Carbon Nanotubes Carrying Cell-Adhesion Peptides do not Interfere with Neuronal Functionality

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This paper is dedicated to the 70th birthday of Professor Gianfranco Scorrano

The potential application of nanoscale materials to biomedical science relies on their ability to functionally integrate with cellular and physiological systems.[1–3] One of the most attractive materials employed to develop nano–bio hybrid systems is represented by carbon nanotubes (CNTs), due to their unique characteristics. In addition, the chemical and electrical properties of CNTs can be exploited in neurology for the development of novel neuroimplantable devices.[4,5]

It has been recently demonstrated that chemically modified CNTs immobilized in layers are compatible substrates with neurons favoring neuronal adhesion, their survival and growth, and supporting neurites elongation.[6,7] CNT interfaces also promote spontaneous synaptic activity in neuronal networks, and can be successfully employed to deliver electrical stimulation to neuronal pathways.[8,9] The first example of electrodes coated with CNTs and implanted into different brain areas in rats or monkeys has been reported. Such electrodes were able both to enhance the detection of the neuronal signals and to perform as optimal stimulators,[10] highlighting the potential of CNTs for the development of electrical brain interfaces.[11,12] Recently, CNT substrates have been reported to change the responsiveness of neurons by forming tight contacts with the cell membranes. Such contacts might favor electrical shortcuts between the proximal and distal compartments of the neurons, affecting neuronal information processing.[13]

Other types of carbon nanomaterials have displayed variable characteristics. While CNT sheets and yarns were able to stimulate cell migration in comparison to plastic and glass substrates, carbon nanofibers reduced cell adhesion and limited cell functions.[14,15] These results suggest that further investigations are necessary to select and conceive new carbon-based neural biomaterials.

Another intriguing possibility is the use of chemically functionalized water-soluble CNTs in biomedical applications to the nervous system. Soluble CNTs can modulate the outgrowth of neuronal processes,[16] suggesting the possibility to selectively enhance neuritis elongation directly at the site of nerve injury, to sustain functional recovery. Water-soluble CNTs can be modified via chemical functionalization, allowing their binding to selective therapeutics or biologically relevant molecules, acting as specific signals presented via a CNT-based drug-delivery system.[17–19]

Despite being highly electrically conductive, pristine CNTs can be chemically modified with different biomolecules while maintaining their intrinsic properties.[20] A few years ago, we developed a powerful method for the functionalization of CNTs with antigenic peptides.[21,22] These functionalized carbon nanotubes (*f*-CNTs) were able to generate specific antibody responses, while the CNT support was non-immunogenic.

In this work, we describe the functionalization of multiwalled carbon nanotubes (*f*-MWNTs) with cell-adhesion peptides, and the study of the effect of these conjugates on different types of cells, including tumor cells (Jurkat) and, most importantly, primary splenocytes and neurons. This study is of fundamental importance for the future integration of peptide-nanotubes into innovative microsystems, that is, soluble functionalized CNTs to be delivered at the site of nerve injury to promote local tissue repair.

For this purpose, we have decided to functionalize MWNTs. We have already interfaced MWNTs with neuronal cells,[8,13] and this type of tubes, in comparison to single-walled carbon nanotubes (SWNTs), offers a wider external surface per single tube for their organic functionalization. In addition, MWNTs are commercially available in larger quantities, so that, for large-scale biological applications, they can be considered more suitable than SWNTs. Initially, the tubes have been cut and oxidized using strong acid conditions, as previously reported (Scheme 1).[23] The importance of obtaining short nanotubes

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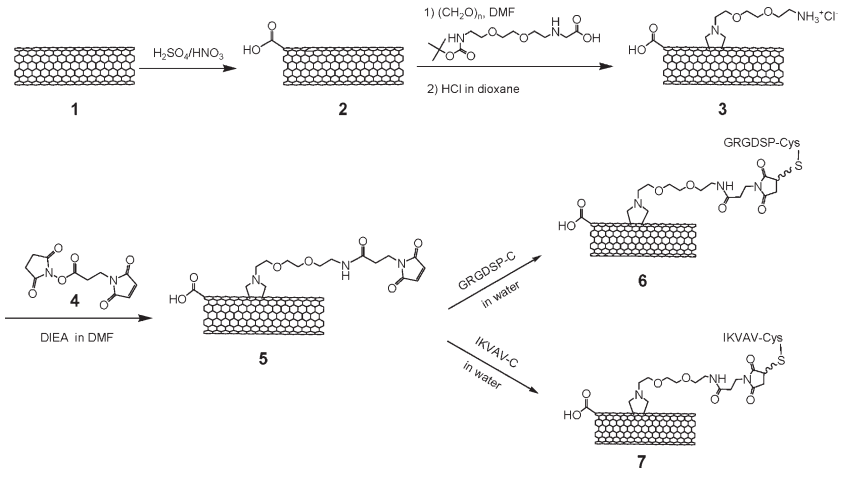

Scheme 1. Synthesis of MWNT–peptide conjugates.

is essential, as concerns about the toxicity of long non-functionalized nanotubes have been reported.[24–26] The tubes subsequently underwent the 1,3-dipolar cycloaddition reaction of azomethine ylides, to afford MWNTs **3**.[27,28] Following this reaction, the amount of amino groups was established with the quantitative Kaiser test, and corresponded to 150 μmol per gram of conjugate. The amino groups of **3** have been coupled to a maleimido function, which is necessary to selectively link the cell-adhesion-promoting peptides. For our purpose, we have selected two peptide sequences. The first sequence corresponds to Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP, **Pep 1**), which is a fibronectin-derived peptide capable of increasing integrin-mediated cell adhesion and spreading on a variety of substrates via the cell-binding domain RGD residues.[29–31] Coating surfaces with RGD-based sequences promotes not only cell adhesion but also neurite outgrowth.[32] In addition, RGD-containing peptides intervene in the mechanism of integrin regulation of neuronal gene expression.[33] Similarly, peptides from different domains of protein laminin have been used to stimulate neuronal growth and axon regeneration.[34–36] The second selected sequence corresponded to Ile-Lys-Val-Ala-Val (IKVAV, **Pep 2**) contained in laminin.[34]

A cysteine residue was added to the C-terminal part of both sequences for the selective chemical ligation to the maleimido MWNTs **5** in water. These two peptides have been prepared in turn using a solid-phase peptide-synthesis approach. The coupling between the peptides and the tubes was followed by HPLC. A decrease of the peak relative to the peptide was monitored during the reaction time (see Supporting Information, Fig. S1). No specific interaction between the nanotubes and the peptides was observed by simply mixing MWNTs **3** and each single peptide (see Supporting Information, Fig. S2). The mixture was analyzed by HPLC at different time points, and no decrease of the peak of the peptide was observed. The coupling reaction was completed within 2 h. The traces of peptide, due to the slight excess used, were eliminated by dialysis. A further experiment, to prove that all maleimido groups around the tubes were saturated with the peptides, was performed repeating the first peptide coupling and by adding, after 24 h, an additional equivalent of peptide. No variation of the amount of this excess of peptide was detected in the HPLC analysis at different times (see Supporting Information, Fig. S3). The excess of peptide was again eliminated by dialysis. The final conjugates MWNTs **6** and **7** have been recovered as black fluffy powders after lyophilization. All nanotube conjugates and precursors have been characterized using thermogravimetric analysis (TGA, see Supporting Information, Fig. S4) and transmission electron microscopy (TEM).

Figure 1 shows representative TEM images of the tubes following the different steps of functionalization. No structural differences in morphology were observed between each *f*-MWNT. The presence of the peptide covalently attached to the tubes was then confirmed by aminoacid sequencing. Figure 2 displays the chromatograms obtained during the Ellman degradation of peptide–nanotube conjugate **7**, proving the presence of all aminoacids. Similarly, MWNTs **6** also underwent peptide sequencing, proving the presence of the peptide attached to the tubes (see Supporting Information, Fig. S6).

With the idea of integrating these peptide–nanotube conjugates into advanced biomedical devices, we started to explore their effects on the viability of tumor cells (Jurkat cell line) and primary immune cells (splenocytes). For this study, we have compared the behavior of MWNTs **6** and **7** to the nanotubes devoid of peptides (MWNTs **3**) and to the peptides alone (GRGDSPC (**Pep 1**) or IKVAVC (**Pep 2**)). Functionalized nanotubes were highly soluble in pure water at 1 mg mL^{-1} concentration. Homogenous dispersions were obtained after sonication. The black solutions were further diluted into cell-culture medium to the final concentrations of 1, 10, and

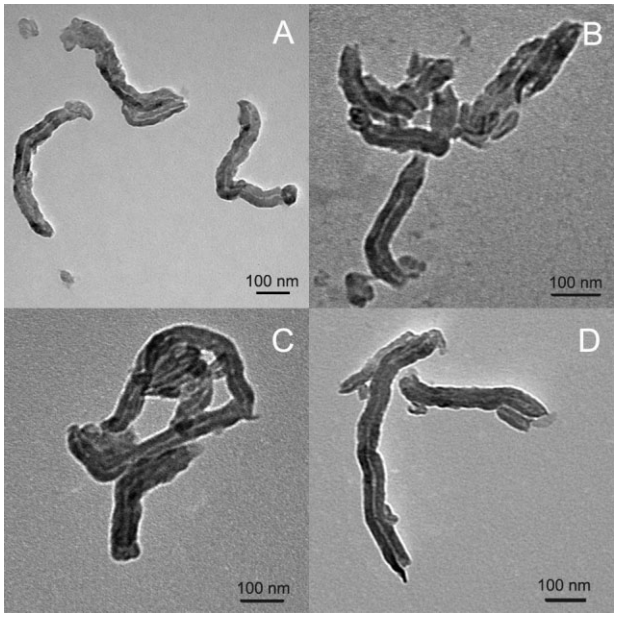

Figure 1. TEM images of A) MWNTs **2**, B) **3**, C) **5**, and D) **7**.

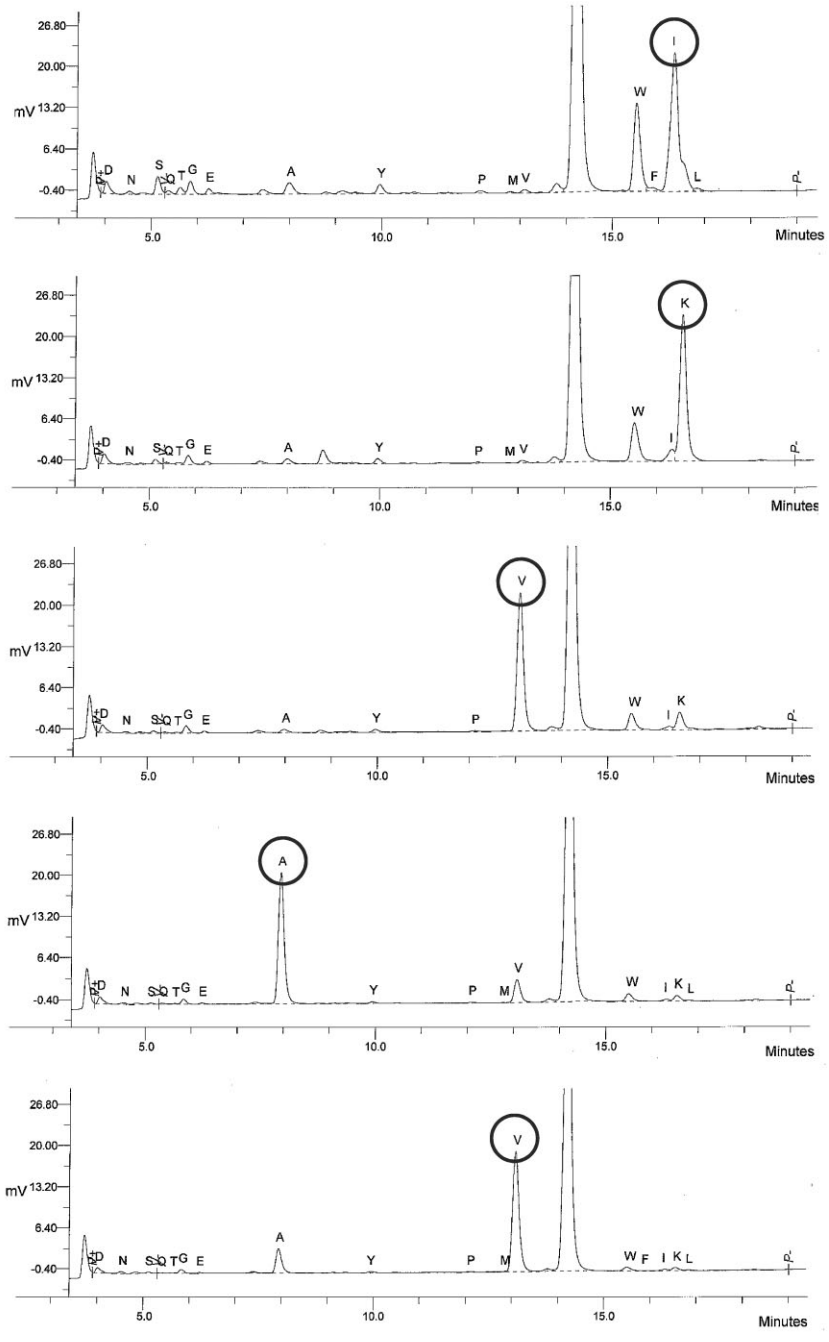

Figure 2. Aminoacid analysis following Ellman degradation of MWNTs **7**. The peaks corresponding to each aminoacid of the peptide sequence (I, K, V, A, V) are circled.

$100\ \mu g\ mL^{-1}$, respectively. The samples were immediately added to human Jurkat lymphoma T cells. Cells were incubated for 22 h in the presence of different doses of nanotubes. They were then washed with culture medium and stained with propidium iodide before flow cytometry analysis. We did not observe any significant loss of cell viability upon incubation of Jurkat cells with 1, 10, or $100\ \mu g\ mL^{-1}$ for all preparations, as compared to untreated cells and control peptides (Fig. 3, top). Only at the highest dose, MWNTs **6** and **7** decreased slightly the number of living cells that remain to 80%, in comparison to untreated cells.

We subsequently tested the cytotoxic effects of the peptide–nanotube conjugates on spleen cells isolated from healthy BALB/c mice. Upon incubation for 22 h, cell viability was assessed by flow cytometry as described above. As shown on Figure 3 (bottom), we detected no effect on cell viability after treatment of splenocytes with the different doses of *f*-MWNTs or peptides alone. We could observe only an insignificant percentage of dead cells at $100\ \mu g\ mL^{-1}$ of MWNTs **3**. Therefore, the results on Jurkat and spleen-cell viability are in full agreement with previous studies on different types of functionalized carbon nanomaterials.[26,37,38] Indeed, more and more analyses of cytotoxic effects of carbon nanotubes highlight the importance of chemical functionalization of CNTs, but also that a clear distinction needs to be done between the use of purified, non-functionalized nanotubes and those instead rendered biocompatible by chemical manipulations.[39–41]

As we designed peptide–MWNT conjugates for interacting with neuronal cells, we then performed a functional analysis of cultured neurons incubated with our peptide–nanotube conjugates or in control conditions. We characterized the electrophysiological responses of rat dissociated hippocampal neurons in culture treated with soluble MWNTs **6** and **7** functionalized with GRGDSPC (**Pep 1**) or IKVAVC (**Pep 2**) peptide sequences, respectively, the control peptides alone, and MWNTs **3** without peptides. Cortical primary neuronal cultures display prominent spontaneous electrical activity after the first 6 days in vitro[8,9], thus we first investigated by single cell-voltage clamp recordings whether incubating neurons in the presence of MWNTs functionalized with peptides modifies neuronal spontaneous activity. Neurons, after 8 days in vitro, were incubated (37 °C; 8 h) with medium containing i) MWNTs **3** ($1\ \mu g\ mL^{-1}$); ii) MWNTs functionalized with one of the two peptides ($1\ \mu g\ mL^{-1}$ MWNTs **6** or $1\ \mu g\ mL^{-1}$ MWNTs **7**); and iii) peptides alone ($1\ \mu g\ mL^{-1}$ peptide GRGDSPC or $1\ \mu g\ mL^{-1}$ peptide IKVAVC). At the end of the incubation time, the culture medium was replaced with a fresh one to remove all nanoparticles, and the electrophysiological recordings were performed at two follow-up time points: 24 and 48 h after the beginning of the incubation.

We recorded spontaneous activity in voltage clamp configuration from single neurons: in each tested treatment, the neuronal activity was detected as inward currents, corresponding to synaptic events, of variable amplitude, and characterized by

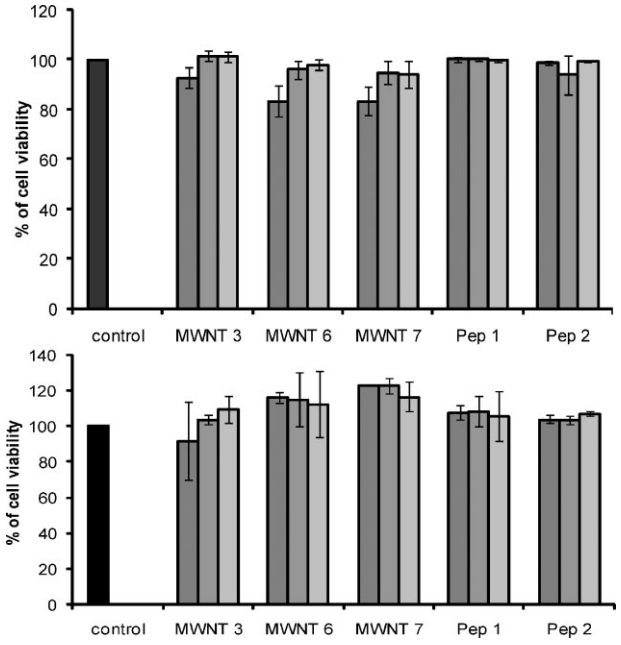

Figure 3. Viability of (top) Jurkat cells and (bottom) splenocytes. Cells were either left untreated or incubated with 1 μg mL^{-1} (light-grey bar), 10 μg mL^{-1} (grey bar), or 100 μg mL^{-1} (dark-grey bar) of the different MWNT–peptide conjugates, peptides alone, and unconjugated MWNTs **3**. 22 h later, cell death was assessed by flow cytometry using propidium iodide.

heterogeneous kinetic properties (see representative traces for MWNTs **6** in Fig. 4A). Such events represent a mixed population of inhibitory and excitatory spontaneous post-synaptic currents (sPSCs), as reported in our previous studies on cultured hippocampal neurons.[8] In Figure 4B, the frequency of sPSCs (see Methods in the Supporting Information) was quantified and did not significantly differ among the three different treatments (MWNTs **3**, MWNTs **6**, and GRGDSPC), both at 24 and 48 h (at 24 h: (1.1 ± 0.2) Hz $n = 15$, (1.1 ± 0.1) Hz $n = 20$, (0.8 ± 0.2) Hz $n = 18$; at 48 h: (1.1 ± 0.3) Hz $n = 5$, (1 ± 0.4) Hz $n = 8$, (1.4 ± 0.3) Hz $n = 9$; MWNTs **3**, MWNTs **6**, and GRGDSPC, respectively). Neurons visualized under bright-field microscopy at 24 and 48 h usually displayed the morphology and shape of healthy cells.[8,9] We further investigated this issue by analyzing some neuronal passive properties, commonly used as indicators of neuronal functional conditions: membrane capacitance and input resistance were measured under voltage clamp and were on average (54 ± 4) pF and (653 ± 54) MΩ in MWNTs **3**-treated neurons ($n = 21$), (48 ± 3) pF and (579 ± 40) MΩ in MWNTs **6**-treated neurons ($n = 40$), and (48 ± 2) pF and (623 ± 46) MΩ in GRGDSPC-treated neurons ($n = 40$) (pooled data for 24 and 48 h, Fig. 4C). We next stimulated cells via depolarizing voltage steps, preceded by a hyperpolarizing stimulus to remove possible inactivation of voltage-gated channels (Fig. 4D, see Methods in the Supporting Information). We quantified peak amplitude of inward and outward currents to the membrane capacitance value of each recorded cell to obtain densities of current and compare these values among the different treatments. Inward and outward current densities were similar under the three treatment conditions, with values on average of (19 ± 3) pA pF^{-1}, (20 ± 3) pA pF^{-1}, and (16 ± 2) pA pF^{-1} ($n = 17$, $n = 27$, $n = 30$; MWNTs **3**, MWNTs **6**, and GRGDSPC, respectively) for the inward components and of (27 ± 3) pA pF^{-1}, (29 ± 2) pA pF^{-1}, and (26 ± 2) pA pF^{-1} ($n = 18$, $n = 31$, $n = 34$; MWNTs **3**, MWNTs **6**, and GRGDSPC, respectively) for the outward current (pooled data for 24 and 48 h, Fig. 4E). All values are in good agreement with previous studies on cultured hippocampal neurons.[42,43] The same electrophysiological experiments, performed in parallel on MWNTs **7** and IKVAVC, gave exactly overlapping results (see Table S1 in the Supporting Information). To prove that the data were obtained in the presence of the MWNT conjugates inside the neurons, we have incubated the cells with two fluorescently labelled MWNTs. Indeed, part of the tubes has been functionalized with fluorescein isothiocyanate (FITC), producing MWNTs **8** (see Supporting Information, Scheme S1). In addition, we have prepared a new doubly functionalized peptide–nanotube conjugate containing FITC (MWNTs **11**, see Supporting Information for details). The fluorescent probe mainly located at the tips of the nanotubes does not modify the properties and, as a consequence, does not influence the uptake of the conjugate, as we already demonstrated for a large series of carbon nanotubes functionalized using a similar approach.[44] This novel conjugate was again characterized by TGA and TEM (see Supporting Information, Figs. S2 and S7). Neurons were able to uptake MWNTs **8** and **11**, and the fluorescent signal was observed inside the cells up to 48 h (see Supporting Information, Figs. S8 and S9), thus proving the presence of the tubes during the electrophysiology recordings. These experiments strongly indicate that incubation of neuronal cells with soluble *f*-MWNTs and MWNT–peptide conjugates and peptides alone does not affect neuronal morphology, viability, and function. At a glance, our data seem to differ from some very recent results showing that pegylated SWNTs were able to block stimulated membrane endocytosis in neurons,[45] an effect that should progressively decrease the frequency in spontaneous sPSCs. Such discrepancy might be due to the different type of treatment, purification, and chemical modification introduced on those nanotubes, namely the coupling with polyethylene glycol. This underlies the importance of investigating the impact of the different kinds of CNT functionalizations on the activity of neuronal circuits.

In summary, the data resulted from this study provide comprehensive evidence of the biocompatibility of soluble *f*-MWNTs with different cell types (Jurkat cells, splenocytes, and neurons). As *f*-MWNTs do not appear to alter neuronal morphology, viability, and basic functions, they represent a promising candidate for the exploitation of novel drug-delivery systems or for designing new generations of self-assembling nerve ''bridges''. In this context, we are currently modifying the surfaces for neuronal cultures with our peptide-modified tubes to study the cell-adhesion properties and growth. In parallel, we are performing in vivo experiments to assess the immunogenic properties of the peptide–nanotubes conjugates.

Experimental

Purified MWNTs **1** were purchased from Nanostructured & Amorphous Materials Inc. Experimental details on their functionalization to generate

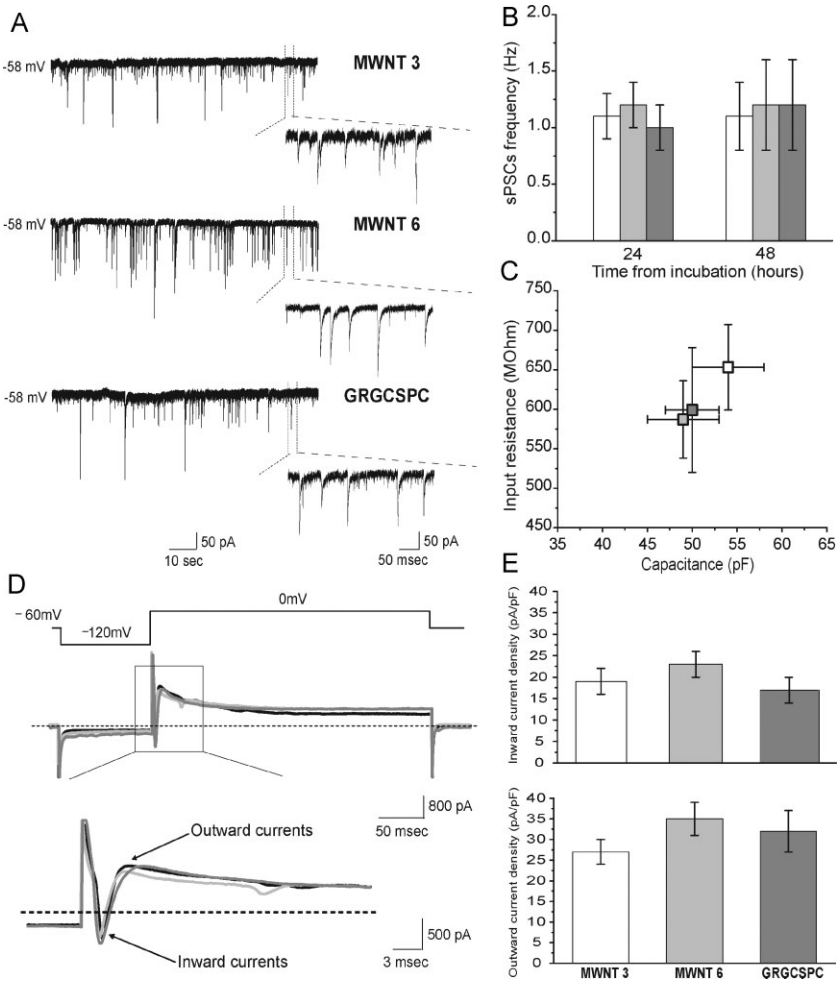

Figure 4. MWNTs **3**, **6**, and peptide GRGDSPC (**Pep 1**) alone do not affect neuronal survival and activity. A) Tracings represent spontaneous synaptic activity recorded from neurons (8 days in vitro) after MWNTs **3**, **6,** and GRGDSPC incubation at 24 h washout. Below each recording, on the right, the magnifications show the presence of heterogeneous events (inward currents), representing the activation of mixed synapses impinging on the recorded neurons. B) sPSCs frequency quantified at 24 and 48 h washout from each treatment with MWNTs **3** (white), **6** (light grey), and GRGDSPC (dark grey), respectively. C) Membrane capacitance and input resistance measured from neurons after treatment with MWNTs **3** (white), **6** (light grey), and GRGDSPC (dark gray), respectively. Note that there are no differences in these values. D) Stimulating protocol (upper trace, voltage steps) and superimposed current recordings obtained from representative neurons treated with MWNTs **3** (black), **6** (light grey), and GRGDSPC (dark grey). Bottom tracings: magnification of the recording area indicating (arrows) the presence of inward and outward currents elicited by the voltage step. E) Mean peak values of inward (top) and outward (bottom) currents, pooled data after 24 and 48 h treatments.

MWNTs **6** and **7**, fluorescently labeled MWNTs, the characterization, and the complete description of the experiments on the uptake, cell viability on tumor cells, splenocytes, and neurons are reported in the Supporting Information.

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