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Carbon nanotube-coupled cell adhesion peptides are non-immunogenic: a promising step toward new biomedical devices‡§

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Carbon nanotubes functionalized with cell adhesion peptides can be considered as novel, promising candidates for the development of advanced drug delivery systems or for designing new generation of self-assembling nerve 'bridges'. An important step toward the integration of these types of conjugates in living bodies is the assessment of their impact on the immune system. In this direction, an integrin-derived peptide has been covalently conjugated to carbon nanotubes. Following intraperitoneal administration, peptide–carbon nanotubes do not trigger an anti-peptide antibody production. Demonstration of the immune neutrality of peptide–carbon nanotubes reinforces their potential use as substrates for neuronal regeneration *in vivo*.

Keywords: carbon nanotubes; peptides; functionalization; immunogenicity

Introduction

Carbon nanotubes (CNTs) are two-dimensional objects made of graphene layers that are rolled up into a hollow cylindrical form, closed or opened at the ends. They can be either single-walled carbon nanotubes (SWCNTs) or multi-walled carbon nanotubes (MWCNTs) depending on the number of rolled sheets. Both types of nanotubes have a diameter in the nanoscale range and lengths that can reach several microns. Although discovered in the middle of the 20th century, they were described at the atomic level only in 1991 [1,2]. These nanomaterials have mechanical, thermal, chemical, and electronic properties that make them unique in nanoscience and nanotechnology [3]. CNTs also assume an important role in the emerging field of nanomedicine [4]. CNTs can be considered novel and innovative tools for the development of alternative methodologies for the delivery of therapeutic molecules.

One important issue with CNTs is related to their insolubility in any solvent. However, organic functionalization has remarkably improved their solubility as well as their biocompatibility profile, thus rendering easier their integration into biological systems [5–7]. Within the wide range of functionalized CNTs with different classes of bioactive molecules, peptide–CNT conjugates display interesting applications as tumor-cell-targeting carriers, cell-growth substrates, diagnostic tools, and new vaccines [8,9]. In addition, these conjugates are also promising substrates to treat diseases associated with the nervous system [10]. Chemically functionalized, water-soluble CNTs can modulate the outgrowth of neuronal processes, suggesting the possibility of selectively enhancing neuritis elongation directly at the site of nerve injury, to sustain functional recovery [11–13]. Alternatively, peptide–nanotubes can be integrated into innovative microsystems or they can be delivered at the site of nerve injury to promote local tissue repair [13,14]. In this context, we have recently prepared MWCNTs functionalized with cell adhesion peptides and studied the effect of these conjugates on different types of cells, including tumor lymphoid cells and primary spleen cells and neurons [14]. The tested conjugates resulted to be highly biocompatible as they altered neither cell viability nor neuronal morphology or basic cell functions. Therefore, they can be considered as promising candidates for the exploitation of novel drug delivery systems or for designing new generation of self-assembling nerve 'bridges'. Following these *in vitro* studies [14], we now report some preliminary experiments with mice, which aimed at assessing the immunogenic properties of cell adhesion peptides when conjugated to nanotubes. Indeed, it is of fundamental importance to verify their immune neutrality when considering the potential implantation of such conjugates *in vivo* as nerve regeneration devices.

Materials and Methods

Materials

Purified MWCNTs were purchased from Nanostructured & Amorphous Materials Inc. (Houston, USA). Regular MWCNTs used in this study were 95% pure, stock no. 1240XH. Outer average diameter was between 20 and 30 nm, and the length was between 0.5 and 2 μm before oxidative treatment. MWCNTs **1** and **2** (Figure 1) were functionalized following the protocol described in Ref. 14. Transmission electronic microscopy (TEM) characterization (Figure 2) showed that the tubes have a length between

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§ *This article is dedicated to the 70th birthday of Professor Claudio Toniolo.*

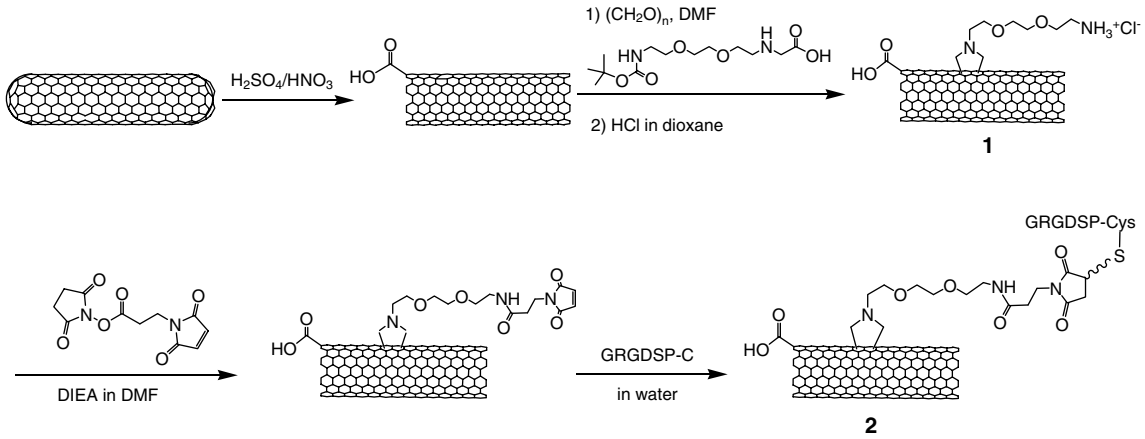

Figure 1. Synthesis of functionalized MWCNTs **1** and **2,** which are used for mouse immunization. Experimental details are reported in Ref. 14.

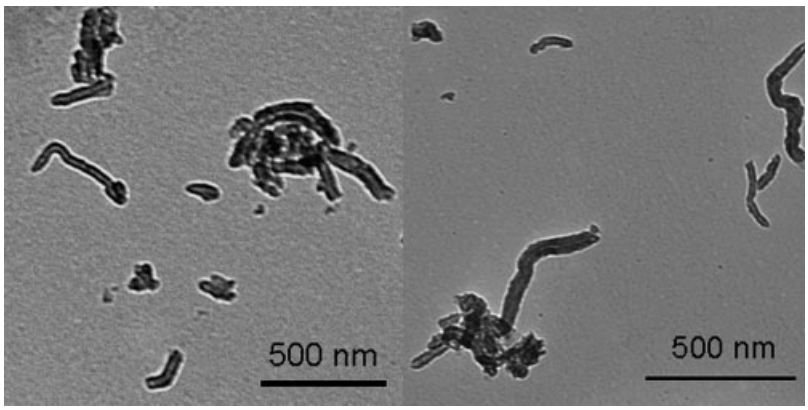

Figure 2. TEM images of MWCNTs **1** (left) and **2** (right).

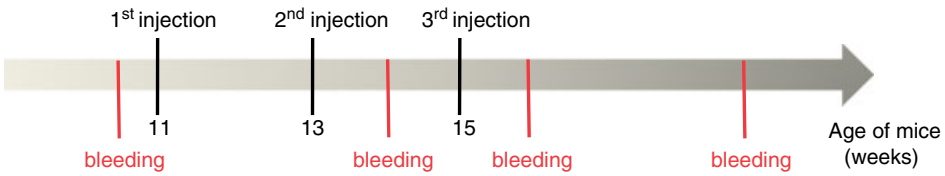

Figure 3. Mouse injection and bleeding protocol. This figure is available in colour online at wileyonlinelibrary.com/journal/jpepsci.

50 and 500 nm. The degree of functionalization was determined by the Kaiser test. The purified nanotubes conjugated with the peptide were characterized by thermogravimetric analysis (TGA) and TEM.

Peptide Synthesis and Coupling to Carrier Proteins

GRGDSPC peptide was prepared on a solid support as reported in Ref. 14. The obtained peptide was then conjugated to keyhole limpet hemocyanin (KLH) and to bovine serum albumin (BSA) for injection and coating purposes, respectively. Coupling was performed according to the manufacturer's protocol (Imject® Maleimide Activated Carrier Proteins, Pierce).

Immunization Protocol

Female BALB/c mice were purchased from Harlan and maintained in our animal facility. Mice (2–4/group) received three intraperitoneal injections: the first one when they were 11-week old and then after every two weeks (Figure 3). Each injection consisted of 200 µg of peptide–CNT conjugate (corresponding to 20 µg of peptide = 10% weight of the conjugate), or 20 µg of peptide (either free or conjugated to KLH), diluted in 0.9% NaCl and emulsified in Freund's adjuvant (Sigma; FA: complete for the first injection and incomplete for subsequent ones). Mice were bled at weeks 14, 16, and 19, and serum was collected upon clotting and centrifugation steps. A pre-bleeding of each animal was performed before the first injection and used as the control in each assay.

ELISA Procedure

Anti-peptide antibodies contained in mouse sera were detected by an enzyme linked immunosorbent assay (ELISA). Shortly, polyvinyl 96-well plates (Falcon) were coated overnight with BSA–peptide conjugate (2.5 µM of peptide) in 0.05 M carbonate/bicarbonate buffer, pH 9.6, at 37 °C (100 µl/well). Plates were blocked with 0.9% (w/v) BSA in phosphate-buffered saline (PBS) containing

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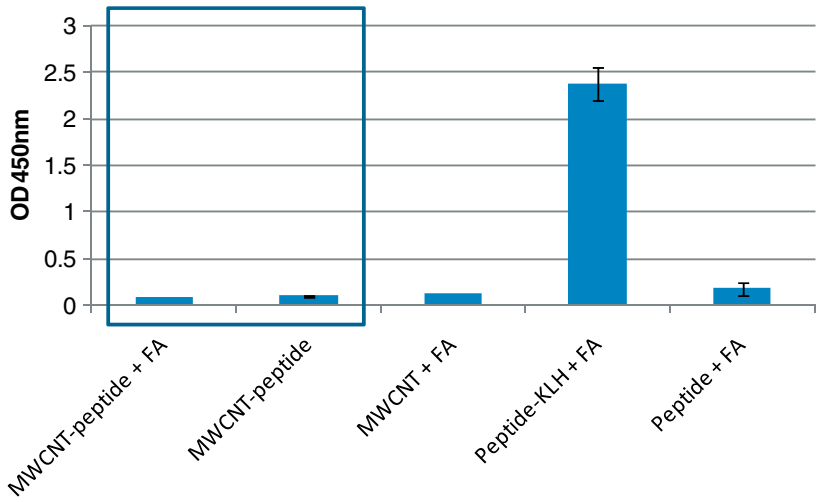

Figure 4. Anti-peptide antibody responses generated in mice following injection of MWCNTs, peptide, or peptide–MWCNT conjugate, in the presence or absence of Freund's adjuvant (FA). Data shown (OD measured at 450 nm) are derived from one representative ELISA test performed with the third bleeding. Bars represent the mean OD +/− SD of the values obtained for mice of each group ($n = 2–4$). This figure is available in colour online at wileyonlinelibrary.com/journal/jpepsci.

0.05% Tween 20 (PBS-T) at 37 °C, for 1 h (200 µl/well). Mouse sera (dilution 1/500 in PBS-T–BSA) were then added (100 µl/well, 1 h, 37 °C), followed by goat anti-mouse IgG (1 : 20 000; Jackson ImmunoResearch) supplemented with goat anti-mouse IgG_3 (1 : 7500; Nordic Immunology), both conjugated to horse radish peroxidase (100 µl/well, 30 min, 37 °C). After thorough washings, the enzymatic activity was assessed by adding 75 µl/well of a substrate solution containing H_2O_2 and 3,3′,5,5′-tetramethyl benzidine as chromogen. Fifteen minutes later, the reaction was stopped with the addition of 25 µl/well of 1 N HCl and the absorbance was measured at 450 nm.

Results and Discussion

To assess the immunogenic properties of cell adhesion peptide–nanotubes, we decided to functionalize MWCNTs with the sequence corresponding to Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP). This peptide 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 [15–17]. Coating surfaces with RGD-based sequence promotes both cell adhesion and neurite outgrowth [18]. In addition, RGD-containing peptides intervene in the mechanism of integrin regulation of neuronal gene expression [19]. Initially, the MWCNTs were treated using strong acids as previously reported (Figure 1) [20]. This procedure affords short nanotubes. This is an important issue as concerns over the toxicity of long nonfunctionalized nanotubes have been reported [21,22]. The tubes were subsequently modified by the 1,3-dipolar cycloaddition reaction of azomethine ylides to afford MWCNT **1** [23,24]. The amount of amino groups was calculated using the quantitative Kaiser test and corresponded to 150 µmol per gram of conjugate. The amino groups of MWCNT **1** have been linked to a maleimido function, which is necessary to couple the peptide via a selective chemical ligation.

For our purpose, a C-terminal cysteine residue was added to GRGDSP. The peptide has been prepared in turn using a solid-phase peptide synthesis approach. The coupling between the peptides and the tubes was followed by HPLC as previously reported [14]. The coupling reaction was completed within 2 h. The traces of peptide, due to the slight excess used, were eliminated by dialysis. The final conjugate MWCNT **2** has been recovered as a black fluffy powder after lyophilization and characterized using TGA and TEM (Figure 2).

The obtained conjugates were intraperitoneally injected into BALB/c mice according to the protocol described in Figure 3. Two control groups were immunized either with the peptide alone (short peptide, negative control) or with the peptide conjugated to KLH as a carrier protein (positive control).

ELISA tests, using the GRGDSP(C)–BSA conjugate as the solid-phase antigen, were then performed in order to analyze whether anti-peptide antibodies were produced by the animals and detected in the collected sera. Of note, the GRGDSP(C) peptide has to be conjugated to BSA for coating, as the peptide by itself is too small to efficiently adhere to plastic. Upon one single injection, no anti-peptide antibody was detected in any group (first bleeding, data not shown). On the contrary, upon two and three injections (second bleeding, data not shown; data for third bleeding is shown in Figure 4), one group of mice, namely, the group that received injections of the peptide coupled with KLH in presence of adjuvant, developed a strong IgG antibody response toward the GRGDSP(C) peptide. This was expected as it was our positive control, the uncoupled peptide being too small to be immunogenic. Most interestingly, animals that were injected with peptide–MWCNT conjugates did not produce detectable levels of anti-peptide antibodies, even when administered together with adjuvant.

The latter result is extremely important as immune neutrality is a critical parameter to envisage *in vivo* implantation of such carbon nanotube-based devices in the future. It should be mentioned that the observed lack of immunogenicity of the peptide–MWCNT conjugate described in this study does not question the exciting possibility of using CNTs as a platform for vaccine delivery. It is also not contradictory to the data previously published by our group and others, which showed immunogenicity using long peptides and/or co-injection of proteins, which provide bystander help [8,25,26]. The GRGDSP(C) peptide is, on the contrary, very short, and although it obviously contains a B cell epitope (since it is able to induce specific antibodies when coupled to the KLH carrier protein), it very likely does not contain any T cell epitope, therefore

leading to the absence of T cell help, which is required for antibody production by B cells. Moreover, contrary to the carrier proteins such as KLH, CNTs are known as *non-immunogenic* and do not harbor intrinsic 'helper' properties [8], which is likely to explain the lack of antibody response to our CNT-based conjugates.

Conclusions

With the aim of using CNTs as a functional matrix for neuron outgrowth and stimulation, we have developed MWCNTs conjugated to an integrin-derived peptide, which favors cell adhesion. The potential *in vivo* implantation of such nanodevices implies that they should not be 'visible' to the immune system, in order to avoid any reject and associated immune responses. In that sense, the results we have presented here are very encouraging as the GRGDSP(C) – MWCNT conjugates do not elicit specific antibodies in mice, even in the presence of a strong adjuvant, which mimics bacterial infection. This demonstration of their immune neutrality reinforces the interesting potentiality of such conjugates as substrates for neuronal regeneration *in vivo*.

Acknowledgements

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