mV/s.

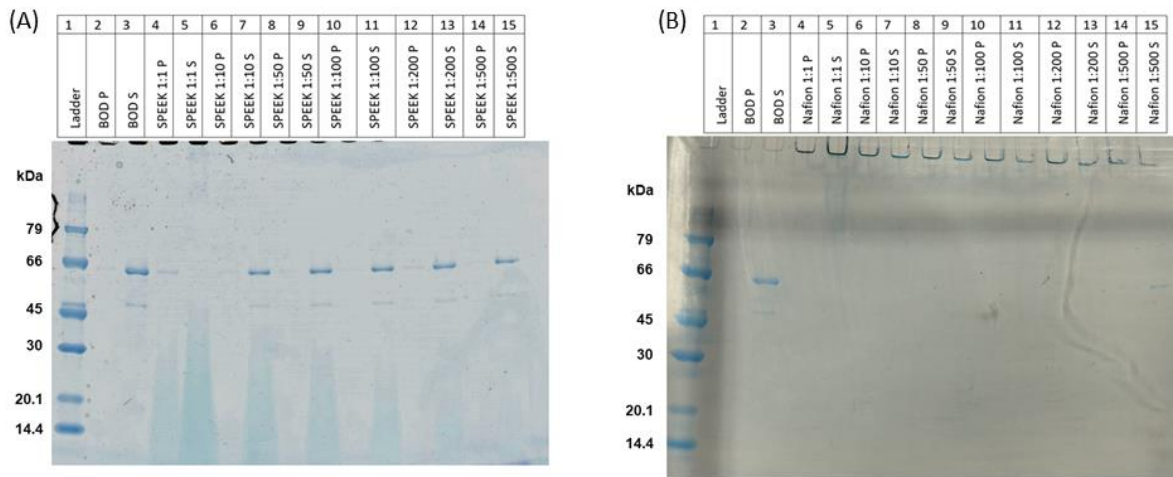


Figure 8. Visualization of *M_v* BOD mixtures incubated with different ratios of SPEEK (A) or Nafion (B) by SDS-PAGE. P and S means pellet and supernatant respectively.

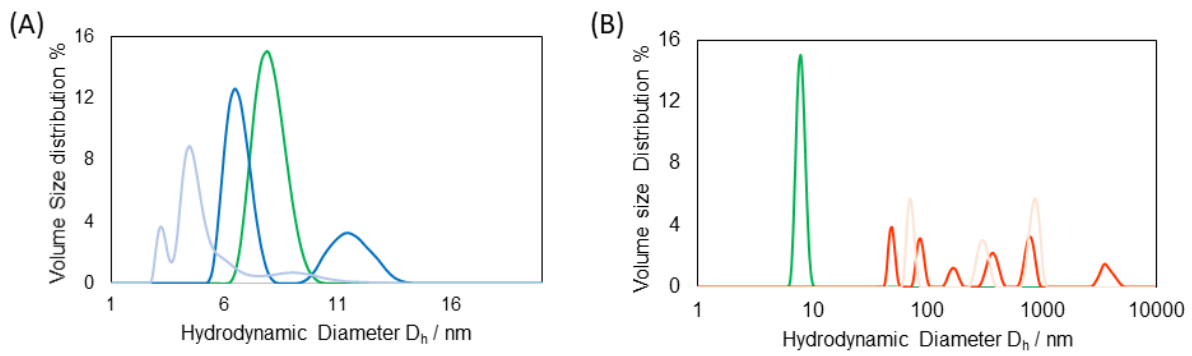


Figure 9. DLS analysis of *Mv* BOD with or without SPEEK (A) or Nafion (B) at 1:10 dilutions. Green curve: 10 μ M BOD; Blue dark curve: BOD + SPEEK 1:10; Blue light curve: 1:10 SPEEK; Red dark curve: BOD + Nafion 1:10; Red light curve: 1:10 Nafion.