Electronic Supplementary Information (ESI†)

Chitosan improves stability of carbon nanotube biocathodes for glucose biofuel cells

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Materials and methods

1. *Enzymes and chemicals*

Laccase from *Tramates versicolor* (12.9 U.mg^{-1}), NaCl, Na_2HPO_4 (> 99 %), NaH_2PO_4 (> 99 %), ferrocyanide ($Fe(CN)_6$) , sodium fluoride and highly viscous chitosan from crab shells were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). An electrode storage solution that contained phosphate buffer (0.02 mole L^{-1}), NaCl (0.14 mole L^{-1}), and KCl (0.0027 mole L^{-1}) at pH 7 was prepared and stored at 4 °C until usage. Silicone (AS310) was purchased from SILICOMET. All aqueous solutions were prepared using ultrapure water from Millipore system. MWCNTs were purchased from Nanocyl (> 95 % purity, 9.5 nm diameter).

2. *Preparation of biocathodes*

Laccase from *Tramates versicolor* (100 mg) was homogenized with multi-walled carbon nanotubes (MWCNTs) (100 mg). Viscous chitosan (1 % in 0.5 % acetic acid) was obtained after 2 hours stirring at ambient temperature. Then the laccase and MWCNT mixture was added to 200 µL of the viscous chitosan solution. The resulting mixture was divided into 5 equal quantities for formation into disks which were compacted using a manual press. In order to make the electrical connection, a wire was embedded in carbon paste covering one side of each disk (surface: 0.5 cm^2, thickness: 1 mm). The perimeter and the covered side of the disc were isolated with silicone.

3. *Laccase activity measurement*

Laccase activity was monitored by measuring the oxidation of ABTS determined by the increase in absorbance at 420 nm (ε_{420} = 3.6 × 10^4 M^{-1} cm^{-1}) [1]. The reaction mixture contained 0.5 mM substrate (ABTS), 2.8 mL of 0.1 M sodium acetate buffer of pH 4.5 and 100 µL of sample solution and incubated for 5 min at ambient temperature (20 ± 3 °C). Measurements were performed with Spectrophotometer Beckman DU®530. Enzyme activity was expressed in µmol of ABTS oxidized per minute [2]. The enzyme activity was monitored

using sample volumes from the cathode buffer storage solution in the beginning of the storage period and after 4 months. Measurements were performed in duplicate.

During the first three months of storage, no laccase activity was detected in the buffer solution. If the release of enzyme is very slow and the laccase activity in solution is too low to be quantified by absorbance measurements, then small quantities of enzyme cannot be detected. Moreover, the released enzyme activity is considered as negligible. The enzyme which is no longer protected by the microenvironment in the 3D electrode matrix can be inactivated in solution over a period of time at neutral pH. The measured activity can be attributed to freshly released enzyme which remains active. It's difficult to quantify and to follow with precision the enzyme molecules loss since inactive ones can coexist but are not detectable. These measurements allow only evaluating the loss of residual active laccase. The conducting chitosan nanofibres without any crosslinking agent or stabilizer improve the enzyme entrapment. In spite of possible disconnection, denaturation or release of some enzyme molecules, the remaining active molecules entrapped in the electrode matrix insured a stable current response. It can be assumed that the initial amount of laccase is largely in excess.

4. ***Scanning electronic microscopy (SEM)***

The structure of the biocathodes was characterized by SEM using an ULTRA 55 FESEM based on the GEMENI FESEM column (Nanotechnology Systems Division, Carl Zeiss NTS GmbH). Samples were sputter-coated with 1 nm gold-palladium using precision etching coating system (PECS 682- Gatan, Inc., CA).

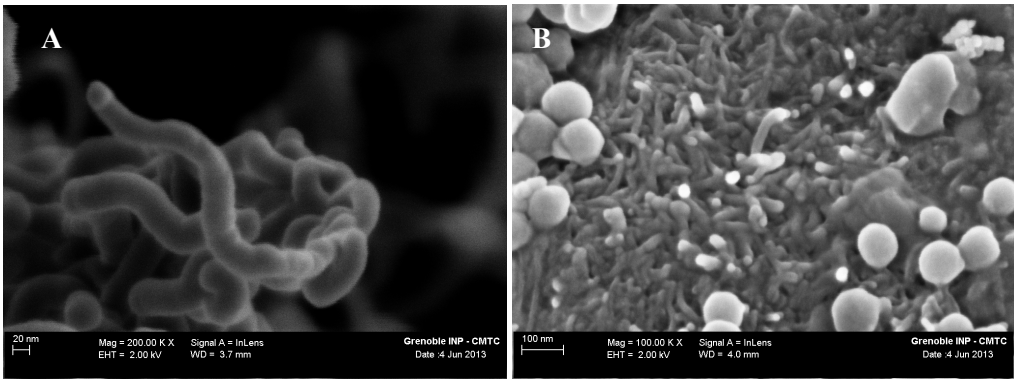

Fig. 1S SEM images of (A) chitosan nanofibres, (B) laccase molecules agglomerates in chitosan matrix

5. ***FTIR analysis***

In order to confirm the chemical composition of the nanofibres, FTIR analysis was performed. Fourier transformed infrared (FTIR) spectra of biocathodes samples were carried out on a PERKIN ELMER PARAGON 1000 spectrometer. The scans were performed at 4 cm^{-1} resolution. Measurements were recorded between 4000 and 600 cm^{-1}. The FTIR spectrum was taken in a transmittance mode.

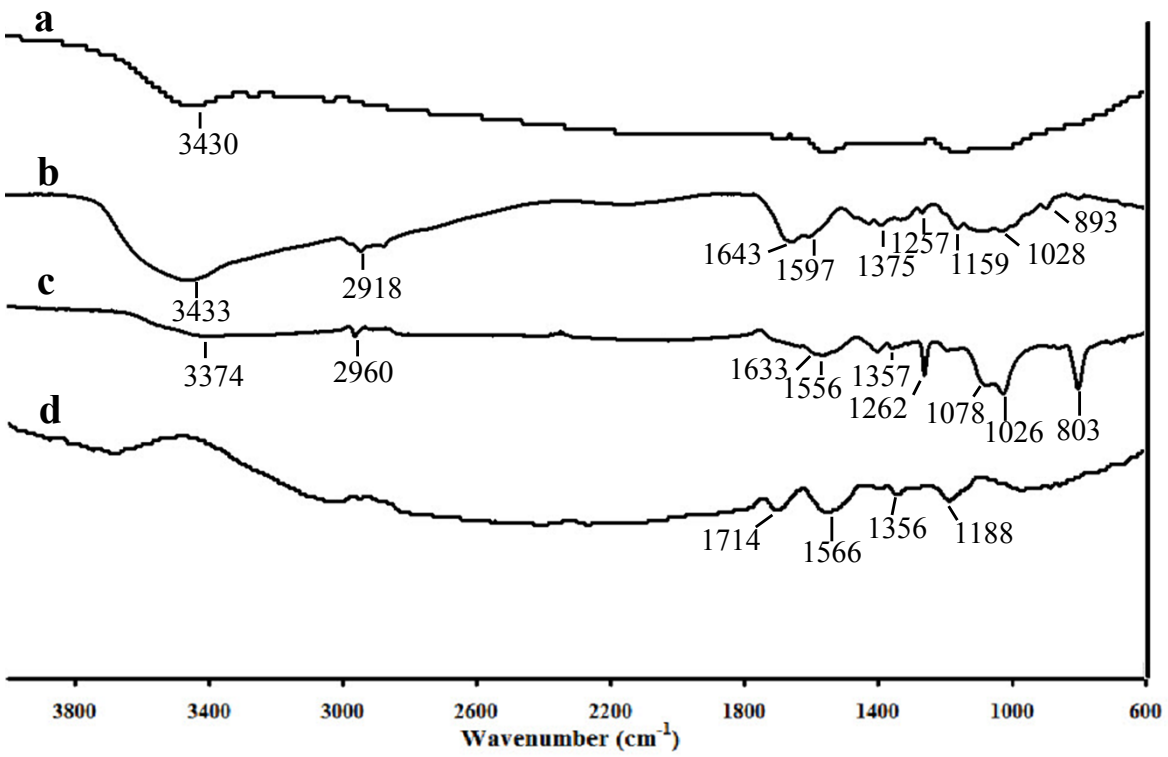

Fig. 2S FTIR spectra of, (a) MWCNT, (b) chitosan, (c) compressed Chit-MWCNT, (d) compressed Chit-MWCNT-laccase

The spectra of (a) MWCNT and (b) chitosan powders were recorded as reference. The MWCNT spectrum (a) display mainly two peaks at 3430 cm^{-1} and 1044 cm^{-1}, corresponding to OH and C-O stretching vibrations, respectively, which is in agreement with characteristic peaks known for MWCNT [3]. Chitosan (spectrum (b)) presents OH stretching band at 3433 cm^{-1}, C-H stretching band at 2918 cm^{-1}, C-O-C stretching band at 1028 cm^{-1}, C-O-C bridges and glycosidic linkage at 893 cm^{-1}. This is also in accordance with IR characteristic bands of chitosan found in literature [4]. The peak at 1597 cm^{-1} is characteristic of chitosan with high deacetylation degree. The chitosan modified with MWCNT (spectrum (c)) points out the presence of both substances, since the fingerprint signals of MWCNT and chitosan are present. Moreover, the peak at 1375 cm^{-1} (curve (b)) is assigned to the $-CH^3$ symmetric deformation shifted to 1357 cm^{-1} and 1356 cm^{-1} in the spectra (c) and (d), corresponding to Chit-MWCNT and Chit-MWCNT-laccase.

Concerning chitosan (spectrum (b)), the amide I band is present, as witnesses the presence of absorption peak at 1633 cm^{-1} (C-O stretching) and 1556 cm^{-1} (N-H stretching). The presence of characteristic peaks of chitosan which remain intact after mixing with MWCNTs as well as the intensity of amide I band confirm that the nanofibres observed on SEM images correspond to chitosan.

The amide I band shifted to 1566 cm^{-1} (curve (c)) in the presence of laccase in the 3D matrix. A broader peak at 1566 cm^{-1} can be attributed to the reaction between the free amino groups of chitosan and laccase. Broad peaks at 1633 cm^{-1} and 1262 cm^{-1} (C-N stretching of amide III) were noticed in spectrum (c), indicating that new crosslinking reactions have indeed occurred. Two broader peaks appeared in the curve (d). The peak

at 1714 cm^{-1} corresponds to an aldehyde II (C=O aldehyde stretching). The peak at 1188 cm^{-1} corresponds to the secondary amine/ C-N stretch. The amino group is represented by the large absorption band in the region 3500-3350 cm^{-1} (curve (b)). The intensity of this band decreased in curve (c) which indicates that some of the amino groups of chitosan have reacted. The absence of the peak at 3374 cm^{-1} indicates that all free amino groups of chitosan were linked to laccase molecules (curve (d)).

6. ***Electrochemical measurements***Electrodes were analyzed in 100 mL of a solution in three electrodes electrochemical cell with a saturated calomel electrode (SCE) as reference electrode and a platinum mesh as a counter electrode.

6.1. Determination of the electrode surface area

The biocathode was analyzed in 100 mL of a solution that contained phosphate buffer (0.02 mole L^{-1}), NaCl (0.14 mole L^{-1}), KCl (0.0027 mole L^{-1}) at pH 7. A saturated calomel electrode (SCE) was used as reference electrode and a platinum mesh as a counter electrode. The chronoamperometric response of the biocathode was recorded at 0.2 V *vs*. SCE. All measurements were performed at ambient temperature (20 ± 3 °C).

We measured the transient oxidation current of ferrocyanide ($Fe(CN)_6$) on the electrode surface at 0.3 V. The corresponding transient cell current is described by the following equation:

$$I = i_d + i_c$$

Where I is the total cell current, i_d is the diffusion limiting current and i_c is the capacitive current of the electrochemical double layer. The diffusion limiting current is given by the Cottrell relationship:

$$i_d = \frac{nFAC\sqrt{D}}{\sqrt{\pi t}}$$

Where n is the number of electron(s) transferred per $Fe(CN)_6$ molecule, F is Faraday's constant, A is the area of the electrode surface in cm^2, D is the diffusion coefficient in $cm^2\ s^{-1}$, C is the concentration of the electroactive species in mol cm^{-3}, and t is time in seconds. The capacitive current is given by the equation:

$$i_c = \frac{E}{R} e^{\left(\frac{-t}{RC_d}\right)}$$

Where E is the applied voltage, R is the resistance of the cathode, C_d is the double layer capacitance and t is the time. For short times, the capacitive current has a considerable contribution to the total cell current. However, for long periods of time its contribution is negligible and the total current is given by the Cottrell relationship and hence the value of $it^{1/2}$ as a function of t should be constant. Since, F= 96400, and n=1, D = 7.7

x 10^{-6} $cm^2.s^{-1}$ for $Fe(CN)_6$,the electrode surface area can be deducted from the plot $it^{1/2}$ *vs*. *t*. Figure 2SA shows the chronoamperometry measurement realized on the Chit-MWCNTs electrode (without laccase) in 5 mM $Fe(CN)_6$ at pH 7. After the suppression of the zone corresponding to the capacitive current, we observed that the current evolved with time according to Cottrell equation. Figure 2SB shows that $it^{1/2}$ as function of *t* is constant, which indicates that transient current is diffusion limited in this zone. The surface area of our electrode is 2 cm^2 for 1 cm^2 geometric area and 25 mg of MWCNTs, providing an electrochemical specific surface area of 40 $cm^2.g^{-1}$. This value is four times lower than the one obtained with the MWCNTs pellet (160 $cm^2.g^{-1}$) [5]. This difference can be attributed to the swelling effect of the electrode since we observed that the volume of Chit-MWCNTs pellet doesn't changed significantly after swelling, in opposite to the case of MWCNTs where the interconnection of MWCNTs is insured only by the initial mechanical compression during the fabrication and electrostatic adsorption.

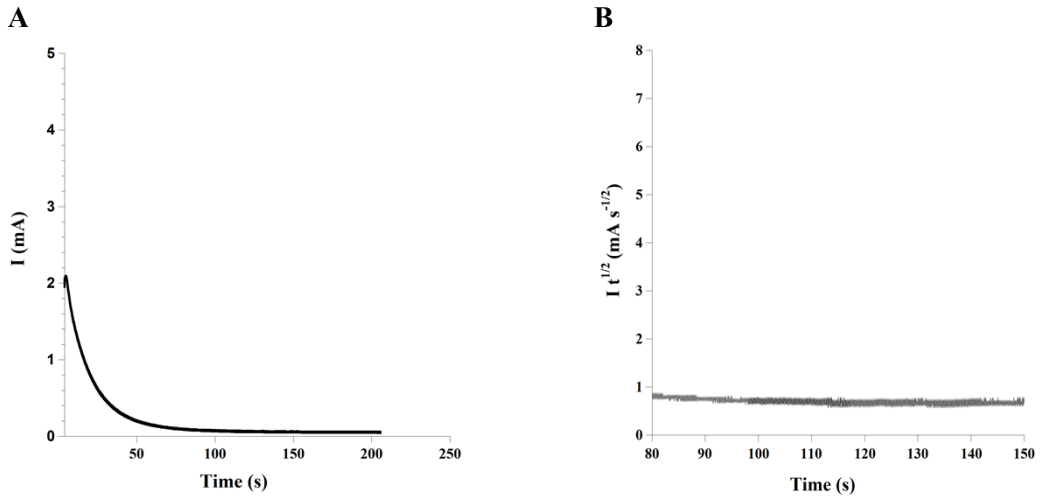

Fig. 3S (A) Chronoamperometric response of Chit-MWCNTs electrode in phosphate buffer containing 0.005 mole L^{-1} ferrocyanide, (B) $it^{1/2}$ as function of time.

6.2. ***Chronoamperometry measurements***

The biocathodes were analyzed in 100 mL of a solution containing NaCl (0.14 mole.L^{-1}), KCl (0.0027 mole.L^{-1}) at pH 7. The chronoamperometric response of the biocathode was recorded at 0.2 V *vs*. SCE. All measurements were performed at ambient temperature (20 ± 3 °C).

In order to study the storage stability, the biocathodes were stored in individual urine collection containers in 3 mL solution of phosphate buffer (0.02 mole.L^{-1}), NaCl (0.14 mole.L^{-1}), and KCl (0.0027 mole.L^{-1}) at pH 7 and at ambient temperature (20 ± 3 °C). Each container was perforated (hole diameter: 3 mm) for air supply and equipped with filter to minimize bacterial contamination. The buffer solution was renewed monthly. The storage stability was evaluated by periodically measuring current density at 0.2 V *vs*. SCE every two weeks during 12 h continuous discharge. The biocathodes were stored at neutral pH and room temperature.

Two Chit-MWCNT electrodes were prepared without adding laccase in the mixture. Chronoamperometric measurements were performed as described above.

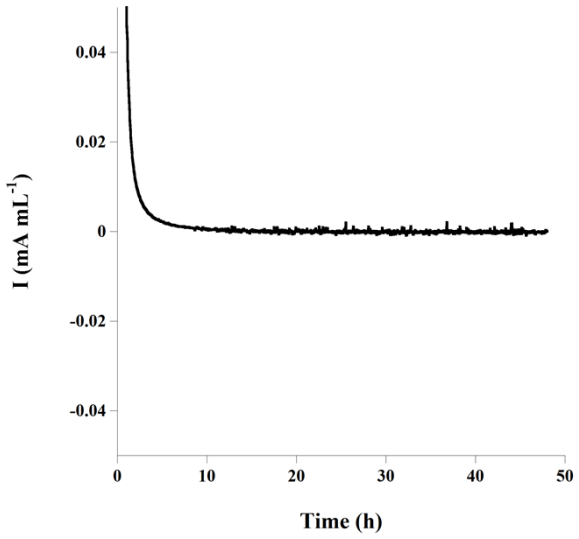

Fig. 4S Chronoamperometric response of Chit-MWCNT electrode at 0.2 V *vs*. SCE.

7. ***Inhibition of the current response by sodium fluoride***

The chronoamperometric response of the Chit-MWCNT-laccase biocathode was also monitored in presence of sodium fluoride (10 mM) which is a strong inhibitor of laccase. The inhibitor which binds the T2/T3 site prevents the electron transfer from the T1 site onto the T2/T3 cluster. The fluoride ion influences the laccase activity by lowering the redox potential of the T3 site [6].

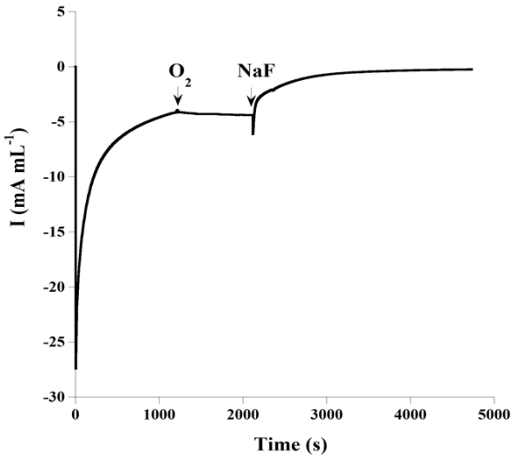

Fig. 5S Chronoamperometric response at 0.2 V of the biocathode in presence of sodium fluoride in phosphate buffered saline solution pH 7

8. ***Measurements of bioelectrode function in sheep serum***

In order to demonstrate the potential use of our bioelectrode as implantable electrode, we assayed the chronoamperometric response of the biocathode in serum at 37°C under continuous discharge for 48 h (Fig. 6S). Three electrodes were used for the reproducibility testing. The experiment duration wasn't extended

because of the contamination risks of the serum sample. It is important to maintain a constant flow of fresh serum which is why an animal model is more appropriate for the long term stability testing.

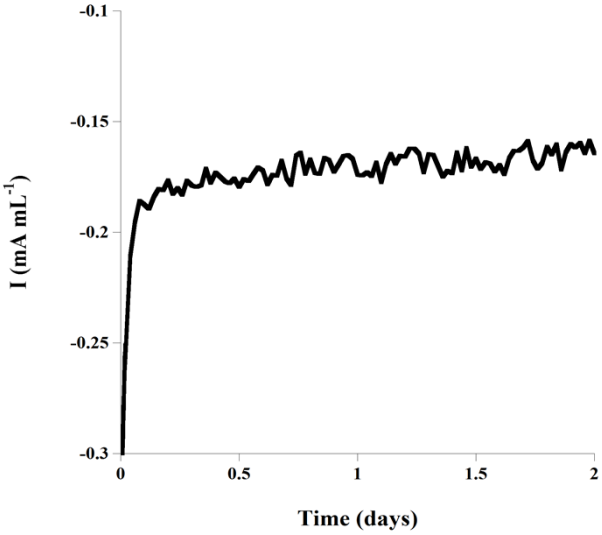

Fig. 6S Chronoamperometric response at 0.2 V vs. SCE of the biocathode in sheep serum at 37°C.

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