Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B

Supporting Information for Biosensing with electroconductive biomimetic soft materials

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S.1 Supporting Methods

Chemical polymerization

1/100 v/v Ammonium Persulfate, 5% solution (APS, Sigma-Aldrich, Italy) and 1/1000 v/v N,N,N,N-Tetramethylethylenediamine (TEMED, Sigma-Aldrich, Italy), are added to the prepolymer solution.

APS in water produces $S_2O_8{}^{2-}$ $2SO_4{}^{-}$ free radicals that initiate the reaction. TEMED catalyzes the reaction because it exists as free radical in solution. The higher is the concentration of TEMED, the faster will be the polymerization. It also would be better to degas the pre-polymer solution before adding APS and TEMED because oxygen can inhibit the initiator. However in this case it is not necessary because the concentration of monomer is high. So the ready solution has to be rapidly transferred to the PDMS mold and then covered with a coverslip like in photopolymerization. In this way it takes about 15 minutes to reach the complete polymerization of little cylindrical hydrogels at room temperature.

Water suspension of SWNTs

Sodium cholate (SC) is an ionic surfactant, it can be removed by dialysis and can also be successfully used for carbon nanotubes dispersion. At concentrations above 2mM, SC will form micelles having M.W. 900 to 1200. The small size of the micelles allows themselves to be easily removed by dialysis

or by gel filtration.

The compound was ultra-sonicated with a 13 mm diameter titanium tip (Bandelin Sonopuls HD 2200) for 60 min at 40W in an ice bath to prevent the overheating of suspension. The power of the ultra-sonicator was kept not too high to prevent an excessive shortening of nanotubes and consequently a deep modification of their bulk properties. Then the resulting suspension was decanted for some days. It appeared of a black color.

Deposition processes

Photolithography

Squared glass slides (borosilicate glass, 40x40 mm, 1 mm thick, Vetrotecnica, Italy) are immersed in a piranha solution (3:1 v/v, concentrated H_2SO_4 : 30% H_2O_2) for 5 minutes.

Because the mixture is a strong oxidizer, it removes most organic matter and it also hydroxylates the surface making it hydrophilic. Then they are rinsed with distilled water and acetone and dried in an oven in air at 400 K till drying is complete. Each glass slide is then positioned on the spin coater rotating plate (WS-650 29MZ-23, Laurell) and covered with 1mL of photoresist (SU-8 2000, MicroChem, USA) every 25 mm of substrate diameter. The speed of the spin coater is set at 2000 rpm for 30 seconds to obtain a final thickness of 6 μm. The SU-8 product consist of chemically amplified, epoxy-based negative resists with high functionality, high optical transparency and

are sensitive to near UV radiation. Cured films or topography are highly resistant to solvents, acids and bases and have excellent thermal stability, making it well suited for permanent use applications.

After that the resist is soft baked on a level hotplate set at 368 K for 2 minutes to relax the polymer structure and thus to reduce internal tensions.

So the photoresist is exposed to UV light ($\lambda = 365$ nm, OAI 150) at 110 - 140 mJ/cm^2. At this stage a photo-mask is put over the glass slide. In the black regions UV light will not reach the photoresist and so it will not crosslink.

Slides are then newly put on a level hotplate set at 368 K for 2 minutes. In this post exposure bake stage the regions that were exposed to UV light can reticulate. After 1 minute an image of the mask should be visible on the surface.

To develop the photoresist, i.e. to remove it from the not lighted regions, the slide is completely immersed in 1-methoxy-2propanol-acetate (Sigma-Aldrich, Italy) for 3 minutes. The development is blocked with isopropyl alcohol. At the end slides are rinsed with distilled water and dried.

Plasma-Enhanced Chemical Vapor Deposition (PECVD)

PECVD is a process used to deposit thin films from a gas state (vapor) to a solid state on a substrate. Chemical reactions are involved in the process, which occur after creation of a plasma of the reacting gases. The plasma is generally created by RF (AC) frequency or DC discharge between two elec-

trodes, the space between which is filled with the reacting gases.

Two different layers are deposited on the glass slides covered with the photoresist mask, using PECVD (PORTA 900s, Plasma Electronics-Antec): first a 50 nm thick titanium layer is deposited on the glass to provide a good mechanical adhesion to glass. Then a second gold layer of about 15-20 nm is deposited over the titanium layer. PECVD can provide a gold surface for these homemade electrodes much purer than that of commercial gold electrode. Parameters used for the depositions are displayed below (Fig. S1). After the deposition, the gold-coated glass slides are treated with the remover

	Titanium layer	**Gold layer**
Current on target	2A	30mA
Atmosphere of deposition	93 sccm Ar	93 sccm Ar
Current on substrate	150 W	150 W
Rotational speed of sample holder	4 rpm	4rpm
Deposition time	6 min	100 s
Distance between target and substrate	12 cm	12 cm
Thickness (by profilometer)	50 nm	15-20 nm

Figure S1: **Deposition parameters for PECVD steps.** -

through sonication at 350 K and rinsed with distilled water to eliminate the photoresist. Thus only the gold layer remains on the glass surface (Fig. S2). In the available configuration these homemade gold electrodes provides five separated lines that can be used as five different working electrodes. Gold electrodes have a diameter of 3 mm. Gold lines are covered with polyamide insulating tape (Kapton) to prevent line shorting once the glass slide is im-

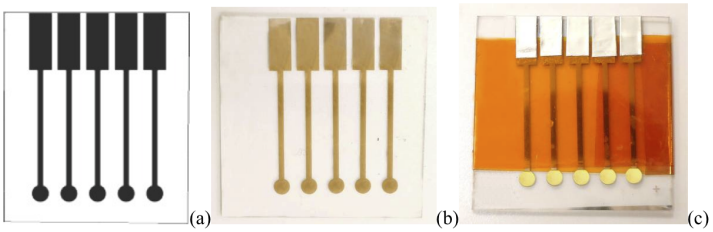

Figure S2: **Patterning of the homemade electrode** - a) photomask; b) gold coated glass; c) finished homemade gold electrode

mersed in the electrolyte solution. Moreover the ends of lines are covered with small strips of electro-conductive tape (aluminium foil coated with Ni NPs-doped glue) that allows connecting it to the potentiostat-galvanostat through a clip for each line. A line-selector allows to chose the desired line without opening or moving the electrochemical cell.

HYs preparation

100 mg of AA powder were dissolved in 100 ?L of milliQ-water resulting in a final volume of 180 μL.

To further increase the SWNTs concentration, the 1wt% carbon nanotubes suspension was heated in order to evaporate water. A 1.5 mL Eppendorf was filled with 1mL of 1wt% nanotubes suspension and put on a hot place. The resulting nanotubes suspensions had a maximum 1.33wt% concentration because a too long heating could damage nanotubes.

Firstly Eppendorf were filled with BIS powder, AA solution and SC solution in the right quantities (Fig. S3).

Then SWNTs suspension were added to the pre-polymer solution (Fig.

CNTs wt%	AA 40% (µL)	AA 100% (µL)	BIS(mg)	2wt% SC in H_2O (µL)
0.1	250		3.5	200
0.25	250		3.5	125
0.375	250		3.5	62.5
0.5	250		3.5	
0.64		180	3.5	
0.85		180	3.5	

Figure S3: **Compositions doped hydrogel prepolymer solutions** -

CNTs wt%	1wt% CNTs in H_2O (µL)	1.33wt% CNTs in H_2O (µL)
0.1	50	
0.25	125	
0.375	187.5	
0.5	250	
0.64	320	
0.85		320

Figure S4: **Additions of carbon nanotubes suspensions** -

S4).

SWNTs-doped HYs were then chemically polymerized on glass coverslips pre-treated with APTES and glutaraldehyde. Photo-polymerization was not applicable to the SWNTs-doped HYs because nanotubes acted as a black filter absorbing light radiation and preventing polymerization even after a long exposure time. This caused burns and excessive drying on the surface while not complete polymerization of lower layers of hydrogel.

Preparation of GOx/SWNTs-doped poly(AA/BIS)/gold biosensor

0.5 mm thick cylindrical SWNTs-doped HYs were polymerized on homemade gold electrodes. Homemade gold electrodes were pre-treated with APTES-

Glut but only on the glass surface that surrounds the gold spot to improve adhesion of HYs without damaging the gold surface.

Biocompatibility tests - Seeding and Culture of C2C12 cells

Cultures of C2C12 cells were used to test two different types of SWNTs-doped hydrogels:

- hydrogels with 20% w/v AA, AA/BIS 29:1 and 0.1% SWNTs
- hydrogels with 20% w/v AA, AA/BIS 29:1 and 0.85% SWNTs

In both cases chemical polymerization method with APS and TEMED was used.

After polymerization, cylindrical hydrogels with 0.5 ?m thickness and 20 mm, were rinsed with distilled water for three times to remove all residual monomer and free nanotubes traces.

Moreover they were rinsed with PBS 1X to cause the swelling of the hydrogel useful to remove further traces of nanotubes from hydrogel pores. After that hydrogels were sterilized with an UV lamp for 15 min working inside a sterile hood.

Subsequently, every hydrogel was put in a compartment of a multiwell and sprinkled with some drops of a protein accession solution (laminin-collagen) on the top face. After 2-3 minutes the protein solution was removed. After-

wards 3 ml of cell culture medium with 3x105 C2C12 cells (murine myoblast cell line) were put in every well, primarily sprinkling the top face of the hydrogel to favour the cell seeding on the surface of the hydrogel.

Samples were maintained in the incubator (Heraeus BBD 6220) at 310 K in an atmosphere of 5% CO_2 and 95% relative humidity for three days, and then they were analyzed with a LIVE/DEAD assays to have information about the cell viability, and labelled with antibodies for C2C12 myoblasts and their functional differentiated myotube state.

LIVE/DEAD assay

Live cells are distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the nonfluorescent cell-permeant Calcein AM to the intensely fluorescent Calcein. The polyanionic dye Calcein is well retained within live cells, producing an intense uniform green fluorescence in live cells (EX/EM $\lambda = 495$ nm/$\lambda = 515$nm).

Ethidium homodimer-1 (EthD-1) enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells (EX/EM 495 nm/ 635 nm). EthD-1 is excluded by the intact plasma membrane of live cells. The determination of cell viability depends on these physical and biochemical properties of cells. It was found that Calcein AM and ethidium

homodimer are optimal dyes for this application.

Cell culture medium were completely removed from the wells and the cell cultures were rinsed with PBS 1X twice. Wells were then filled with PBS 1X solution of 3 μM calcein AM and 3 μM ethidiumhomodimer-1. After 45 minutes at room temperature cell culture were washed twice in PBS 1X and analyzed with a fluorescence microscope (Leica DMI6000-B).

Immunochemistry

C2C12 are precursor cells called myoblasts able to mature into myotubes, the functional unit of muscle fibers. Cells were fixed in 4% paraformaldehide (Sigma-Aldrich) and permeabilized with 0.1% triton-x100 (Sigma-Aldrich) in phosphate buffer (PBS) and 5% foetal bovine serum. Desmin and myosin, which are citoskeletal proteins selectively expressed by myoblast and myotubes respectively, were labelled by specific primary antibodies (Abcam) for 1 hour. Primary antibodies were detected with a fluorophore-conjugated secondary antibody (Invitrogen) and nuclei were stained with a Hoechst dye (Invitrogen).

Electrical signaling from cells and breeding/culturing protocol specifications Cardyomyocites are from 0-3 days neonatal rats Sprague Dawley that are breeded under legislative regulations in experimental animals after authorization of University Ethical Commitee of Experimentation on Ani-

mals (C.E.A.S.A.) of University of Padua.

Cells were seeded on sterilized HYs (sterilization occurs with ethanol 100% immersion, subsequent rinsing with milliQ water and storing in sterile PBS for 12 h) at a cellular density of 300'000 cells/HY. Since polyacrilamide is cellular repellent, it was needed to functionalise HY surface with an adhesion protein. It was used laminine (BD, 354232) at 100 μg/mL concentration. Laminine solution was deposited on HY, whose surface was previously dried with vacuum pump and incubated for 2 min at room temperature. The protein is then removed.

EIS measurements and chronoamperometries with cells were conducted in recording solution (NaCl 125 mM, KCl 5 mM, Na_3PO_4 1 mM, $MgSO_4$ 1 mM, Hepes 20 mM, $CaCl_2$ 2 mM, Glucosio 5.5 mM a pH 7.4 con NaOH).

S.1.1 Supporting Results

Raman measurements

Raman spectroscopy was carried out to get information about the dispersion of SWNTs. A 15x15 μm squared area was scanned on three humid hydrogels with three different concentrations of SWNTs (0.1wt%, 0.5wt% and 0.85wt%). A Raman/SNOM confocal spectrometer (Alpha 300S, Witec) equipped with a He-Ne laser ($\lambda = 633$ nm) and a 10X lens was used.

Spectra were integrated 10 times for each point in the G-band peak interval (between 1585 and 1597 cm^{-1}). Then data were normalized on the maximum and minimum peak intensity collected throughout the three HYs and plotted as a function of x-y spatial coordinates obtaining three comparable microRaman maps of the surface of the hydrogels.

The specific Raman activity of carbon nanotubes allows a qualitative comparison between their concentrations in different doped-hydrogels, by considering the Raman radiation coming from the volume near the surface.

Surface maps (Fig. S5 were easily obtained for humid hydrogels. The same maps for the transversal sections were not obtained because the cut surfaces were too rough to achieve a good focus on them at 10X magnification.

The intensity of the G-band peak provided by CNTs were used to compare the amount of nanotubes present in the three samples. Maps in 5 are normalized on the maximum intensity of the G-band throughout the three HYs. So darkest regions correspond to zone at low level of doping. All maps

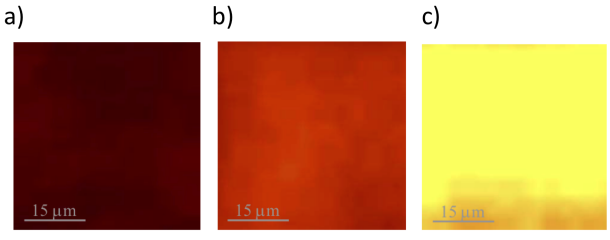

Figure S5: **MicroRaman maps** - SWNT HY 0.1wt% a), SWNT HY 0.5wt% b) and SWNT HY 0.85wt%

show a good color uniformity that confirms a good degree of dispersion on a micrometric scale. Only some slight variations to lighter color (i.e. increment in intensity) appear, meaning that CNTs concentration is a little bit higher in those regions. In agreement with micrographs (Fig. S6) these could be regions where CNTs are more aggregated, thus more concentrated than in the rest of the hydrogel surface.

In Fig. S5d the mean intensity of the G-band peak for every map is plotted as a function of CNTs theoretical concentration. The graph shows that increasing nanotubes concentration, the surface concentration increases too as it was expected.

Micrographs (Fig. S6) of HYs were taken with a OLYMPUS inverted steromicroscope coupled with a camera, at a magnification of 10X and fixing light intensity and other optical parameters. This gave the possibility to compare the microstructure of HYs with different SWNTs concentrations. Micro pictures of both the surface and of the transversal section of HYs were taken.

EIS measurements

Below we present summarizing table referring to Fig. 3 in the manuscript (Fig. S7). Standard errors are calculated on the base of three repetitive measurements.

Electrodes treated within the manuscript were also statistically characterized by means of analysis of variance (ANOVA).

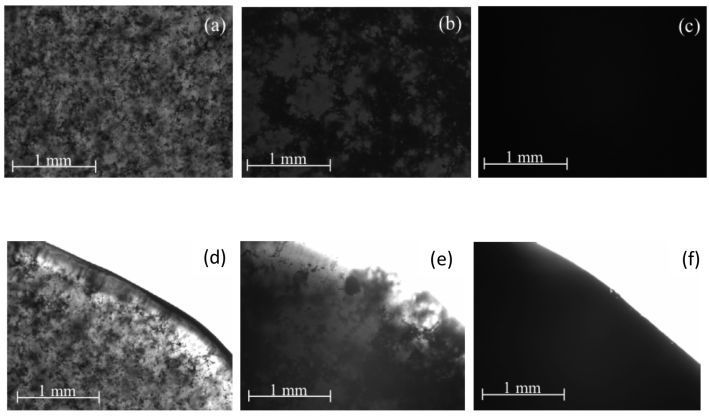

Figure S6: **Optical micrographs** - SWNT HY 0.1wt% a), SWNT HY 0.5wt% b) and SWNT HY 0.85wt% c). Top view and transversal view.

ANOVA test in fact provides a statistical test of whether or not the means of several groups are all equal. It generalizes t-test to more than two groups. Box chart plot of Fig. S7b shows mean values, the smallest observation (sample minimum), lower quartile, median, upper quartile, and largest observation (sample maximum) for every sample.

CV measurements

Referring to Fig. 4a of the main manuscript, we show in the figure below (Fig. S8) the evolution of the anodic peaks with time for the four samples. The same curves show that for 0 wt%, 0.1 wt% and 0.85 wt% HYs, the capacitive current increases with nanotubes loading and with time. Also for 0.5 wt% sample the area gets larger but it is much greater than others from the beginning. Since a linear shape like 0.5wt% is specific for an insulating layer (a quasi ohmic response is given), thus confirming the fact that in such

a)

SWCNTs conc. (wt%)	Circuit	R_{P1} mean (Mohm)	St. Dev.	R_{P2} mean (Mohm)	St. Dev.
0	$R_S(R_{P1}Q)$	3.0	0.47	Not present	
0,1	$R_S(R_{P1}Q)$	1.6	0.53	Not present	
0,25	$R_S(R_{P1}Q)$	0.96	0.53	Not present	
0,25	$R_S(R_{P1}Q)(R_{P2}C)$	0.021		0,30	
0,375	$R_S(R_{P1}Q)(R_{P2}C)$	0.026	0.014	0,22	0,075
0,5	$R_S(R_{P1}Q)(R_{P2}C)$	0.026	0.027	0,11	0,027
0,64	$R_S(R_{P1}Q)$	0.22	0.065	Not present	
0,85	$R_S(R_{P1}Q)$	0.19	0.040	Not present	
GOLD	$R_S(R_{P1}Q)$	0.32	0.16	Not present	

b)

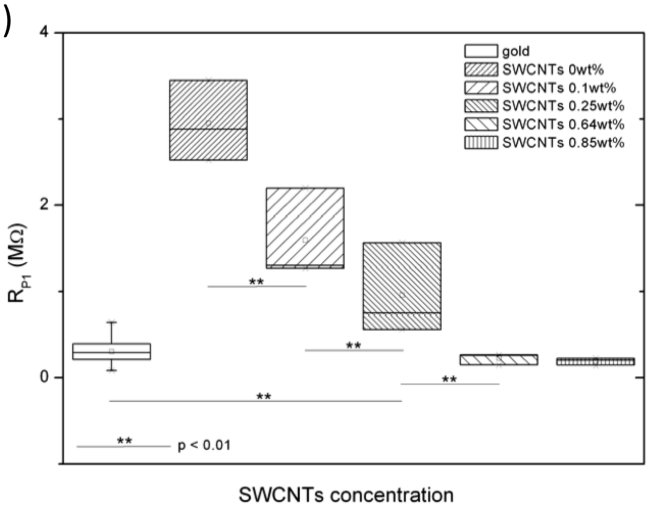

Figure S7: **Statistics for electric measurements.** - a) Mean resistances for modified HYs studied in the work referring to Fig.3 of the main manuscript; b) ANOVA box plot for the samples.

conditions these samples are less conductive than the others as EIS results predicted.

CA measurements

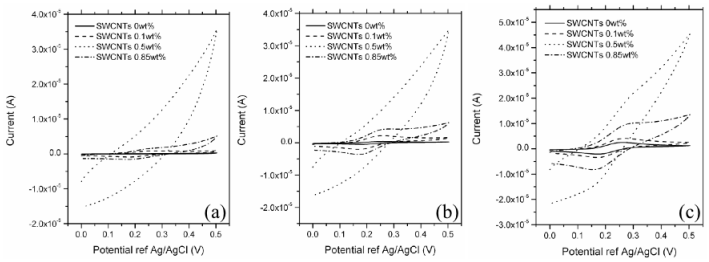

Figure S8: **Comparison between voltammograms.** - CVs in 10mM KNO3, 500?M FcMetOH aqueous solution for SWNTs-doped hydrogels - gold surface composite electrodes after 6 min (a), 30 min (b) and 150 min (c)

As shown in Fig. S9, a glucose biosensor made with HY without SWNTs does not provide any current peak both with low glucose addition even with higher glucose concentration. In biosensor without nanotubes, H2O2 cannot reach immediately the gold surface and so any current is provided.

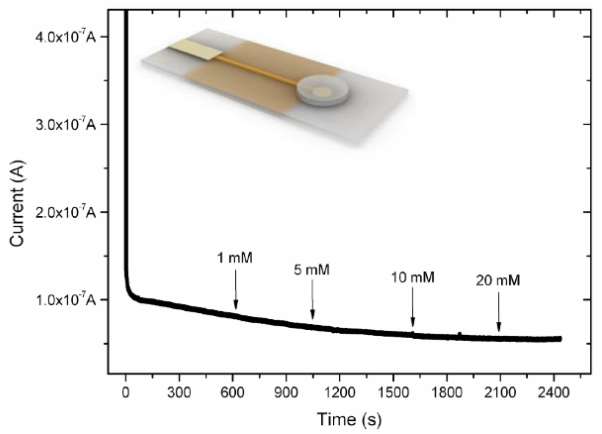

Figure S9: **Potentiostatic measurement on GOx-HY with 0wt% SWNTs concentration.** - Successive glucose additions are reported on the graph with arrows.