



SCUOLA INTERNAZIONALE SUPERIORE DI STUDI AVANZATI

SISSA Digital Library

Chemically Cross-Linked Carbon Nanotube Films Engineered to Control Neuronal Signaling

This is a pre print version of the following article:

Original

Chemically Cross-Linked Carbon Nanotube Films Engineered to Control Neuronal Signaling / Barrejón, Myriam; Rauti, Rossana; Ballerini, Laura; Prato, Maurizio. - In: ACS NANO. - ISSN 1936-0851. - 13:8(2019), pp. 8879-8889.

Availability:

This version is available at: 20.500.11767/98816 since: 2019-07-23T09:44:01Z

Publisher:

Published

DOI:10.1021/acsnano.9b02429

Terms of use:

openAccess

Testo definito dall'ateneo relativo alle clausole di concessione d'uso

Publisher copyright

(Article begins on next page)

Chemically Cross-Linked Carbon Nanotube Films Engineered to Control Neuronal Signaling

Myriam Barrejón, Rossana Rauti, Laura Ballerini, and Maurizio Prato

ACS Nano, Just Accepted Manuscript • DOI: 10.1021/acsnano.9b02429 • Publication Date (Web): 22 Jul 2019

Downloaded from pubs.acs.org on July 22, 2019

Just Accepted

“Just Accepted” manuscripts have been peer-reviewed and accepted for publication. They are posted online prior to technical editing, formatting for publication and author proofing. The American Chemical Society provides “Just Accepted” as a service to the research community to expedite the dissemination of scientific material as soon as possible after acceptance. “Just Accepted” manuscripts appear in full in PDF format accompanied by an HTML abstract. “Just Accepted” manuscripts have been fully peer reviewed, but should not be considered the official version of record. They are citable by the Digital Object Identifier (DOI®). “Just Accepted” is an optional service offered to authors. Therefore, the “Just Accepted” Web site may not include all articles that will be published in the journal. After a manuscript is technically edited and formatted, it will be removed from the “Just Accepted” Web site and published as an ASAP article. Note that technical editing may introduce minor changes to the manuscript text and/or graphics which could affect content, and all legal disclaimers and ethical guidelines that apply to the journal pertain. ACS cannot be held responsible for errors or consequences arising from the use of information contained in these “Just Accepted” manuscripts.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Chemically Cross-Linked Carbon Nanotube Films Engineered to Control Neuronal Signaling

*Myriam Barrejón^{†a}, Rossana Rauti^{‡b}, Laura Ballerini^{*b} and Maurizio Prato^{*a,c,d}*

^aDepartment of Chemical and Pharmaceutical Sciences, Università degli Studi di

Trieste, Via Licio Giorgieri 1, Trieste 34127, Italy

^bInternational School for Advanced Studies (SISSA/ISAS), Trieste 34136, Italy

^cCarbon Bionanotechnology Group, CIC biomaGUNE, Paseo Miramón 182, San

Sebastián, 20014 Guipúzcoa, Spain

^dBasque Foundation for Science, Ikerbasque, Bilbao 48013, Spain

ABSTRACT

In recent years, the use of free-standing carbon nanotube (CNT) films for neural tissue-engineering have attracted tremendous attention. CNT films show large surface area and high electrical conductivity that combined to flexibility and biocompatibility may promote neuron growth and differentiation while stimulating neural activity. Besides, adhesion, survival, and growth of neurons can be modulated through chemical modification of CNTs. Axonal and synaptic signaling can also be positively tuned by these materials. Here we describe the ability of free-standing CNT films to influence neuronal activity. We demonstrate that the degree of cross-linking between the CNTs has a strong impact on the electrical conductivity of the substrate, which, in turn, regulates neural circuit outputs.

KEYWORDS: single-walled carbon nanotube, cross-linking, functionalization degree, neuronal behavior, synaptic activity.

1
2
3 Carbon nanotubes (CNTs) have long been recognized for owning the perfect
4
5
6
7 combination of strength and conductivity, both thermal and electrical, along with
8
9
10 large specific surface area characterized by low density.^{1,2} However, in general
11
12
13 CNTs are not suitable for practical applications, since they are produced in powder
14
15
16 and show poor processability for use in the macroscopic scale. In this sense, the
17
18
19 research on CNT films (often called buckypapers) has blossomed during the past
20
21
22 years,³⁻⁶ yielding materials with improved properties. Films of pure CNTs can be
23
24
25 prepared by vacuum filtration methods,⁴ however the mechanical performance and
26
27
28 stability of pristine CNT films are still limited, as the interaction between adjacent
29
30
31 CNTs takes place through weak π - π stacking and van der Waals forces.⁷ Chemical
32
33
34 cross-linking of CNTs is a promising strategy for increasing the mechanical features
35
36
37 and overcoming the aforementioned weaknesses of CNT films. A buckypaper made
38
39
40 by covalently cross-linked CNTs would result in stronger interactions among CNTs
41
42
43 featuring higher tensile strength of the CNTs films.^{6,8,9} In addition, networks of CNTs
44
45
46 generated by cross-linking are expected to exhibit interesting porous structure,
47
48
49
50
51
52
53
54
55
56
57
58
59
60 beneficial for a wide number of applications.

1
2
3
4 Nowadays, chemical modification and assembly of CNTs into higher order
5
6
7 constructs preserving CNTs founding features is still a major challenge. The cross-
8
9
10 linking process is obtained, in general, at the expense of a reduction in electrical
11
12
13 conductivity, due, at least in part, to the disruption of the CNT structure *via* covalent
14
15
16 bonding. Therefore, a compromise between the mechanical and electrical properties
17
18
19 may be necessary, and the optimal crosslinking degree should be determined
20
21
22 according to the desired final properties and applications.
23
24
25

26
27
28 Several methods have been proposed for cross-linking CNTs, including the use of
29
30
31 long and flexible poly-ethylene glycol chains,¹⁰ 1,4-benzoquinones,¹¹ aryl diazonium
32
33
34 salts¹² and polymer coating.¹³ More recently, Ku *et al.* demonstrated, through
35
36
37 molecular dynamics and experimental studies, that the mechanical properties
38
39
40 strongly depend on the degree of cross-linking, length of cross-linkers and the
41
42
43 diameter of the employed CNTs¹⁴ and they proved that the incorporation of aromatic
44
45
46 cross-linkers between carbon nanotube fibers, enhances the mechanical properties
47
48
49 without a significant loss of electrical conductivity. Hence, this method appears as a
50
51
52
53
54
55
56
57
58
59
60

1
2
3 promising methodology for the preparation of CNT films with interesting structural
4
5
6
7 and electrical properties for a wide number of applications.
8
9

10 Among the potential applications of CNT films,¹⁵ an emerging field is that of
11
12 neuroscience. CNT substrates promote cell viability and adhesion¹⁶ and during the
13
14 last decade many studies reported that CNTs enhance and guide axon growth and
15
16 to potentiate synapse formation and network activity¹⁶⁻²² as well as single neuron
17
18 excitability, by providing a cell-substrate electrical coupling.¹⁷ When used to coat
19
20 brain implants, CNTs are able to outperform the traditional electrodes by enhancing
21
22 recordings and stimulation from and to brain tissues, both *in vitro* and *in vivo*.^{23,24}
23
24
25 Interestingly, CNTs films contribute in reducing astrogliosis, affecting astrocytes
26
27 morphology and proliferation,²¹ ultimately limiting glial scar formation.²²
28
29
30
31
32
33
34
35
36
37
38
39
40

41 To successfully promote neuronal growth, the CNT substrates should show high
42
43 mechanical stability in order to provide a stable neural interface.¹⁶ High electrical
44
45 conductivity is also crucial to achieve good stimulation of the neural cells.¹⁶ However,
46
47
48 the development of CNT substrates with appropriate mechanical stability and good
49
50
51 conductivity, applicable to the design of neuro-implantable devices, still remains a
52
53
54
55
56
57
58
59
60

1
2
3 challenge. In this direction, the chemical modification of CNTs can be used to
4
5
6 influence the CNT/neuronal interaction and modify cell behavior.²⁵ Most of the
7
8
9 studies involving neural substrates that consist of covalently modified CNTs reported
10
11
12 so far, are based on oxidized materials.^{26–30} CNTs covalently functionalized with
13
14
15 protein growth factors have been also described.³¹ Conducting polymers have been
16
17
18 also used for the chemical modification of carbon nanotubes and their application for
19
20
21 neuronal growth.^{32–34} However, this kind of hybrid materials normally shows reduced
22
23
24 electrical conductivity, a CNT feature shown to be relevant in neuronal
25
26
27 interfacing.^{17,35,36} In other cases, patterned CNTs are used as scaffolds to support
28
29
30 the neurite outgrowth,³⁷ yielding fragile substrates that may lead to detachment of
31
32
33 the CNTs during the implantation process exposing scaffolds materials potentially
34
35
36 toxic.
37
38
39
40
41
42
43
44

45 In this framework, one of the major requirements to allow successful design of
46
47
48 CNT-based devices in neurology, is the development of conductive and freestanding
49
50
51 structures with high CNT content, covalently cross-linked materials with improved
52
53
54 mechanical stability. Such freestanding CNT substrates could be simultaneously
55
56
57
58
59
60

1
2
3 used to deliver controlled and precise electrical stimulus and to promote cell growth
4
5
6
7 on a physical template of controlled porosity.
8
9

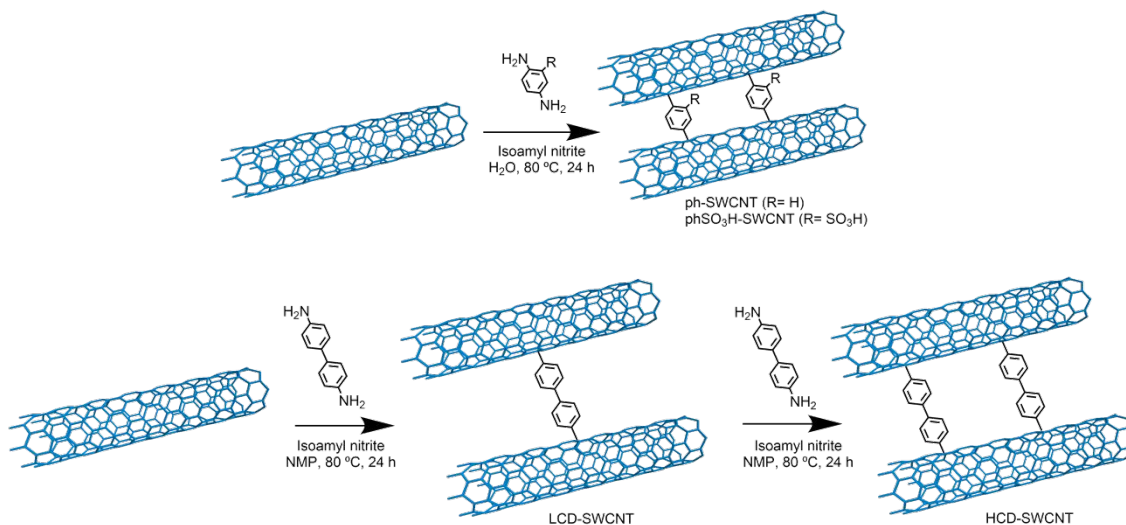
10 Herein we focus on the chemical cross-linking of single-walled carbon nanotubes
11
12 (SWCNTs) through aromatic compounds, with the aim of achieving CNT substrates
13
14 with upgraded mechanical properties, still maintaining interesting electroconductive
15
16
17 behavior. We challenged the cross-linked SWCNT scaffolds, showing different
18
19
20
21 conductive properties, with neuronal networks growth and function.
22
23
24
25
26

27 28 **RESULTS**

29 30 31 **Features of manufactured CNT-based substrates**

32
33
34
35
36 Single-walled carbon nanotubes (SWCNTs) were chosen for the formation of the
37
38
39 cross-linked materials because of their high flexibility, feature that could make the
40
41
42 formation of the desired CNT meshes easier. Aryl diazonium salt chemistry, which
43
44
45 has been widely used in the literature for grafting aryl groups to the surface of
46
47
48 graphitic materials,^{38,39} was applied for the cross-linking of SWCNTs. For this
49
50
51
52
53 purpose, *p*-phenylene diamino derivatives with different lengths were employed as
54
55
56
57
58
59
60

1
2
3
4 cross-linkers, while the degree of entanglement was checked by varying the degree
5
6
7 of functionalization. In detail, the crosslinking procedure was performed using *p*-
8
9
10 phenylenediamine, 2,5-diaminobenzenesulfonic acid and benzidine, yielding the
11
12
13 products **ph-SWCNT**, **phSO₃H-SWCNT**, **LCD-SWCNT** and **HCD-SWCNT**,
14
15
16
17 respectively (Figure 1). **LCD** and **HCD** refer to the products resulting from the cross-
18
19
20 linking with benzidine at low and high degrees of cross-linking, as reported in a
21
22
23 previous work,⁴⁰ where the synthesis and characterization of **LCD-SWCNT** and
24
25
26
27
28 **HCD-SWCNT** have been described.



50 **Figure 1.** Diazonium salt cross-linking reaction routes to provide the interlinked SWCNTs.
51
52
53
54
55
56
57
58
59
60

1
2
3 The successful formation of the interlinked materials was followed by Raman
4 spectroscopy, in combination with UV-Vis-NIR absorption spectroscopy,
5
6 thermogravimetric analysis (TGA), atomic force microscopy (AFM), transmission
7
8 electron microscopy (TEM), scanning electron microscopy (SEM) and conductivity
9
10 measurements, to obtain fully detailed information about the structural, electronic
11
12 and chemical properties of the synthesized nanohybrids.
13
14
15
16
17
18
19
20
21
22
23

24 UV-Vis-NIR absorption spectra were recorded for each material and compared
25
26 with *p*-SWCNTs (Figure S1). The spectrum corresponding to *p*-SWCNTs exhibits
27
28 the typical Van Hove singularities attributed to the different optical transitions. As
29
30 expected, the cross-linking procedure resulted in the loss of the van Hove
31
32 singularities. This loss of features is due to the alteration of the electronic structure,
33
34
35 which is particularly indicative of side-wall functionalization.
36
37
38
39
40
41
42
43
44

45 Raman spectroscopy provided also efficient support to the successful cross-linking
46
47 of SWCNTs (Figure S2 and Table 1). The spectra performed using the 785-nm line
48
49 laser show the typical peaks corresponding to the CNTs, that is, the radial breathing
50
51 mode (RBM), between 180 cm^{-1} and 280 cm^{-1} , and the two peaks corresponding to
52
53
54
55
56
57
58
59
60

1
2
3 the disorder-induced D mode and the C-C stretching G mode, at 1295 cm⁻¹ and 1595
4
5
6
7 cm⁻¹, respectively. As observed, the intensity of the I_D/I_G ratio was higher for the
8
9
10 cross-linked materials when compared to *p*-SWCNTs, as a consequence of the
11
12
13 increase of the structural defects on the CNT sidewall after the cross-linking process.
14
15

16
17 To get a deeper insight into the types of CNTs that were involved in the
18
19
20 functionalization process (metallic or semiconducting) the RBM regions were
21
22
23 analyzed. To this end, laser lines 532-nm and 633-nm were employed, since the
24
25
26
27 785-nm laser line is only in resonance with semiconducting CNTs. As shown in
28
29
30
31 Figure S2d, where the RBM spectra are normalized to the mode of the nanotube
32
33
34 with the largest diameter that was not affected by the functionalization (smallest
35
36
37 Raman shift), the RBM intensities indicate that metallic CNTs are affected by the
38
39
40 cross-linking process to a larger extent when compared to semiconducting CNTs.
41
42
43
44
45 These results are in good agreement with previous studies that have revealed that
46
47
48 the covalent functionalization of SWCNTs with diazonium salts takes place with high
49
50
51 selectivity towards metallic CNTs.^{41,42}
52
53
54
55
56
57
58
59
60

1
2
3 The degree of functionalization was determined by TGA under nitrogen flow and
4
5
6 consisted on heating ramps of 10°C/min up to 800°C (Figure S3 and Table 1). In
7
8
9
10 order to facilitate the determination of the decomposition temperatures of the cross-
11
12
13 linking agents, differential thermogravimetric (DTG) curves were superimposed
14
15
16
17 (Figure S3).
18
19

20
21 From DTG curves, the initial decomposition temperatures of the weightlessness of
22
23 the cross-linking agents were estimated as follows: around 280 °C for **ph-SWCNT**,
24
25
26
27 363 °C for **phSO₃H-SWCNT** and 230 °C for **LCD-SWCNTs** and **HCD-SWCNTs**, and
28
29
30 these are completely removed at around 600 °C, 580 °C and 380 °C for **ph-SWCNT**,
31
32
33
34 **phSO₃H-SWCNT** and **LCD-/HCD- SWCNTs**, respectively. The residual mass, once
35
36
37 the cross-linking agents have been removed, is 68 % for **ph-SWCNT**, 78 % for
38
39
40
41 **phSO₃H-SWCNT**, 91 % for **LCD-SWCNTs** and 86 % for **HCD-SWCNT**, which might
42
43
44
45 be attributed to remaining mass of CNTs, probably corresponding to those CNTs
46
47
48 with larger diameters. TGA analysis allowed also the estimation of the functional
49
50
51 group coverage (FGC) for each sample, that was estimated from the weight loss
52
53
54
55 percentages (wt%) of the cross-linking agents (wt%_{-linker}) and SWCNTs (wt%_{-SWCNTs})
56
57
58
59
60

1
2
3 in each sample, and the molecular weights of the cross-linking agents ($Mw_{\text{-linker}}$) and
4
5
6
7 atomic mass of carbon according to the following equation:⁴³
8
9

$$10 \quad FCG = \frac{wt\%_{\text{-SWCNTs}}/12.01}{wt\%_{\text{-linker}}/Mw_{\text{-linker}}}$$

11
12
13
14 For this purpose, the wt% was determined from the mass loss in the TGA curves
15
16
17
18 under N_2 , at 600 °C for **ph-SWCNT**, at 550 °C for **phSO₃H-SWCNT**, and 500 °C for
19
20
21 **LCD-SWCNTs** and **HCD-SWCNTs**, where is considered that the cross-linking agent
22
23
24 has been completely removed from the CNT sidewall. According to this, the TGA
25
26
27
28 curves developed a group coverage of 1 group every 23 carbon atoms (2023 $\mu\text{mol/g}$)
29
30
31 and 1 group every 77 carbon atoms (1045 $\mu\text{mol/g}$), for **Ph-SWCNT** and **PhSO₃H-**
32
33
34
35 **SWCNT**, respectively, in agreement with the results from the Raman studies
36
37
38 presented above. For the longest linker consisting of two phenyl rings, the calculated
39
40
41
42 group coverages were 1 group every 234 carbon atoms (536 $\mu\text{mol/g}$) and 1 group
43
44
45 every 98 carbon atoms (944 $\mu\text{mol/g}$) for **LCD-SWCNT** and **HCD-SWCNT**,
46
47
48
49 respectively, as previously described in the literature.⁴⁰ These latter results, along
50
51
52
53
54
55
56
57
58
59
60

1
2
3 with the I_D/I_G values observed for both materials, confirmed the higher loading of
4
5
6
7 linkers reached through the second round of functionalization.
8
9

10 Electrical conductivity studies were also performed for the synthesized materials
11
12 and compared to those of ***p*-SWCNTs**. For this purpose, the van der Pauw method
13
14 was employed.⁴⁴ Thin films were prepared using the vacuum filtration method and
15
16
17 the average membrane thickness was measured. The sheet resistance (R_s) was
18
19
20
21 evaluated by means of a 4-point probe conductometer. For ***p*-SWCNTs**, **Ph-SWCNT**
22
23
24 and **PhSO₃H-SWCNT**, which did not show the free-standing structure required to be
25
26
27
28 manipulated by tweezers, the conductivity was calculated without peeling off the
29
30
31
32 materials from the surface of the PTFE filter. In order to avoid inaccurate reading
33
34
35 due to the direct contact between the conductometer points and the filter, thick films
36
37
38 of material were prepared. Electrical conductivity (σ) was then calculated from the
39
40
41
42 measured R_s and the sample thickness (t), according to the formula $\sigma = 1/(R_s t)$. The
43
44
45 data reported in Table 1 collects the results obtained from Raman, TGA and
46
47
48 conductivity measurements. As expected, ***p*-SWCNTs** presented the highest values
49
50
51
52 of conductivity which decreased after the disruption of the π - π structure by means
53
54
55
56
57
58
59
60

of functionalization, and more so, with the increase of the functionalization degree, as clearly observed in the case of **LCD-SWCNT** relative to **HCD-SWCNT**. These results are consistent with the analysis previously performed on the RBM Raman regions (Figure S2d). The change toward less metallic behaviour after the cross-linking process explains the decrease of conductivity observed for the cross-linked materials that is higher when the loading of cross-linking agent increases.

Table 1. TGA, Raman and Conductivity data for the resulting cross-linked materials.

Material	TGA ($\mu\text{mol/g}$)*	Raman I_D/I_G	σ (S/m)
<i>p</i> -SWCNTs	-	0.06	3389
Ph-SWCNT	2023	0.27	56
PhSO ₃ H-SWCNT	1045	0.23	102
LCD-SWCNT	536	0.24	1083
HCD-SWCNT	944	0.39	81

* μmol of functionalizing groups per gram of material.

To better understand the topography and structure of the cross-linked materials, TEM and SEM studies were performed. TEM images revealed the existence of clear differences before and after the interlinking process. For **Ph-SWCNT** and **PhSO₃H-**

1
2
3 **SWCNT** (Figure S4), larger meshes of nanotubes were observed after the
4
5
6
7 functionalization process, indicating a successful covalent cross-linking of the CNTs.
8
9

10 Considerable differences were also observed by TEM between the **LCD-SWCNT**
11
12
13 and **HCD-SWCNT**.⁴⁰ According to its paper-like physical appearance, the density of
14
15
16
17 the meshwork was higher for **LCD-SWCNTs** in comparison with **p-SWCNTs**, and
18
19
20
21 most notable, a dense meshwork of CNTs characterized by large surface
22
23
24 roughness was observed for **HCD-SWCNTs**.⁴⁰ Similarly, a change in the surface
25
26
27 morphology was observed by SEM between the **p-SWCNTs** and the interlinked
28
29
30
31 materials. As observed, **ph-SWCNT** and **phSO₃H-SWCNT** (Figure S5) exhibited a
32
33
34
35 more compacted rough surface when compared to the *pristine* material. In the case
36
37
38 of **LCD-SWCNT** and **HCD-SWCNT**, the interlinked SWCNTs exhibited a network-
39
40
41
42 like structure with homogeneous distribution leading to a large continuous wrinkled
43
44
45 film of nanotubes.⁴⁰
46
47
48

49 To estimate the cross-linked SWCNTs potential as substrates for neuronal growth,
50
51
52 dissociated hippocampal cultures^{35,45} were seeded on **LCD-SWCNTs** and **HCD-**
53
54
55
56 **SWCNT**. The choice of **LCD-SWCNT** and **HCD-SWCNT** for the study was motivated
57
58
59
60

1
2
3 by the easy preparation of free-standing substrates in these cases, which is a
4
5
6 prerequisite for their future application as substrates for neural growth in the injured
7
8
9 tissue. It is important to note that **Ph-SWCNT** and **PhSO₃H-SWCNT** were obtained
10
11
12 as powder samples after the functionalization process. Free-standing substrates
13
14
15 were not achieved in these cases. Therefore, their manipulation in future
16
17
18 implantation processes would not be a straightforward task. This, together with the
19
20
21 fact that the electrical conductivity of **Ph-SWCNT** and **PhSO₃H-SWCNT** was very
22
23
24 similar to that of **HCD-SWCNT** (see Table 1), led us to the decision of using only
25
26
27 **LCD-SWCNT** and **HCD-SWCNT** for neural interfacing. On the other hand, the
28
29
30 existence of considerably different values of electrical conductivity between **LCD-**
31
32
33 **SWCNT** and **HCD-SWCNT** (1083 S/m *vs* 81 S/m, respectively) allowed an
34
35
36 interesting comparison about how this parameter affects neuronal behavior. These
37
38
39 conductivity values are considerably better than those reported previously for
40
41
42 MWCNTs chemically modified through the same approach,²⁵ which is probably
43
44
45 attributable to the intrinsic conductivity of the *pristine* materials, that is much better
46
47
48 in the case of the *p*-SWCNTs employed in the current work ($\sigma = 3389$ S/m for *p*-
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 SWCNTs *vs.* $\sigma = 625$ S/m for p -MWCNTs (calculated from the sheet resistance in
4
5
6
7 ref 25)). After the functionalization process, higher loading of functional groups is
8
9
10 achieved in the present work (944 $\mu\text{mol/g}$ for the SWCNTs with higher degree of
11
12
13 functionalization *vs.* 230 $\mu\text{mol/g}$ for the MWCNTs with higher degree of
14
15
16 functionalization reported in ref. 25). Even so, the electrical conductivity for the
17
18
19 functionalized SWCNTs is around 200 times better than that reported for the highly
20
21
22 functionalized MWCNTs ($\sigma = 0.341$ S/m and $\sigma = 81$ S/m for functionalized- MWCNTs
23
24
25 and SWCNTs, respectively), which suggest the promise of our cross-linked CNT-
26
27
28 based substrates for the improvement of neuronal activity.
29
30
31
32
33

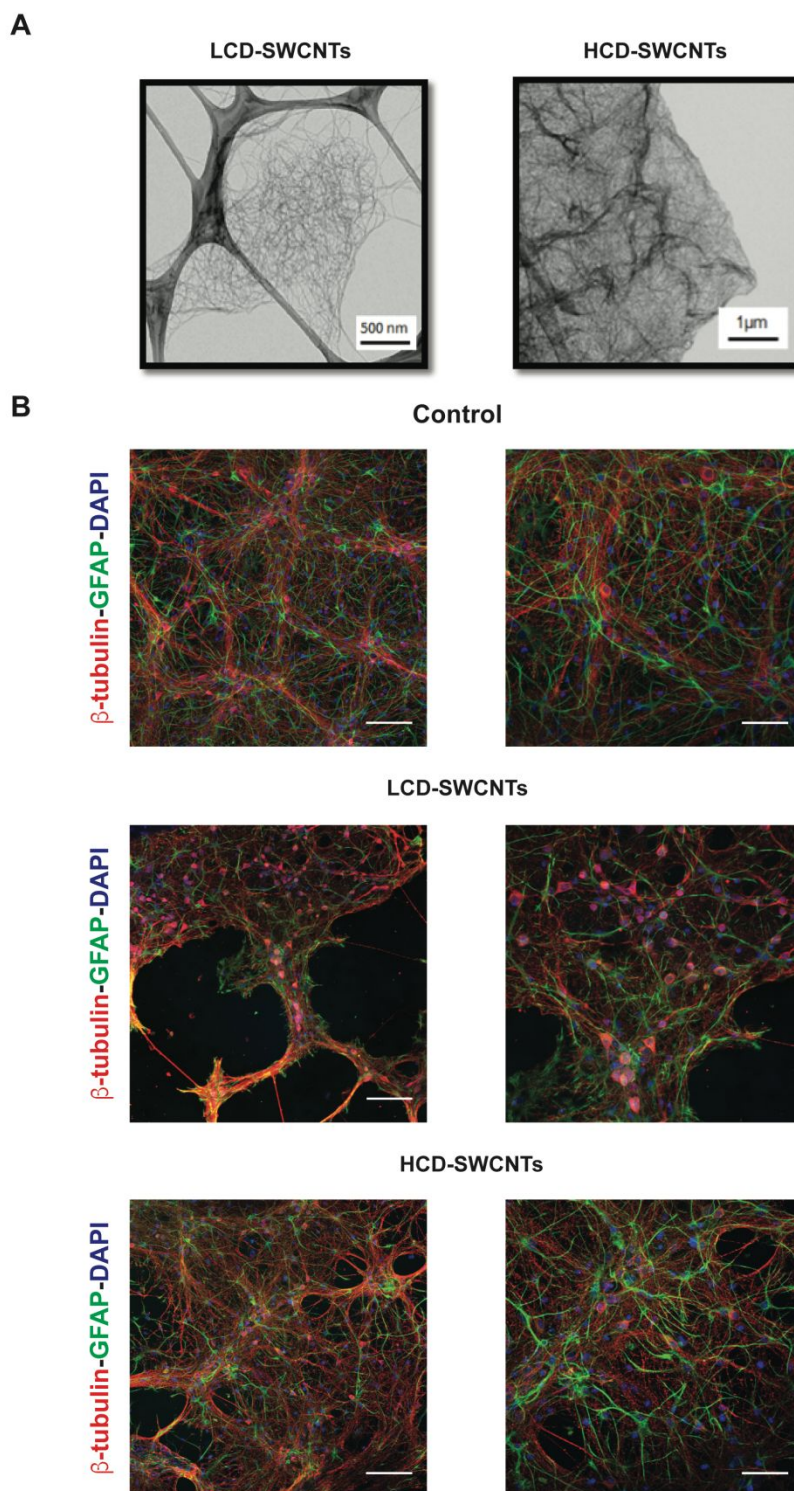
34
35 Confocal reconstructions and calcium imaging experiments were then performed
36
37
38 to assess the behavior of the neural cells when seeded on the conductive CNT
39
40
41 substrates.
42
43
44

45
46 **Confocal reconstructions of hippocampal cultures interfaced to LCD-SWCNTs and**
47
48
49 **HCD-SWCNTs substrates.**
50
51
52
53
54
55
56
57
58
59
60

1
2
3 To investigate neuronal networks formation and growth when integrated with the
4
5
6
7 SWCNTs films, we used rat dissociated hippocampal cultures.^{17,35,36} Cultured bio-
8
9
10 systems allow reliable *in vitro* reconstruction of synaptic connections among
11
12
13 heterogeneous neuronal phenotypes in the presence of neuroglial cells, and are
14
15
16 brain-circuit models instrumental to investigate ad hoc manufactured materials for
17
18
19 interfacing neuronal activity.
20
21
22
23

24 Primary neurons from rat hippocampus were plated on LCD- and HCD-SWCNTs
25
26
27 films and, as control condition, on poly-L-ornithine treated glass slides. Cells were
28
29
30 cultured into functional synaptic networks. Cell morphology and adhesion to
31
32
33 SWCNTs substrates were assessed by immunofluorescence labeling and confocal
34
35
36 microscopy. Figure 2A shows representative TEM images of the two different
37
38
39 substrates. We compared neurons grown on poly-L-ornithine (Control) substrates
40
41
42 with those grown on SWCNTs scaffolds, after 9-11 days *in vitro* (DIV). To prove the
43
44
45 compatibility of both LCD- and HCD-SWCNTs substrates, we imaged by
46
47
48 immunofluorescence the specific cytoskeletal components β -Tubulin III, to identify
49
50
51 neurons, and glial fibrillary acidic protein (GFAP) for astrocytes.^{36,46,47} In all cultures
52
53
54
55
56
57
58
59
60

1
2
3 tested (n = 33 visual fields for the three different conditions), before fixation for
4
5
6
7 confocal microscopy, live neuronal calcium activity was monitored.
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



1
2
3 **Figure 2.** In (A) TEM images for LCD-SWCNTs (left) and HCD-SWCNTs (right) substrates.
4
5 In (B) confocal micrographs show hippocampal cultures grown (10 DIV) on Control (top),
6
7 LCD-SWCNTs (middle) and HCD-SWCNTs (bottom) substrates immune-stained for β -
8
9 tubulin III (in red), GFAP (in green) and DAPI (blue) at lower (left panel) and higher
10
11 magnification (right panel). Scale bar: 100 μ m (left) and 50 μ m (right).
12

13
14 Figure 2B shows low (left panels) and high (right panels) magnification confocal
15
16 micrographs with β -Tubulin III positive cells (in red) and GFAP positive ones (in
17
18 green) developed in control (top) or in the synthesized LCD-SWCNTs (middle) and
19
20 HCD-SWCNTs (bottom) substrates; in all images nuclei are visualized by DAPI (in
21
22 blue). TEM images for LCD- and HCD- SWCNTs are shown in A to provide further
23
24 insight into the morphology of each substrate.
25
26
27
28
29
30
31
32

33
34 Both neurons and neuroglia cells matured once on SWCNTs supports, as shown
35
36 in Fig 2B (high magnification) by the outgrowth of axons and dendrites in the case
37
38 of neurons^{36,46} and by the stellate shape of GFAP+ cells,^{36,48} leading to the
39
40 development of a complex network covering the entire SWCNTs surface. We further
41
42 compared cell densities among LCD-SWCNTs, HCD-SWCNTs and control cultures.
43
44
45 LCD- and HCD-SWCNTs and controls supported neuron and neuroglia growth in a
46
47 similar manner, as highlighted by cell density measures (for neurons, in controls:
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 140 ± 35 β -tubulin positive cells/mm²; in **LCD-SWCNTs**: 110 ± 29 β -tubulin positive
4
5
6
7 cells/mm²; in **HCD-SWCNTs**: 138 ± 35 β -tubulin positive cells/mm²; for neuroglia, in
8
9
10 controls: 110 ± 20 GFAP positive cells/mm²; in **LCD-SWCNTs**: 120 ± 25 GFAP
11
12
13 positive cells/mm²; in **HCD-SWCNTs**: 118 ± 20 GFAP positive cells/mm²; n = 11
14
15
16
17 visual fields per condition, three independent culture series). Thus, both **LCD-** and
18
19
20 **HCD-SWCNTs** membranes allowed attachment and growth of rat hippocampal
21
22
23
24 neurons displaying mature morphology.
25
26
27

28 **Live imaging of hippocampal cultures interfaced to LCD-SWCNTs and HCD-** 29 30 31 **SWCNTs substrates.**

32
33
34
35 To study the emergence of neuronal activity by the networks formed interfaced to
36
37
38 **LCD-SWCNTs** and **HCD-SWCNTs** substrates, we used fluorescent calcium
39
40
41 imaging.^{49,50} After 8-10 DIV, neurons mature and generate synaptic signaling
42
43
44
45 spontaneously, involving the activation of glutamate and GABA_A receptor mediated
46
47
48 synapses,^{45,51} the major central nervous system (CNS) neurotransmitters for fast
49
50
51 synaptic communication. We compared activity emerging from neurons, cultured on
52
53
54
55
56
57
58
59
60

1
2
3 Control, **LCD-** and **HCD-SWCNTs** substrates (Figures 3 and 4). Neurons loaded with
4
5
6
7 the membrane permeable Ca^{2+} dye Oregon green 488 BAPTA-1 were
8
9
10 simultaneously visualized within the sampled area and on average 20 ± 4 fluorescent
11
12
13
14 cells were analyzed in each visual field ($680 \times 680 \mu\text{m}^2$; Fig. 3A and 4A, left panels).
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

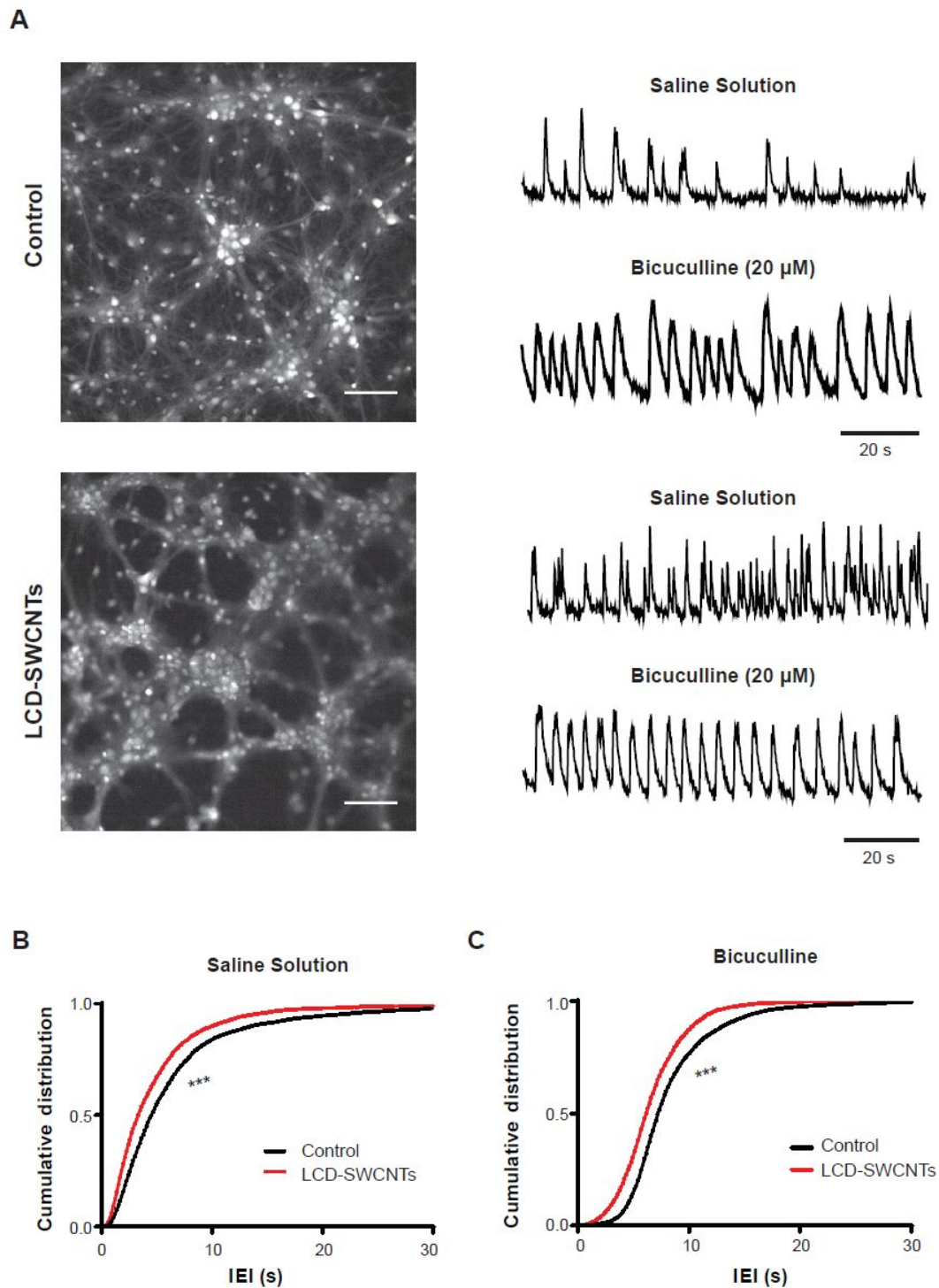
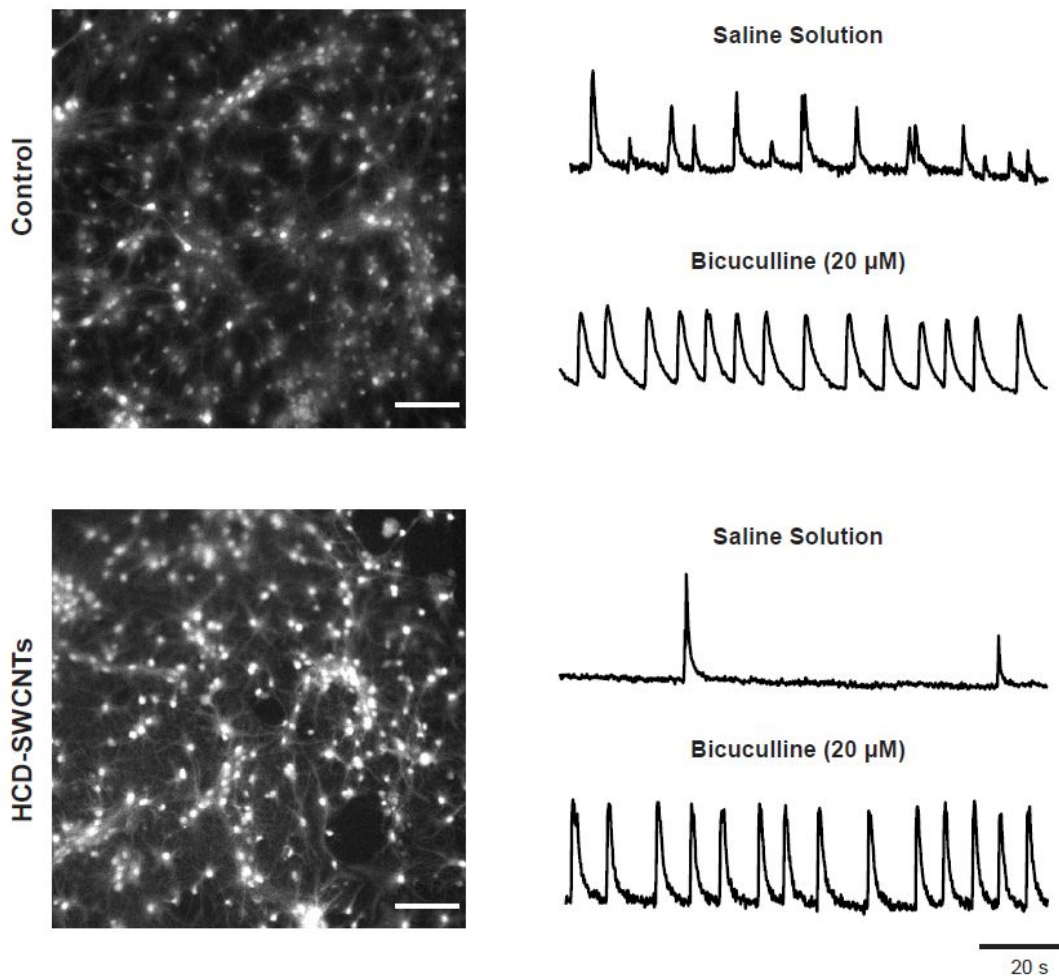


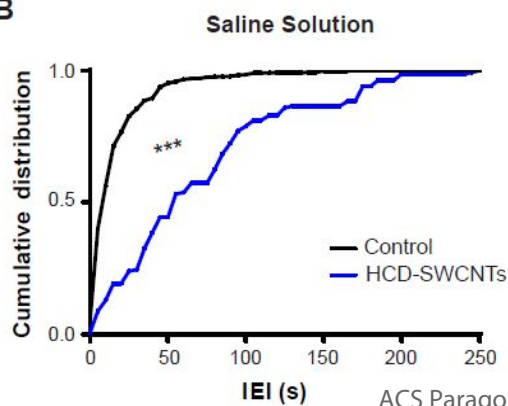
Figure 3. In (A) (left) snapshots of representative fields of neuronal cultures grown on Control (top) and LCD-SWCNTs (bottom) substrates, stained with the Oregon Green 488-BAPTA-1 AM. Scale bar: 50 μm . In (A) (right) repetitive Ca^{2+} -events spontaneously (top) or bicuculline

1
2
3 induced (bottom) recorded in hippocampal cultures of 10 DIV. In (B) cumulative distribution
4 of IEI values recorded in saline solution of Control (black) and LCD (red) cells (** $p < 0.0001$;
5 Kolmogorov-Smirnoff test). In (C) cumulative distribution of bicuculline-induced IEI values of
6 Control (black) and LCD (red) cells (** $p < 0.0001$; Kolmogorov-Smirnoff test).
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

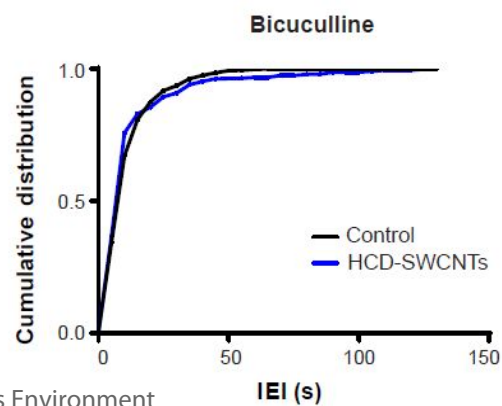
A



B



C



1
2
3 **Figure 4.** In (A) (left) snapshots of representative fields of neuronal cultures grown on Control
4 (top) and HCD-SWCNTs (bottom) substrates, stained with the Oregon Green 488-BAPTA-
5 1 AM. Scale bar: 50 μm . In (A) (right) repetitive Ca^{2+} -events spontaneously (top) or
6 bicuculline induced (bottom) recorded in hippocampal cultures of 10 DIV. In (B) cumulative
7 distribution of IEI values recorded in saline solution of Control (black) and HCD (blue) cells
8 (***) $p < 0.0001$; Kolmogorov-Smirnoff test). In (C) cumulative distribution of bicuculline-
9 induced IEI values of Control (black) and HCD (blue) cells (***) $p < 0.0001$; Kolmogorov-
10 Smirnoff test).
11
12
13
14
15
16
17
18

19 The experiments were performed at 9-11 DIV when neurons, intensely connected
20 by synapses, display spontaneous activity in the form of bursts of activity, emerging
21 by irregular synchronized firing events,^{17,36,52} generating transient episodes of
22 calcium rise from the baseline (Figures 3 and 4).^{36,52-54} In Control, spontaneous Ca^{2+}
23 activity was detected in 67 % of the recorded neurons (172 out of 257 neurons),
24 visualized in each field (n = 10 fields). Notably, in LCD-SWCNTs virtually all (> 98
25 %, 133 out of 136 neurons; n = 6 fields) were active and generated spontaneous,
26 repetitive Ca^{2+} episodes. In Figure 3A (right) shows examples of fluorescent
27 recordings of active cells sampled from Control and LCD-SWCNTs substrates.
28 Spontaneous episodes of activity appear as bursts of synaptic, action potential,
29 dependent events and are blocked by application of tetrodotoxin (TTX, 1 μM ; a toxin
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 that targets voltage dependent sodium channels). We measured the amount of Ca^{2+}
4
5
6
7 episodes occurring in spontaneously active cells and we quantified the inter-event
8
9
10 interval (IEI), the interval of time between the onset of a calcium wave and the
11
12
13 beginning of the next one, that is significantly (***) $p < 0.0001$; Kolmogorov-Smirnoff
14
15
16 test) shorter in **LCD-SWCNTs** substrates (on average 8 ± 4 s, $n = 133$ cells, from 3
17
18
19 different series of cultures) when compared to control ones (14 ± 4 s, $n = 172$ cells,
20
21
22 from 3 different series of cultures; see the IEI cumulative distribution in Figure 3B),
23
24
25 strongly indicative of a different functional organization due to the presence of **LCD-**
26
27
28
29
30
31 **SWCNTs**.

32
33
34
35 In a second group of experiments we blocked inhibitory synaptic transmission by
36
37
38 bicuculline ($20 \mu\text{M}$) application. This antagonist of GABA_A receptors is known to alter
39
40
41 the profile of network activity^{55,56} from random bursting to synchrony, characterized
42
43
44 by more intense and regular bursting.^{53,54} As shown in Figure 3A, active cells upon
45
46
47 bicuculline exposure, generate Ca^{2+} events with IEI of, on average, 4 ± 0.9 s in **LCD-**
48
49
50
51 **SWCNTs** neurons ($n = 133$), a value significantly ($p < 0.001$) lower when compared
52
53
54 to that of Control (on average 8 ± 3 s; $n = 172$ cells; see the cumulative distribution
55
56
57
58
59
60

1
2
3 plot in Figure 3C). Such disinhibited episodes were completely abolished by TTX
4
5
6 applications, confirming their neuronal origin. Figure 4A (right panel) shows
7
8
9 representative fluorescent tracings from active cells recorded in **HCD-SWCNTs**,
10
11
12 notably Ca^{2+} spontaneous activity was detected only in 21 % (34 out of 162 neurons;
13
14
15
16
17 **HCD-SWCNTs**) of the cells visualized in each field (n = 6 fields), a very low value
18
19
20 when compared to their relative Control (65 %; 97 out of 149 neurons; n = 6 fields).
21
22
23
24 When we measured the spontaneous Ca^{2+} activity, the IEI was significantly (***) $p <$
25
26
27 0.0001; Kolmogorov-Smirnoff test) longer in **HCD-SWCNTs** substrates (on average
28
29
30 72 ± 10 s n = 34 cells, from 3 different series of cultures) when compared to control
31
32
33
34
35 ones (on average 17 ± 7 s, n = 97 cells, from 3 different series of cultures; see also
36
37
38 cumulative distribution in Figure 4B). However, upon pharmacological removal of
39
40
41 synaptic inhibition (Figure 4C) a IEI similar to control was restored (**HCD-SWCNTs**
42
43
44
45 substrates on average 8 ± 5 s, n = 34 cells; control substrates on average 10 ± 3 s,
46
47
48 n = 97 cells). Thus, apparently, the different substrates promote the formation of
49
50
51
52 diverse networks, in particular the inhibitory control on **HCD-SWCNTs** appeared to
53
54
55
56 be strongly developed in these cultured brain circuits, when compared to **LCD-**
57
58
59
60

1
2
3 **SWCNTs** and Control. While **LCD-SWCNTs**, as expected, boosted network activity
4
5
6
7 in all network states (resting and disinhibited).
8
9

10 **DISCUSSION**

11
12
13
14 The present study provides important insight into how the electrical conductivity of
15
16
17 the CNT free-standing substrates affects the growth and electrical properties of
18
19
20 cultured hippocampal networks. The cross-linking of SWCNT with diaminophenyl
21
22
23 derivatives has provided an efficient route to design free-standing CNT substrates
24
25
26 with tunable electrical properties, promising for applications in neural tissue
27
28
29 engineering. Raman and electrical studies demonstrated that the electrical
30
31
32 conductivity of the final materials is directly related to the type of cross-linker and the
33
34
35 degree of functionalization (Table 1). In the particular case of benzidine as cross-
36
37
38 linker, an important decrease of the electrical conductivity was observed when
39
40
41 increasing the degree of functionalization by 40 % (1083 S/m for **LCD-SWCNT** vs.
42
43
44 81 S/m for **HCD-SWCNT**), allowing the preparation of free-standing neuronal
45
46
47
48
49
50
51 substrates with different electrical properties. Both, **LCD-SWCNT** and **HCD-SWCNT**,
52
53
54
55
56
57
58
59
60

1
2
3 have been employed as substrates for interfacing neural cells. The most important
4
5
6 finding of the study is that neuronal circuits chronically interfaced to SWCNTs
7
8
9
10 substrates can be effectively affected by the CNTs degree of functionalization.

11
12
13 Cultured brain circuits represent the simplest *in vitro* model of brain network. The
14
15
16 morphology and cell phenotype distribution of hippocampal networks did not differed
17
18
19
20
21 when developed on control glass surfaces or on **LCD-** and **HCD-SWCNTs** carpets.

22
23
24 The two cellular networks are made of healthy and functional cells, indicating the
25
26
27 cytocompatibility of both SWCNTs substrates (Figure 2B).³⁵

28
29
30
31 Conversely, when investigating network dynamics by monitoring intracellular
32
33
34 calcium activity, we observed significant differences comparing **LCD-SWCNTs**
35
36
37 substrates with **HCD** ones. Live calcium episodes, whose neuronal and synaptic
38
39
40 nature was supported by TTX experiments,⁵⁴ reflected the spontaneous activation of
41
42
43 synapses, typical of these preparations.¹⁷ Specifically, such synchronized synaptic
44
45
46 activation are accepted measure of neural network dynamics.⁵³

47
48
49
50
51
52 We speculated that **LCD-SWCNTs** substrates within their range of conductivity
53
54
55 and functionalization could enhance neuronal activity (Figure 3), in a way similar to
56
57
58
59
60

1
2
3 what happens with MWCNTs.^{17,25,35,57} In fact, interfacing hippocampal neurons with
4
5
6 **LCD-SWCNTs** substrates, we reproduced CNTs ability to support signaling and
7
8
9
10 synchronization in cultured circuits.^{17,45} Our results confirmed that neuronal
11
12
13 physiological and synaptic efficacy could be tuned by specific neurons-surface
14
15
16 interactions, in particular when using electrically conductive growth
17
18
19
20
21 interfaces.^{25,35,36,58}
22
23

24 We, in fact, observed that neurons grown on **LCD-SWCNTs** generate bursts with
25
26
27 higher rate, with or without inhibition, potentially due to an improved efficiency in
28
29
30 axonal signaling and to the increase in synaptic connections^{17,35,36,58} (Figure 3). We
31
32
33 favor the hypothesis that the **LCD-SWCNTs** ability to alter cellular excitability could
34
35
36 significantly contribute to the described results. In fact, the higher the cellular
37
38
39 excitability the higher would be the probability of finding active cells.^{36,59,60}
40
41
42
43
44

45 As degree of functionalization increases, this effect on the emerging neuronal
46
47
48 activity decreased (Figure 4). This effect is not attributable to differences in neuronal
49
50
51 survival or morphology (Figure 3), but possibly represents a consequence of the
52
53
54
55
56
57
58
59
60

1
2
3 properties of the SWCNTs substrates and could primarily result from specific
4
5
6
7 synaptic interactions.⁶¹
8
9

10 In fact, reducing the conductivity of the substrates, we observed a significant
11
12
13 decrease in terms of active cells and the emerging spontaneous activity. We cannot
14
15
16
17 exclude that **HCD-SWCNTs** interfere with the maturation of inhibitory neurons and
18
19
20 the network composition. In this hypothesis, **HCD-SWCNTs** may favor more mature
21
22
23 chloride ion fluxes through GABA_A receptors. In neurons, the amplitude of the
24
25
26
27 inhibitory currents is governed by the intracellular chloride concentration and, in
28
29
30
31 more mature stages of development, goes from higher to lower values, in respect to
32
33
34 the extracellular milieu.⁶² Thus, in **HCD-SWCNTs**, there could be a higher fraction of
35
36
37
38 effective inhibitory neurons, thus biasing the spontaneous network activity.
39
40

41
42 Based on our results, we may propose that **HCD-SWCNTs** properties are
43
44
45 responsible of a different maturation in cellular organization. However, even under
46
47
48 these conditions, the block of GABA_A receptor-mediated inhibition boosted Ca²⁺
49
50
51
52 episodes as in control conditions, suggesting a similar role of inhibition in
53
54
55
56 counteracting excitability in the synaptic network.
57
58
59
60

1
2
3 Hu and collaborators³⁰ found that the chemical functionalization of CNTs with
4
5
6
7 different molecules is able to control the outgrowth and branching of neuronal
8
9
10 processes. Accordingly, Malarkey and co-workers reported that SWCNT scaffolds
11
12
13 with different conductivities affected rat hippocampal neuron neurite outgrowth.²⁷ Our
14
15
16 current results are also in agreement with our recent report²⁵ where the impact of
17
18
19
20
21 MWCNTs growth substrates on cell development and signaling depended on their
22
23
24 degree of functionalization and on the functionalized groups. In that work,²⁵ despite
25
26
27 the absence of changes in network size in all the functionalization conditions tested,
28
29
30
31 we measured an increased synaptic activity only in those neurons interfaced to the
32
33
34
35 MWCNTs with the lowest functionalization degree.

36
37
38 Although we cannot exclude such modulations, the simple tuning of neurite
39
40
41 outgrowth would not explain the similar activity obtained in bicuculline when applied
42
43
44
45 in control and **HCD-SWCNTs**, thus we favour the hypothesis that the conductivity of
46
47
48
49 CNTs might regulate network excitability and synapse formation.^{17,35}
50

51
52 Overall, our results demonstrate that SWCNTs can be systematically manipulated
53
54
55
56 and functionalized to display different electrical properties.
57
58
59
60

1
2
3
4 These results show that SWCNTs structuring *via* cross-linking is a key factor to
5
6
7 successfully use CNT-based substrates for biomedical applications or tissue
8
9
10 engineering. For their peculiar chemical and electrical properties, as well as their
11
12
13 functionalization degree, **LCD-SWCNTs** seem to be the most promising cross-linked
14
15
16 substrate for the design of neuronal interfaces of the future.
17
18

19
20
21 Characterizing the interactions between CNTs and brain cells and their circuitry
22
23
24 together with how the quality of conductivity affects their functionality offer
25
26
27 developments for applying nanotechnology to and for the nervous system.
28
29

30 31 **CONCLUSIONS** 32 33 34

35
36 In this work, a facile and promising method based on chemical cross-linking of
37
38
39 carbon nanotubes is employed for controlling the electrical conductivity of CNT-
40
41
42 based substrates. We have developed a method to simultaneously obtain free-
43
44
45 standing CNT-based substrates and tune the electrical properties of the final
46
47
48 materials. Immunofluorescence studies and calcium imaging demonstrated that
49
50
51 neural activity can be regulated by modifying the electrical properties of the CNT-
52
53
54
55
56
57
58
59
60

1
2
3 based substrates, while maintaining neuronal growth unaffected. These findings
4
5
6
7 may provide possibilities for controlling the electrical properties of future implantable
8
9
10 neuronal interfaces.
11
12
13

14 EXPERIMENTAL SECTION

18 Materials

19
20
21
22 All the chemicals and solvents were purchased from Sigma Aldrich and used
23
24
25 without any further purification. Single-walled carbon nanotubes HiPco (SWCNTs)
26
27
28 were purchased from Nanointegris (purified grade, length = 100-1000 nm, diameter
29
30
31 ~0.8-1.2 nm, < 15% remaining iron particles) and used without further purification
32
33
34
35
36 treatment.
37
38
39

40 Synthesis

44 General procedure for the synthesis of cross-linked SWCNTs.

45
46
47 100 mg of *pristine* single walled carbon nanotubes (*p*-SWCNTs) were dispersed in
48
49
50
51 150 mL of *N*-methyl-2-pyrrolidone (NMP) and sonicated for 15 minutes. 100 mg of
52
53
54 the corresponding phenyl derivative and 0.5 mL of isopentyl nitrite were added and
55
56
57
58
59
60

1
2
3 the reaction mixture was stirred for 24 hours at 80°C. The crude was filtered through
4
5
6 a PTFE membrane with an average pore size of 0.45 μm and the black precipitate
7
8
9
10 was washed several times with NMP, methanol and diethyl ether. The resulting
11
12
13
14 material was dried under vacuum and weighed.
15
16
17

18 **Characterization methods**

19
20

21 UV-Vis-NIR measurements were carried out on a Cary 5000 Spectrometer
22
23
24 (Varian), using 1 cm path quartz cuvettes. Thermogravimetric analyses were
25
26
27
28 performed using a TGA Q500 (TA Instruments). Raman spectra were obtained on
29
30
31
32 Renishaw inVia Raman microscope at room temperature with an exciting laser
33
34
35 source ($\lambda = 785 \text{ nm}$). Measurements were taken with 10 s of exposure time and the
36
37
38
39 laser spot was focused on the sample surface using a long working distance 50 \times
40
41
42 objective. Raman spectra were collected on numerous spots on the sample and
43
44
45
46 recorded with a Philips SPC1030NC camera. Atomic force microscopy (AFM) images
47
48
49 were acquired in tapping mode using a Multimode V7.30 (Veeco Instruments Inc.,
50
51
52
53 Santa Barbara, USA) with a NanoScope V controller (Digital Instruments, Santa
54
55
56
57
58
59
60

1
2
3 Barbara, USA). The cantilevers (HQ:NSC15/Al BS probes from Mikromasch) were
4
5
6
7 silicon cantilevers with a resonance frequency of 325 kHz and a nominal force
8
9
10 constant of 40 Nm^{-1} . The images were processed using WSxM (freely downloadable
11
12
13 scanning probe microscopy software from (www.nanotec.es). Transmission electron
14
15
16
17 microscopy (TEM) images were recorded on on a Philips EM208 TEM using iTEM
18
19
20 software (Olympus Soft Imaging Solutions GmbH, Münster, Germany). About 1 mg
21
22
23
24 of material was dispersed in 5 mL of EtOH and sonicated for 30 minutes. Then one
25
26
27
28 drop of this solution was deposited on a TEM grid (Lacey carbon Film, Copper, 300
29
30
31 Mesh). Scanning electron microscopy (SEM) images were acquired collecting
32
33
34
35 secondary electrons on a commercial SEM (Gemini SUPRA 40, Carl Zeiss NTS
36
37
38 GmbH, Oberkochen). For the analysis, the different materials were placed on
39
40
41
42 conductive double side carbon tape (Ted Pella, Inc., USA) and imaged at 5 keV. For
43
44
45 the powdered samples (ph-SWCNT and phSO₃H-SWCNT), about 1 mg of material
46
47
48
49 was placed on the double-sided carbon tape previously attached to the SEM stub,
50
51
52
53 and was pressed lightly to have the sample adhere to the tape. In the case of LCD-
54
55
56
57 and HCD-SWCNTs a piece of material was sectioned using a scalpel and mounted
58
59
60

1
2
3 on the SEM stub using the mentioned double sided carbon tape. Resistance was
4
5
6
7 collected using a Jandel four-point probe analyzer (RM-3000) and conductivity was
8
9
10 calculated applying the Ohm's law. For this purpose, the required films were
11
12
13 prepared through filtration of 1 mg of material on a PTFE filter (Millipore 0.45 μm
14
15
16 pores) and the membrane thickness was measured with a micrometer (High-
17
18
19 Accuracy Digimatic Micrometer 293-100, Mitutoyo).
20
21
22
23
24

25 **Preparation of primary hippocampal cultures.**

26
27

28 Primary dissociated cultures were prepared from postnatal (P2-P3) rats as
29
30
31 previously reported.^{17,30,42} All procedures were approved by the local veterinary
32
33
34 authorities and performed in accordance with the Italian law (decree 116/92) and the
35
36
37 EU guidelines (86/609/CE, 2007/526/CE and 2010/63/UE). Animal's use was
38
39
40
41 approved by the Italian Ministry of Health. All efforts were made to minimize animal
42
43
44 suffering and to reduce the number of animals used. Cells were plated on poly-L-
45
46
47 ornithine-coated glass coverslips, on HCD-SWCNTs and on LCD-SWCNTs carpets.
48
49
50
51 Before using for culturing, both SWCNTs substrates were mounted on the glass
52
53
54
55
56
57
58
59
60

1
2
3 coverslips (12×24 mm², 0.13–0.16 mm thick, Kindler, EU) by a thin adhesive layer of
4
5
6
7 PDMS cured at 120 °C. One hour prior to plating, CNTs substrates were treated with
8
9
10 an air-plasma-cleaner in order to facilitate cell adhesion^{36,63} and at the end sterilized
11
12
13
14 with an UV lamp.
15
16
17
18
19
20

21 **Calcium Imaging.**

22
23
24
25 Cultures were loaded with cell permeable Ca²⁺-dye Oregon Green 488 BAPTA-1
26
27
28 AM (Invitrogen). 4 mM stock solution of the Ca²⁺ dye was prepared in DMSO and
29
30
31
32 cultures were incubated with a final concentration of 4 μM for 40 min (37 °C; 5%
33
34
35 CO₂).^{30,42} The samples were then placed in a recording chamber mounted on an
36
37
38
39 inverted microscope (Nikon Eclipse Ti-U) where they were continuously superfused
40
41
42
43 at RT by a recording solution of the following composition (mM): 150 NaCl, 4 KCl, 1
44
45
46 MgCl₂, 2 CaCl₂, 10 HEPES, 10 glucose (pH adjusted to 7.4 with NaOH), to
47
48
49
50 continuously supply nutrients to the tissue. Cultures were observed with a 20×
51
52
53 objective (0.45 NA) and recordings were performed from visual fields (680 × 680
54
55
56
57
58
59
60

1
2
3 μm^2 , binning 4). Ca^{2+} -dye was excited at 488 nm with a mercury lamp; excitation
4
5
6
7 light was separated from the light emitted from the sample using a 395 nm dichroic
8
9
10 mirror and ND filter (1/32). Images were continuously acquired (exposure time 150
11
12
13 ms) using an ORCA-Flash4.0 V2 sCMOS camera (Hamamatsu). The imaging
14
15
16
17 system was controlled by an integrating imaging software (HCLImage Live). In order
18
19
20 to induce rhythmic burst, 20 μM bicuculline methiodide was bath-applied after 10
21
22
23 minutes recording. At the end of each experiment, Tetrodotoxin (TTX, 1 μM , a
24
25
26 voltage-gated, fast Na^+ channel blocker; Latoxan) was applied to confirm the
27
28
29 neuronal nature of the recorded signals. Recorded images were analyzed off-line
30
31
32 both with Fiji (selecting region of interest, ROI, around cell bodies) and Clampfit
33
34
35 software (pClamp suite, 10.2 version; Molecular Devices LLC, US). Intracellular Ca^{2+}
36
37
38 transients were expressed as fractional amplitude increase ($\Delta F/F_0$, where F_0 is the
39
40
41 baseline fluorescence level and ΔF is the rise over baseline); we determine the onset
42
43
44 time of neuronal activation by detecting those events in the fluorescence signal that
45
46
47
48
49
50
51
52 exceed at least five times the standard deviation of the noise.
53
54
55
56
57
58
59
60

Immunocytochemistry and image processing.

After 9-11 days *in-vitro* hippocampal neurons and glial cells were fixed by 4 % formaldehyde (prepared from fresh paraformaldehyde; Sigma) in PBS for 20 minutes, at room temperature (RT). After that, cells were washed three times with PBS and permeabilized with 1% Triton X-100 for 30 min, blocked with 5% FBS in PBS for 30 min at room temperature, and incubated with primary antibodies for 45 min. The primary antibodies used were as follows: rabbit polyclonal anti- β -tubulin III (1:500 dilution) and mouse monoclonal anti-GFAP (1:500 dilution). After PBS washes, cells were incubated for 45 minutes with AlexaFluor 594 goat anti-rabbit (Invitrogen, dilution 1:500) and AlexaFluor 488 goat anti-mouse (Invitrogen, dilution 1:500). Samples were mounted in Vectashield with DAPI in order to stain the nuclei (Vector Laboratories) on 1 mm tick coverslips. Images were acquired using a Nikon C2 confocal microscope (Nikon, Japan) at 40x magnification. Z-stacks were acquired every 500 nm, from seven to ten random fields for control and SWCNTs substrates. Offline analysis was performed using the image-processing package Fiji.

ASSOCIATED CONTENT

The supporting information is available online: Figure S1. UV-Vis plots of the cross-linked materials in comparison with p -SWCNTs; Figure S2. Raman plots of the cross-linked materials in comparison with p -SWCNTs; Figure S3. TGA plots of the cross-linked materials in comparison with p -SWCNTs; Figure S4. TEM images of p -SWCNTs, ph-SWCNTs and $\text{phSO}_3\text{H-SWCNTs}$; Figure S5. SEM images of p -SWCNTs, ph-SWCNTs and $\text{phSO}_3\text{H-SWCNTs}$.

ACKNOWLEDGMENTS

This work was supported by the Spanish Ministry of Economy and Competitiveness MINECO (project CTQ2016-76721-R), by the University of Trieste, Consorzio Interuniversitario Nazionale per la Scienza e Tecnologia dei Materiali (INSTM), Ministero dell'Università e della Ricerca (MIUR) (FIRB prot. RBAP11ETKA and Cofin. Prot. 2010N3T9M4) and by the ByAxon n. 737116. MP, as the recipient of the AXA Chair, is grateful to the AXA Research Fund for financial support. This work

1
2
3 was performed under the Maria de Maeztu Units of Excellence Program from the
4
5
6
7 Spanish State Research Agency – Grant No. MDM-2017-0720
8
9
10

11 **Author Contributions.**

12
13
14 ‡These authors contributed equally to this work.
15
16
17
18
19

20 **REFERENCES**

- 21
22
23 (1) Hirsch, A. The Era of Carbon Allotropes. *Nat. Mater.* **2010**, *9*, 868–871.
24
25
26
27 (2) Tasis, D.; Tagmatarchis, N.; Bianco, A.; Prato, M. Chemistry of Carbon
28
29
30
31 Nanotubes. *Chem. Rev.* **2006**, *106*, 1105–1136.
32
33
34
35 (3) Sreekumar, T. V.; Liu, T.; Kumar, S.; Ericson, L. M.; Hauge, R. H.; Smalley, R.
36
37
38
39 E. Single-Wall Carbon Nanotube Films. *Chem. Mater.* **2003**, *15*, 175–178.
40
41
42
43 (4) Gu, B. H.; Swager, T. M. Fabrication of Free-Standing , Conductive , and
44
45
46
47 Transparent Carbon Nanotube Films. *Adv. Mater.* **2008**, *20*, 4433–4437.
48
49
50
51 (5) Zhang, L.; Zhang, G.; Liu, C.; Fan, S. High-Density Carbon Nanotube
52
53
54
55 Buckypapers with Superior Transport and Mechanical Properties. *Nano Lett.*
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

2012, 12, 4848–4852.

- (6) Jakubinek, M. B.; Ashrafi, B.; Guan, J.; Johnson, M. B.; White, M. A.; Simard, B. 3D Chemically Cross-Linked Single-Walled Carbon Nanotube Buckypapers. *RSC Adv.* **2014**, 4, 57564–57573.
- (7) Behabtu, N.; Green, M. J.; Pasquali, M. Carbon Nanotube-Based Neat Fibers. *Nano Today* **2008**, 3, 24–34.
- (8) Yu, Q.; Alvarez, N. T.; Miller, P.; Malik, R.; Haase, M. R.; Schulz, M.; Shanov, V.; Zhu, X. Mechanical Strength Improvements of Carbon Nanotube Threads through Epoxy Cross-Linking. *Materials* **2016**, 9, 1–12.
- (9) Schirowski, M.; Abellán, G.; Nuin, E.; Pampel, J.; Dolle, C.; Wedler, V.; Fellingner, T. P.; Spiecker, E.; Hauke, F.; Hirsch, A. Fundamental Insights into the Reductive Covalent Cross-Linking of Single-Walled Carbon Nanotubes. *J. Am. Chem. Soc.* **2018**, 140, 3352–3360.
- (10) Holzinger, M.; Steinmetz, J.; Samaille, D.; Glerup, M.; Paillet, M.; Bernier, P.;

- 1
2
3 Ley, L.; Graupner, R. [2 + 1] Cycloaddition for Cross-Linking SWCNTs.
4
5
6
7 *Carbon N. Y.* **2004**, *42*, 941–947.
8
9
10
11 (11) Ventura, D. N.; Stone, R. A.; Chen, K. S.; Hariri, H. H.; Riddle, K. A.; Fellers,
12
13
14 T. J.; Yun, C. S.; Strouse, G. F.; Kroto, H. W.; Acquah, S. F. A. Assembly of
15
16
17
18 Cross-Linked Multi-Walled Carbon Nanotube Mats. *Carbon N. Y.* **2010**, *48*,
19
20
21
22 987–994.
23
24
25
26 (12) Kumar, R.; Rao, C. N. R. Assemblies of Single-Walled Carbon Nanotubes
27
28
29
30 Generated by Covalent Cross-Linking with Organic Linkers. *J. Mater. Chem.*
31
32
33 *A* **2015**, *3*, 6747–6750.
34
35
36
37 (13) Zhang, Y.; Broekhuis, A. A.; Stuart, M. C. A.; Fernandez, T.; Fausti, D.; Rudolf,
38
39
40
41 P.; Picchioni, F. Cross-Linking of Multiwalled Carbon Nanotubes with
42
43
44
45 Polymeric Amines. *Macromolecules* **2008**, 6141–6146.
46
47
48
49 (14) Park, O. K.; Choi, H.; Jeong, H.; Jung, Y.; Yu, J.; Lee, J. K.; Hwang, J. Y.; Kim,
50
51
52
53 S. M.; Jeong, Y.; Park, C. R.; Endo, M.; Ku, B. C. High-Modulus and Strength
54
55
56
57 Carbon Nanotube Fibers Using Molecular Cross-Linking. *Carbon N. Y.* **2017**,
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

118, 413–421.

(15) Hu, L.; Hecht, D. S.; Gru, G. Carbon Nanotube Thin Films: Fabrication , Properties , and Applications. *Chem. Rev.* **2010**, *110*, 5790–5844.

(16) Marchesan, S.; Ballerini, L.; Maurizio, P. Nanomaterials for Stimulating Nerve Growth. *Science* **2017**, *356*, 1010–1012.

(17) Cellot, G.; Cilia, E.; Cipollone, S.; Rancic, V.; Sucapane, A.; Giordani, S.; Gambazzi, L.; Markram, H.; Grandolfo, M.; Scaini, D.; Gelain, F.; Casalis, L.; Prato, M.; Giugliano, M.; Ballerini, L. Carbon Nanotubes Might Improve Neuronal Performance by Favouring Electrical Shortcuts. *Nat. Nanotech.* **2009**, *4*, 126–133.

(18) Bosi, S.; Fabbro, A.; Ballerini, L.; Prato, M. Carbon Nanotubes: A Promise for Nerve Tissue Engineering. *Nanotechnol. Rev.* **2013**, *2*, 47–57.

(19) Sucapane, A.; Cellot, G.; Prato, M.; Giugliano, M.; Parpura, V.; Ballerini, L. Interactions Between Cultured Neurons and Carbon Nanotubes: A

1
2
3 Nanoneuroscience Vignette. *J. Nanoneurosci.* **2009**, *1*, 10–16.
4
5
6

- 7
8 (20) Fan, L.; Feng, C.; Zhao, W.; Qian, L.; Wang, Y.; Li, Y. Directional Neurite
9
10 Outgrowth on Superaligned Carbon Nanotube Yarn Patterned Substrate.
11
12
13
14
15 *Nano Lett.* **2012**, *12*, 3668–3673.
16
17

- 18
19 (21) Gottipati, M. K.; Samuelson, J. J.; Kalinina, I.; Bekyarova, E.; Haddon, R. C.;
20
21
22 Parpura, V. Chemically Functionalized Single-Walled Carbon Nanotube Films
23
24
25
26 Modulate the Morpho-Functional and Proliferative Characteristics of
27
28
29 Astrocytes. *Nano Lett.* **2013**, *13*, 4387–4392.
30
31

- 32
33
34 (22) Usmani, S.; Aurand, E. R.; Medelin, M.; Fabbro, A.; Scaini, D.; Laishram, J.;
35
36
37 Rosselli, F. B.; Ansuini, A.; Zoccolan, D.; Scarselli, M.; Crescenzi, M. De; Bosi,
38
39
40 S.; Prato, M.; Ballerini, L. 3D Meshes of Carbon Nanotubes Guide Functional
41
42
43
44 Reconnection of Segregated Spinal Explants. *Sci. Adv.* **2016**, *2*, e1600087.
45
46

- 47
48 (23) Keefer, E. W.; Botterman, B. R.; Romero, M. I.; Rossi, A. F.; Gross, G. W.
49
50
51
52 Carbon Nanotube Coating Improves Neuronal Recordings *Nat. Nanotech.*
53
54
55
56 **2008**, *3*, 1–6.
57
58

- 1
2
3 (24) Ansaldo, A.; Castagnola, E.; Maggiolini, E.; Fadiga, L.; Ricci, D. Superior
4
5
6 Electrochemical Performance of Carbon Nanotubes Directly Grown on Sharp
7
8
9
10 Microelectrodes. *ACS Nano* **2011**, *5*, 2206–2214.
11
12
13
14 (25) Bosi, S.; Fabbro, A.; Cantarutti, C.; Mihajlovic, M.; Ballerini, L.; Prato, M.
15
16
17 Carbon Based Substrates for Interfacing Neurons: Comparing Pristine with
18
19
20
21 Functionalized Carbon Nanotubes Effects on Cultured Neuronal Networks.
22
23
24
25 *Carbon N. Y.* **2016**, *97*, 87–91.
26
27
28
29 (26) Hu, H.; Ni, Y.; Mandal, S. K.; Montana, V.; Zhao, B.; Haddon, R. C.; Parpura,
30
31
32 V. Polyethyleneimine Functionalized Single-Walled Carbon Nanotubes as a
33
34
35
36 Substrate for Neuronal Growth. *J. Phys. Chem. B* **2005**, *109*, 4285–4289.
37
38
39
40 (27) Malarkey, E. B.; Fisher, K. A.; Bekyarova, E.; Liu, W.; Haddon, R. C.; Parpura,
41
42
43 V. Conductive Single-Walled Carbon Nanotube Substrates Modulate Neuronal
44
45
46
47 Growth. *Nano Lett.* **2009**, *9*, 264–268.
48
49
50
51 (28) Liu, J.; Appaix, F.; Bibari, O.; Marchand, G.; Benabid, A. L.; Sauter-Starace,
52
53
54
55 F.; Waard, M. De. Control of Neuronal Network Organization by Chemical
56
57
58
59
60

1
2
3 Surface Functionalization of Multi-Walled Carbon Nanotube Arrays.
4
5

6
7 *Nanotechnology* **2011**, *22*, 195101.
8
9

- 10
11 (29) Lee, H. J.; Yoon, O. J.; Kim, D. H.; Jang, Y. M.; Kim, H. W.; Lee, W. B.; Lee,
12
13
14 N. E.; Kim, S. S. Neurite Outgrowth on Nanocomposite Scaffolds Synthesized
15
16 from PLGA and Carboxylated Carbon Nanotubes. *Adv. Eng. Mater.* **2009**, *11*,
17
18 B261–B266.
19
20
21
22

- 23
24
25 (30) Hu, H.; Ni, Y.; Montana, V.; Haddon, R. C.; Parpura, V. Chemically
26
27
28 Functionalized Carbon Nanotubes as Substrates for Neuronal Growth. *Nano*
29
30
31
32 *Lett.* **2004**, *4*, 14–16.
33
34
35

- 36
37 (31) Matsumoto, K.; Sato, C.; Naka, Y.; Kitazawa, A.; Whitby, R. L. D.; Shimizu, N.
38
39
40 Neurite Outgrowths of Neurons with Neurotrophin-Coated Carbon Nanotubes.
41
42
43
44 *J. Biosci. Bioeng.* **2007**, *103*, 216–220.
45
46
47

- 48 (32) Luo, X.; Weaver, C. L.; Zhou, D. D.; Greenberg, R.; Cui, X. T. Highly Stable
49
50
51 Carbon Nanotube Doped Poly(3,4-Ethylenedioxythiophene) for Chronic
52
53
54
55 Neural Stimulation. *Biomaterials* **2011**, *32*, 5551–5557.
56
57
58
59

- 1
2
3
4 (33) Abidian, M. R.; Ludwig, K. A.; Marzullo, T. C.; Martin, D. C.; Kipke, D. R.
5
6
7 Interfacing Conducting Polymer Nanotubes with the Central Nervous System:
8
9
10 Chronic Neural Recording Using Poly(3,4-Ethylenedioxythiophene)
11
12
13 Nanotubes. *Adv. Mater.* **2009**, *21*, 3764–3770.
14
15
16
17
18 (34) Alegret, N.; Dominguez-Alfaro, A.; González-Domínguez, J. M.; Arnaiz, B.;
19
20
21 Cossío, U.; Bosi, S.; Vázquez, E.; Ramos-Cabrer, P.; Mecerreyes, D.; Prato,
22
23
24 M. Three-Dimensional Conductive Scaffolds as Neural Prostheses Based on
25
26
27 Carbon Nanotubes and Polypyrrole. *ACS Appl. Mater. Interfaces* **2018**, *10*,
28
29
30
31
32 43904–43914.
33
34
35
36 (35) Lovat, V.; Pantarotto, D.; Lagostena, L.; Cacciari, B.; Grandolfo, M.; Righi, M.;
37
38
39 Spalluto, G.; Prato, M.; Ballerini, L. Carbon Nanotube Substrates Boost
40
41
42
43 Neuronal Electrical Signaling. *Nano Lett.* **2005**, *5*, 1107–1110.
44
45
46
47 (36) Bosi, S.; Rauti, R.; Laishram, J.; Turco, A.; Lonardoni, D.; Nieus, T.; Prato, M.;
48
49
50
51 Scaini, D.; Ballerini, L. From 2D to 3D: Novel Nanostructured Scaffolds to
52
53
54
55 Investigate Signalling in Reconstructed Neuronal Networks. *Sci. Rep.* **2015**, *5*,
56
57
58
59
60

- 1
2
3 9562.
4
5
6
7
8 (37) Jang, M. J.; Namgung, S.; Hong, S.; Nam, Y. Directional Neurite Growth Using
9
10 Carbon Nanotube Patterned Substrates as a Biomimetic Cue.
11
12
13
14 *Nanotechnology* **2010**, *21*, 235102–235108.
15
16
17
18
19 (38) Bahr, J. L.; Tour, J. M. Highly Functionalized Carbon Nanotubes Using *In Situ*
20
21
22 Generated Diazonium Compounds. *Chem. Mater.* **2001**, *13*, 3823–3824.
23
24
25
26
27 (39) Bahr, J. L.; Yang, J.; Kosynkin, D. V; Bronikowski, M. J.; Smalley, R. E.; Tour,
28
29
30 J. M. Functionalization of Carbon Nanotubes by Electrochemical Reduction of
31
32
33 Aryl Diazonium Salts : A Bucky Paper Electrode. *J. Am. Chem. Soc* **2001**, *123*,
34
35
36
37 6536–6542.
38
39
40
41 (40) Barrejón, M.; Syrgiannis, Z.; Burian, M.; Bosi, S.; Fornasiero, P.; Amenitsch,
42
43
44 H.; Prato, M. Cross-Linked Carbon Nanotube Adsorbents for Water
45
46
47
48 Treatment : Tuning the Sorption Capacity through Chemical Functionalization.
49
50
51
52 *ACS Appl. Mater. Interfaces* **2019**, *11*, 12920-12930.
53
54
55
56
57
58
59
60

- 1
2
3
4 (41) Dyke, A.; Stewart, M. P.; Maya, F.; Tour, J. M. Diazonium-Based
5
6
7 Functionalization of Carbon Nanotubes: XPS and GC–MS Analysis and
8
9
10 Mechanistic Implications. *Synlett* **2004**, 155–160.
11
12
13
14
15 (42) Schirowski, M.; Tyborsky, C.; Janina, M.; Hauke, F.; Hirsch, A.; Goclon, J.
16
17
18 Reductive Diazotation of Carbon Nanotubes: An Experimental and Theoretical
19
20
21 Selectivity Study. *Chem. Sci.* **2019**, *10*, 706–717.
22
23
24
25
26 (43) Maeda, Y.; Saito, K.; Akamatsu, N.; Chiba, Y.; Ohno, S.; Okui, Y.; Yamada,
27
28
29 M.; Hasegawa, T.; Kako, M.; Akasaka, T. Analysis of Functionalization Degree
30
31
32 of Single-Walled Carbon Nanotubes Having Various Substituents. *J. Am.*
33
34
35
36
37 *Chem. Soc.* **2012**, *134*, 18101–18108.
38
39
40
41 (44) L. J. van der Pauw. A Method of Measuring the Resistivity and Hall Coefficient
42
43
44 on Lamellae of Arbitrary Shape. *Phys. Tech. Rev.* **1958**, *20*, 220–224.
45
46
47
48
49 (45) Cellot, G.; Toma, F. M.; Kasap Varley, Z.; Laishram, J.; Villari, A.; Quintana,
50
51
52 M.; Cipollone, S.; Prato, M.; Ballerini, L. Carbon Nanotube Scaffolds Tune
53
54
55 Synaptic Strength in Cultured Neural Circuits: Novel Frontiers in
56
57
58
59
60

1
2
3 Nanomaterial-Tissue Interactions. *J. Neurosci.* **2011**, *31*, 12945–12953.

4
5
6
7
8 (46) Alfred, C.; Banker, G. A.; Binder, L. Immunocytochemical Localization of
9
10 Tubulin and Protein 2 During the Development Hippocampal Neurons in
11
12 Culture. *J. Neurosci.* **1986**, *6*, 714–722.

13
14
15
16
17
18 (47) Willmott, N. J.; Wong, K.; Strong, A. J. Intercellular Ca⁺² Waves in Rat
19
20 Hippocampal Slice and Dissociated Glial ^ Neuron Cultures Mediated by Nitric
21
22 Oxide. *FEBS Lett.* **2000**, *487*, 239–247.

23
24
25
26
27
28 (48) Marconi, E.; Nieuw, T.; Maccione, A.; Valente, P.; Simi, A.; Messa, M.; Dante,
29
30 S.; Baldelli, P.; Berdondini, L.; Benfenati, F. Emergent Functional Properties
31
32 of Neuronal Networks with Controlled Topology. *PLoS One* **2012**, *7*, e34648.

33
34
35
36
37 (49) Ross, W. N.; Hole, W. Understanding Calcium Waves and Sparks in Central
38
39 Neurons. *Nat. Rev. Neuroscience* **2015**, *13*, 157–168.

40
41
42 (50) Grienberger, C.; Konnerth, A. Primer Imaging Calcium in Neurons. *Neuron*
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
2012, *73*, 862–885.

- 1
2
3
4 (51) Galante, M.; Nistri, A.; Ballerini, L. Opposite Changes in Synaptic Activity of
5
6 Organotypic Rat Spinal Cord Cultures after Chronic Block of AMPA/kainate or
7
8 Glycine and GABA A Receptors. *J. Physiol.* **2000**, *523*, 639–651.
9
10
11
12
13
14 (52) Rauti, R.; Lozano, N.; León, V.; Scaini, D.; Musto, M.; Rago, I.; Paolo, F.;
15
16 Severino, U.; Fabbro, A.; Vázquez, E.; Kostarelos, K.; Prato, M.; Ballerini, L.
17
18 Graphene Oxide Nanosheets Reshape Synaptic Function in Cultured Brain
19
20
21
22
23
24
25
26
27
28
29 (53) Stetter, O.; Battaglia, D.; Soriano, J.; Geisel, T. Model-Free Reconstruction of
30
31
32
33
34
35
36
37
38
39
40
41 (54) Fabbro, A.; Pastore, B.; Nistri, A.; Ballerini, L. Activity-Independent
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
- (55) Sokal, D. M.; Mason, R.; Parker, T. L. Multi-Neuronal Recordings Reveal a
Differential Effect of Thapsigargin on Bicuculline- or Gabazine-Induced

1
2
3 Epileptiform Excitability in Rat Hippocampal Neuronal Networks.

4
5
6
7 *Neuropharmacology* **2000**, *39*, 2408–2417.

8
9
10
11 (56) Tibau, E.; Valencia, M.; Soriano, J. Identification of Neuronal Network
12
13
14 Properties from the Spectral Analysis of Calcium Imaging Signals in Neuronal
15
16
17
18 Cultures. *Front. Neural Circuits* **2013**, *7*, 1–16.

19
20
21
22 (57) Mazzatenta, A.; Giugliano, M.; Campidelli, S.; Gambazzi, L.; Businaro, L.;
23
24
25 Markram, H.; Prato, M.; Ballerini, L. Interfacing Neurons with Carbon
26
27
28 Nanotubes: Electrical Signal Transfer and Synaptic Stimulation in Cultured
29
30
31
32 Brain Circuits. *J. Neurosci.* **2007**, *27*, 6931–6936.

33
34
35
36
37 (58) Schmidt, C. E.; Shastri, V. R.; Vacanti, J. P.; Anger, R. O. L. Stimulation of
38
39
40 Neurite Outgrowth Using an Electrically. *Proc. Natl. Acad. Sci.* **1997**, *94*, 8948–
41
42
43
44 8953.

45
46
47
48 (59) Aguado, F.; Carmona, M. A.; Pozas, E.; Aguiló, A.; Martínez-guijarro, F. J.;
49
50
51 Alcantara, S.; Borrell, V.; Yuste, R.; Ibañez, C. F.; Soriano, E. BDNF Regulates
52
53
54
55 Spontaneous Correlated Activity at Early Developmental Stages by Increasing
56
57
58

1
2
3 Synaptogenesis and Expression of the K^+ / Cl^- Co-Transporter KCC2.
4
5

6
7 *Development* **2003**, 1267–1280.
8
9

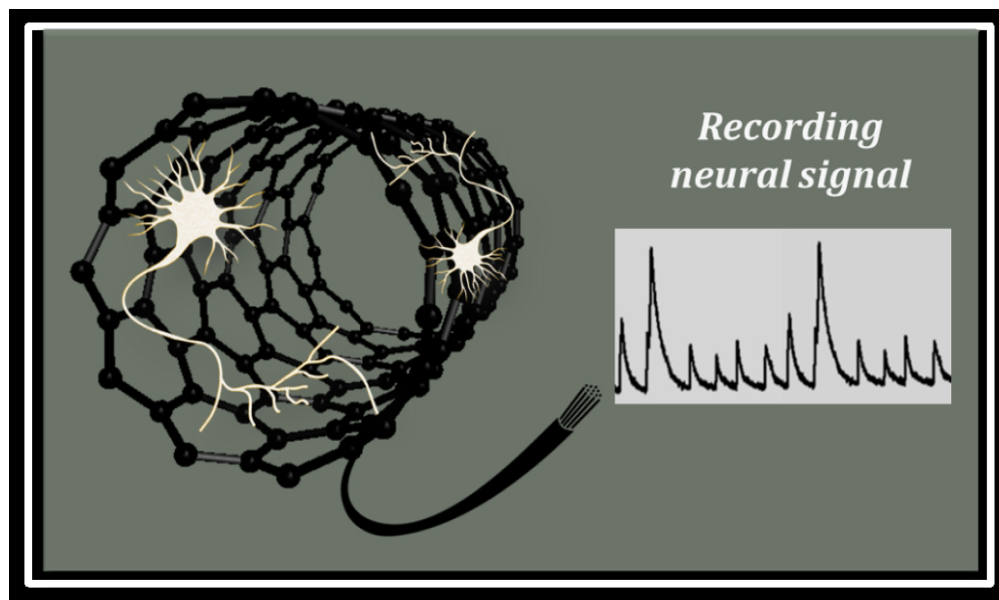
10
11 (60) Yang, L.; Qi, Y.; Yang, Y. Astrocytes Control Food Intake by Inhibiting AGRP
12
13 Neuron Activity *via* Adenosine A_1 Receptors. *Cell Rep.* **2015**, *11*, 798–807.
14
15
16
17

18
19 (61) Marom, S.; Shahaf, G. Development , Learning and Memory in Large Random
20
21 Networks of Cortical Neurons : Lessons beyond Anatomy. *Q. Rev. Biophys.*
22
23 **2002**, *35*, 63–87.
24
25
26
27

28
29 (62) Arosio, D.; Ratto, G. M. Twenty Years of Fluorescence Imaging of Intracellular
30
31 Chloride. *Front. Cell. Neurosci.* **2014**, *8*, 258.
32
33
34
35

36
37 (63) Martín, C.; Merino, S.; González-Domínguez, J. M.; Rauti, R.; Ballerini, L.;
38
39 Prato, M.; Vázquez, E. Graphene Improves the Biocompatibility of
40
41 Polyacrylamide Hydrogels: 3D Polymeric Scaffolds for Neuronal Growth. *Sci.*
42
43
44
45
46
47
48
49 *Rep.* **2017**, *7*, 10942.
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



26
27

TOC

28
29

176x105mm (149 x 149 DPI)