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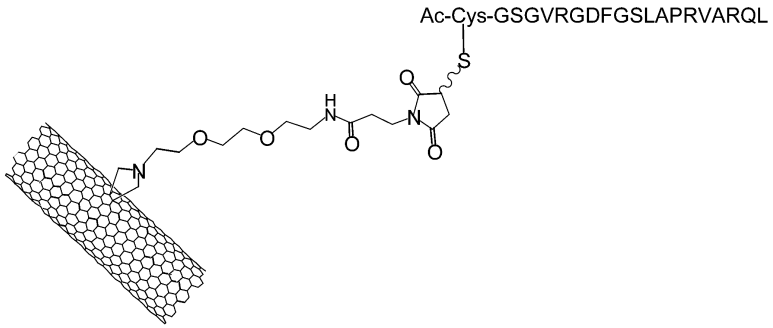

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Synthesis, Structural Characterization, and Immunological Properties of Carbon Nanotubes Functionalized with Peptides

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Abstract: Carbon nanotubes (NTs) are becoming highly attractive molecules for applications in medicinal chemistry. The main problem of insolubility in aqueous media has been solved by developing a synthetic protocol that allows highly water-soluble carbon NTs to be obtained. As a result, biologically active peptides can be easily linked through a stable covalent bond to carbon NTs. We have demonstrated that a bound peptide from the foot-and-mouth disease virus, corresponding to the 141−159 region of the viral envelope protein VP1, retained the structural integrity and was recognized by monoclonal and polyclonal antibodies. In addition, this peptide−NT conjugate is immunogenic, eliciting antibody responses of the right specificity. Such a system could be greatly advantageous for diagnostic purposes and could find future applications in vaccine delivery.

Introduction

The potential applications of carbon nanotubes (NTs) in medicinal chemistry are of great interest, given their capacity to interact with macromolecules such as proteins and oligosaccharides.[1−3] The main difficulty in regard to the integration of such materials into biological systems is derived from their lack of solubility in physiological solutions. Different methods have been proposed to address the problem of carbon NT solubility. Covalent modification by the organic functionalization of end-groups and side walls of carbon NTs allows for a dramatic increase of the solubility of NTs in a range of solvents,[4,5] including water.[6−11] Starch[3] or oligomers, such as poly(vinylpyrrolidone),[6] are able to wrap and transport single-walled nanotubes (SWNTs) into aqueous buffers and create water-soluble nano-hybrids. Oxidized SWNTs can be functionalized at their carboxylic groups with poly(ethylene) oxide derivatives,[7] sugar moieties,[8] oligonucleotides,[9a−d] peptide nucleic acids,[9e] or proteins.[10] We have recently obtained a highly efficient method for solubilizing NTs, based on chemical modification of the side wall through 1,3-dipolar cycloaddition of azomethine ylides.[12,13] By carefully choosing the reactive intermediates, it is possible to obtain highly soluble, derivatized NTs ready for further modifications. SWNTs functionalized with N-protected amino acids can therefore be easily prepared.[13] Here, we report the synthesis and characterization of the first covalently linked peptide−carbon NTs and present evidence of their potential applications in immunology.

Results and Discussion

Peptide−Carbon Nanotube Synthesis. In our studies, we employed two different methods of linking bioactive peptides to SWNTs through a stable bond: (i) the fragment condensation of fully protected peptides[14] and (ii) selective chemical ligation.[15]

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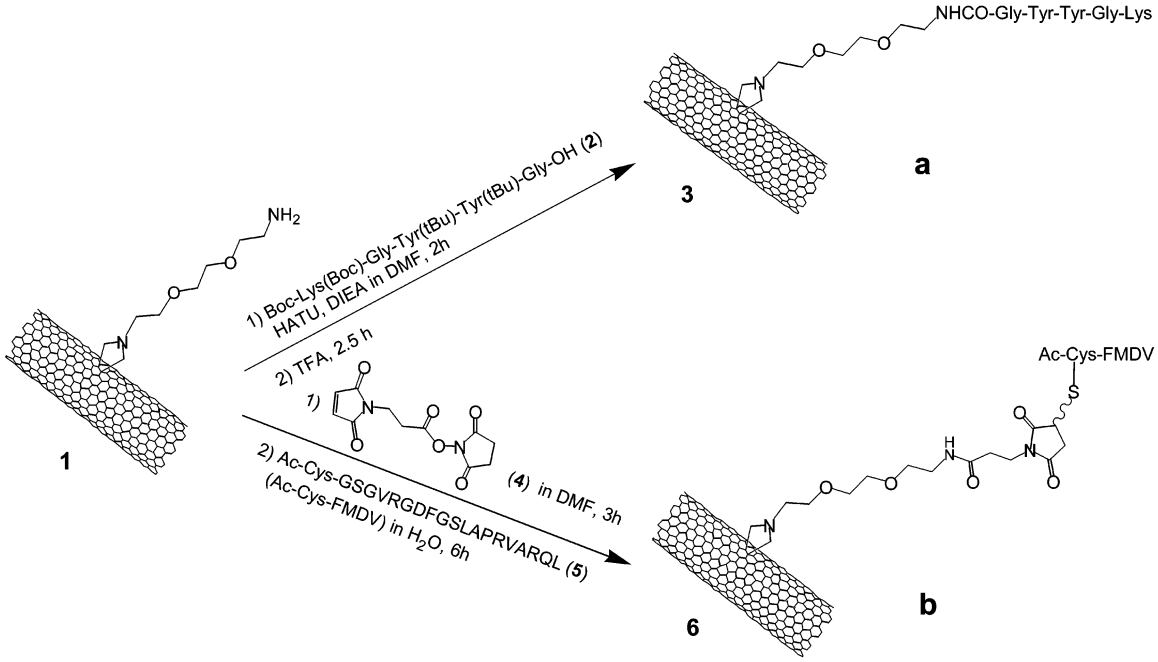
Scheme 1. Synthesis of the Peptide−Carbon Nanotubes via (a) Peptide Fragment Condensation and (b) Chemoselective Ligation



The SWNTs were functionalized as previously described to obtain wires (**1**) with free amino groups uniformly distributed around their side walls.[13] The amount of functional groups per gram of material (loading), measured with a quantitative Kaiser test,[16] was ∼0.30−0.50 mmol/g. For the fragment condensation approach, we have chosen the *N*-terminal and side-chain protected model pentapeptide **2** corresponding to the sequence KGYYG (Scheme 1a). This peptide, which has been prepared by solid-phase synthesis,[14] was coupled to the SWNTs in a threefold excess upon activation with *O*-(7-aza-*N*-hydroxybenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) and diisopropylethylamine (DIEA) in dimethylformamide (DMF) for 2 h. After evaporation of the solvent, peptide-derivatized carbon NT **3** was isolated by repeated precipitation from a methanol/diethyl ether mixture. The protecting groups were subsequently removed by treating the conjugate with trifluoroacetic acid (TFA).

For peptide conjugation onto carbon NTs using chemoselective ligation, we have chosen a B-cell epitope from the foot-and-mouth disease virus (FMDV), corresponding to the 141−159 region of the viral envelope protein VP1 (Scheme 1b).[17] This peptide was shown to elicit neutralizing antibody responses that are sufficient to afford complete protection of cattle against the disease, thus strengthening the possibility of designing peptide-based vaccines.[18] The free amino groups of SWNTs **1** were first derivatized using a fivefold excess of *N*-succinimidyl 3-maleimidopropionate[15c] (**4**) in DMF. After the solution was stirred for 3 h, the excess reagent was eliminated by the addition of amino-PEGA resin[19] as a scavenger, to block the remaining carboxylic active ester. The *N*-terminal acetylated FMDV peptide **5**, bearing a cysteine at position 1, was dissolved in water and linked to the maleimido moiety of the wires in twofold excess, to obtain the peptide−SWNT **6** after stirring for 6 h. Unreacted peptide **5** was eliminated using a scavenger resin. For this purpose, we recycled the previous PEGA resin that was derivatized with the maleimido function.

The formation of the peptide covalent bond to the SWNTs was followed by RP-HPLC (see Supporting Information). Because full-length carbon NTs are not able to pass through a C_{18} chromatographic column—they remain trapped on the precolumn system—we followed the course of the chemical ligation by observing the decrease of peptide **5** concentration in the reaction mixture. After the coupling reaction, a remarkable decrease in the intensity of peptide absorbance was detected. The signal disappeared completely after treating the solution with the scavenger resin. We verified the effectiveness of the purification method using scavenger resins by mixing the SWNTs **1** and peptide **5** in water and subsequently adding a maleimido-PEGA resin to monitor the complete removal of peptide from the solution. We did not observe any decrease of peptide concentration after the addition of the SWNTs, as a result of simple aggregation of peptide around the wires.

Structural Characterization. Both peptide−carbon NTs **3** and **6** were examined by transmission electron microscopy (TEM). Figure 1 shows the presence of peptide-based SWNTs, upon evaporation of the solvent. Bundles with different diameters, in the range of 8−53 nm, can be clearly seen.

A more detailed structural characterization of both peptide−SWNT conjugates was performed by NMR spectroscopy (see Supporting Information). The SWNTs functionalized with KGYYG were studied either with the fully protected peptide

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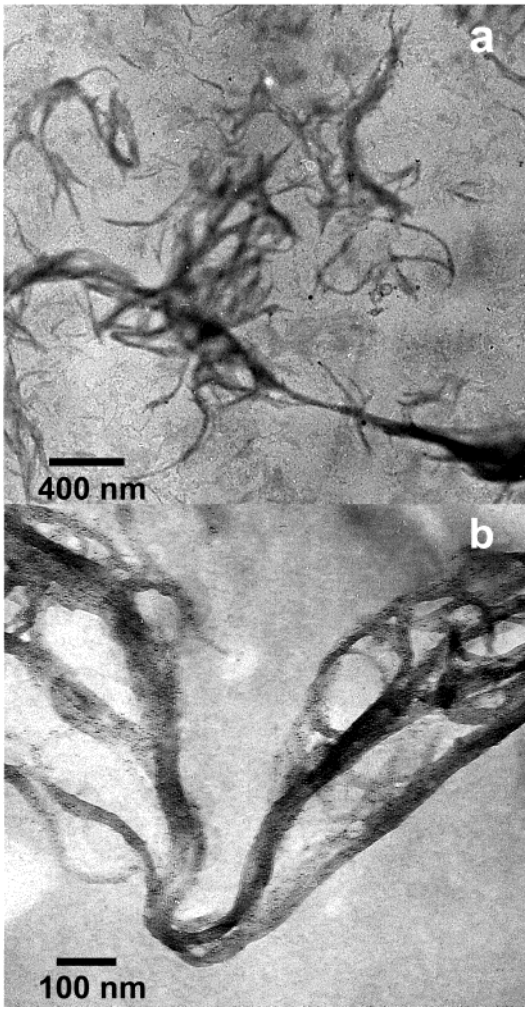

Figure 1. TEM images of peptide−carbon nanotubes (a) **3** and (b) **6**.

or with the N-terminus and side-chain free peptide in CD_3CN and H_2O/*t*-BuOH-d_9 solution, respectively. Because of the presence of a N^{15}-labeled Gly at the C-terminal part, homonuclear and heteronuclear two-dimensional (2D) NMR spectra were recorded. A broad correlation peak in the decoupled $^{15}N-^{1}H$ spectrum of the fully protected compound **3**, with the maximum peak height measured at 119.6/7.40 ppm (Figure S4),[20] was indicative of a homogeneous distribution of peptide around the NT side wall. A series of bi-dimensional experiments then permitted all the resonances of the peptide moiety to be assigned. A decrease and a broadening of the signal intensities were observed for the amino acid residues approaching the aromatic tube walls. In the case of deprotected peptide−carbon NT **3**, we attributed all the residue signals in an aqueous solution (Figure 2a).

All the expected sequential αH_i-NH_{i+1} cross peaks were present in the ROESY spectrum; however, most importantly, a spatial correlation between the αH of glycine at position 5 and the amide proton of the oligoethylene glycol chain confirmed the covalent bond between the peptide and the carbon NTs (Figure 2b). In the case of peptide−NT conjugate **6**, we acquired a series of TOCSY, NOESY, and ROESY spectra in water, which allowed the 20 amino acid residues to be fully assigned

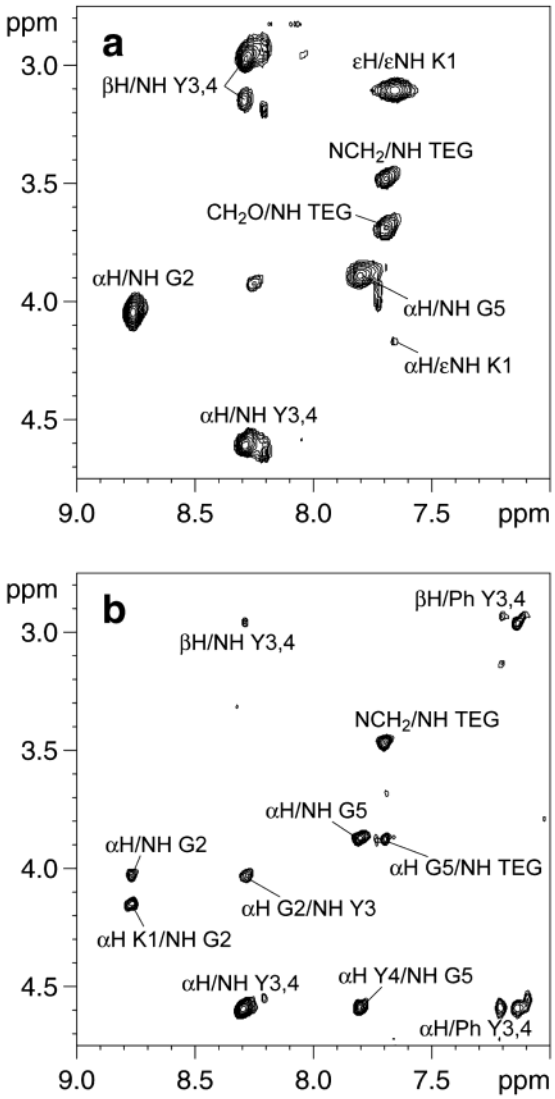

Figure 2. Partial (a) TOCSY and (b) ROESY ^{1}H NMR spectra of peptide−NT **3** in H_2O/*t*-BuOH-d_9 (9:1) solution. Peptide residues are numbered from Lys1 to Gly5. TEG denotes triethylene glycol. TOCSY spectrum has been recorded while decoupling ^{15}N heteronucleus.

(Figure 3). The chemical-shift dispersion, and the intensity and position of the NOE, are very similar (except for some residues at both sequence termini) to those of the same peptide previously studied free in aqueous solution,[21] or bound to, POEPOP resin.[22] This suggests that the peptide, when linked to the carbon NTs, displays the same conformational behavior. We finally characterized the peptide−NTs **3** and **6** by amino acid analysis, and the calculated peptide loading was in accordance with the quantitative Kaiser test.

Immunological Studies. The immunological reactivity of the FMDV peptide coupled to NTs (**6**), with the specific monoclonal antibody (mAb) 21 × 27, was assessed using surface plasmon resonance technology on a Biacore3000 instrument. This device measures the increase in mass on a coated gold film when interaction occurs between an immobilized ligand and an analyte

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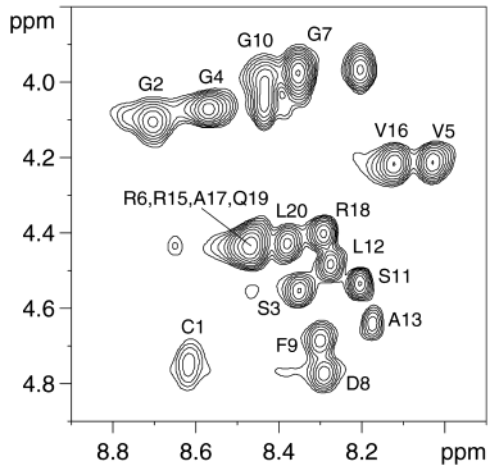

Figure 3. Partial TOCSY ^{1}H NMR spectra of peptide−NT **6** in H_2O/*t*-BuOH-d_9 (9:1) solution. Peptide residues are numbered from Cys1 to Leu20.

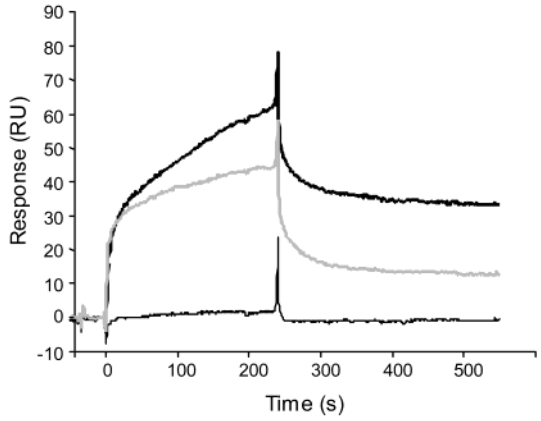

Figure 4. Sensorgrams obtained by allowing the analytes to react on a monoclonal antipeptide antibody. The association phase required 4 min, and the dissociation phase required 5 min. The response with the peptide−NT conjugate **6** (6 μM) is shown as the bold black line, the response with free FMDV peptide **5** (5 μM) is shown as the bold gray line, and the response of the acetylated NT **1** used at the same concentration as the peptide−NT **6** is shown as the thinner black line. RU value corresponds to the resonance unit (1000 RU = 1 ng/mm^2 of analyte).

in constant flow over the surface. Prior to injecting the solution of free or NT-conjugated peptide (**5** or **6**, respectively), the specific mAb was immobilized on a chip.[23] As shown in Figure 4, the antibody recognized the FMDV peptide that was covalently linked to the NT in a manner similar to that of the free peptide. The slower association rate and higher response in resonance units are due to the increase in molecular weight of the peptide−NT complex, compared to that of the free peptide. This is because the increase in response is directly correlated to the mass of the recognized molecule.

In addition, an enzyme-linked immunosorbent assay (ELISA) was performed to compare the recognition of NT-conjugated or free FMDV peptide directly coated onto plastic wells by a polyclonal mouse anti-FMDV peptide serum or the mAb 21 × 27. A horseradish peroxidase-conjugate goat anti-mouse IgG was used to reveal the antibody−antigen interaction.[24] Both solid-phase antigens **5** and **6** were recognized equally well by the polyclonal and monoclonal antibodies (see Supporting Information). This is in agreement with the Biacore results and strongly suggests that the secondary structure of the NT-linked peptide, which is necessary for the spatial interaction with specific antibodies, is properly presented by the carbon wires. Carbon NT-displayed peptides have several advantages for use in diagnostics: (1) their efficient binding on ELISA plates overcomes potential problems that may be encountered with the direct coating of peptides onto a solid support, because of their physicochemical properties; (2) they present peptides in a more accessible way for antibody recognition, in comparison to peptides coated directly onto the ELISA plate; and, finally, (3) they have the potential to present different epitopes, thus allowing higher diagnostic accuracy. Increasing the amount or accessibility of peptides on an ELISA plate can also be obtained by chemical immobilization (using, for example, glutaraldehyde), by coupling the peptide to a carrier protein or by using streptavidin-coated plates to immobilize biotinylated peptides.[25] Covalent linking can result in modification of amino acid residues that are important for epitope recognition, whereas the use of carrier proteins or streptavidin, which are antigenic molecules, can result in an increase of background signal. The use of the antigenically inert carbon NTs overcomes these potential drawbacks.

Finally, peptide−NT conjugates can be exploited for immunogenicity studies. In preliminary experiments, the FMDV peptide−NT **6** elicited strong antipeptide antibody responses after intraperitoneal immunization of mice (data not shown). This emphasizes the effectiveness of carbon NT supports to present peptides to the immune system.

Conclusion

We have applied two different strategies for the preparation of peptide−carbon nanotubes (NTs), on the basis of the fragment condensation and the selective chemical ligation. A model pentapeptide and an antigenic epitope from the foot-and-mouth disease virus (FMDV) were covalently linked to single-walled nanotubes (SWNTs). A detailed structural characterization has been performed, using transmission electron microscopy and 2D NMR spectroscopy. The antigenicity of the FMDV peptide−carbon NT conjugate was then proved by a surface plasmon resonance analysis and an enzyme-linked immunosorbent assay (ELISA) test. The results from both experiments suggested that the peptide bound to the NT support adopts the correct secondary conformation necessary for recognition by specific antibodies. Moreover, a study in vivo has shown that the FMDV peptide−NT is also immunogenic. Our findings highlight the potential use of peptide−carbon NT conjugates for diagnostic purposes and pave the way for their application in vaccine delivery. Finally, functionalized carbon NTs can be considered ideal scaffolds for the multivalent presentation of molecules to modulate ligand−receptor interactions.

Experimental Section

General. Single-walled nanotubes (SWNTs) were purchased from Carbon Nanotechnologies, Inc. (Houston, TX). All reagents and solvents were obtained from commercial suppliers and used without further purification. Wang, 2-chloro-trityl, and PEGA resins were purchased

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from Neosystem (Strasbourg, France), Seen Chemicals (Dielsdorf, Switzerland), and Novabiochem (Läufelfingen, Switzerland), respectively. Matrix-assisted laser desorption ionization−time-of-flight (MALDI−TOF) mass analysis was performed on a linear MALDI−TOF instrument (Bruker), using α-cyano-4-hydroxycinnamic acid as the matrix. Amino acid analysis was performed on an Applied Biosystem model 130A separation system coupled to an Applied Biosystem model 420A derivatizer.

Abbreviations. Symbols and abbreviations for amino acids and peptides are in accordance with the recommendations of the IUPAC−IUB Commission on Nomenclature (*J. Biol. Chem.* **1972**, *247*, 977).

Peptide Synthesis. Peptide KGYYG (**2**) was prepared manually on a 2-chloro-trityl resin, using the standard Fmoc/*t*-Bu solid-phase procedure.[14] The fully protected peptide was cleaved from the resin using 40% 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) in dichloromethane (DCM) and characterized by RP-HPLC chromatography and MALDI−TOF mass spectrometry. The sequence of the peptide **5**, corresponding to the VP1 region 141−159 of FMDV (variant USA), used in this study is [141]GSGVRGDFGSLAPRVARQL.[159] An additional Cys residue was added to the N-terminal part of the peptide for the conjugation to the SWNTs. The N-terminus of the peptide was acetylated. The synthesis of the peptide was performed on the Wang resin, using a multichannel peptide synthesizer working on standard Fmoc/*t*-Bu chemistry.[26]

Synthesis of Peptide−Nanotube 3. Carbon NT (**1**) (10 mg, 4 μmol, based on the loading calculated with the quantitative Kaiser test[16]) suspended in 2 mL of dimethylformamide (DMF) was neutralized with diisopropylethylamine (DIEA) (80 μL, 46 μmol). A solution of **2** (61.3 mg, 6.7 μmol) in 2 mL of DMF was activated with *O*-(7-aza-*N*-hydroxybenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) (29.7 mg, 7.8 μmol) for 10 min and subsequently added to carbon NTs **1**. The mixture was stirred for 2 h. The solvent was evaporated, and the product was precipitated from methanol/diethyl ether and dried under vacuum. The fully protected peptide−carbon NT conjugate was solubilized in 500 μL of methanol, and 1 mL of a 4 M HCl solution in dioxane was added. After the solution was stirred for 1 h, the product was obtained by precipitation in cold diethyl ether. Yield: 99% (10 mg).

Synthesis of Peptide−Nanotube 6. Carbon NT (**1**) (7.0 mg, 2.8 μmol, based on the loading calculated with the quantitative Kaiser test[16]) suspended in 2 mL of DMF was neutralized with DIEA (15 μL, 8.5 μmol). *N*-Succinimidyl-3-maleimidopropionate (**4**) (12 mg, 45 μmol) dissolved in 2 mL of DMF was added, and the reaction was stirred for 6 h at room temperature. The excess of **4** was eliminated overnight, by adding 50 mg of PEGA-NH_2 resin. The resin was eliminated by filtration, and the solvent was evaporated. The product was dissolved in methanol and precipitated several times with cold diethyl ether. Ac-Cys-GSGVRGDFGSLAPRVARQL 5 (4.0 mg, 1.91 μmol) was added to a solution of the carbon NTs functionalized with the succinimidyl group (4.0 mg, 1.6 μmol) in 1.5 mL of water. The reaction was stirred for 6 h at room temperature, and 70 mg of PEGA-NH_2 resin previously derivatized with **4** were added to eliminate the excess of peptide **5** overnight. The resin was eliminated by filtration and the solvent was lyophylized, affording compound **6**. Yield: 79% (5.13 mg).

Antibodies. mAb 21 × 27 and polyclonal antibodies have been generated after injecting mice with the conjugate FMDV 147−156 and 141−159 peptide, respectively (manuscript in preparation).

ELISA Test. Polyvinyl (Falcon) or Maxisorp microtiter plates were coated with the peptides and peptide−carbon NT conjugates in a carbonate/bicarbonate buffer at pH = 9.6 overnight at 4 °C. After the plates were washed with PBS containing 0.05% Tween (v/v) (PBS-T), they were blocked with 1% BSA in PBS-T for 2 h at 37 °C. Serial twofold dilutions of serum samples in PBS-T containing 0.3% BSA were made across the plate, and the plates were incubated for 1 h at 37 °C. After the plates were washed, 50 μL of horseradish peroxidase-conjugated goat anti-mouse IgG (1/20000 in PBS-T) Fc-specific (Jackson Immunoresearch, West Grove, PA) were added in each well and plates were incubated at 37 °C for 1 h. Unbound conjugate was removed by washing with PBS-T, and the enzymatic reaction was determined as previously described.[24] Absorbance was measured at 450 nm.

Biacore Measurements. Rabbit anti-mouse Fcγ IgG (Biacore, Uppsala, Sweden) was immobilized on a CM5 carboxylated, dextran-coated chip by the standard amino-coupling procedure recommended by Biacore. Supernatants of hybridoma cultures secreting the 21 × 27 anti-FMDV peptide antibody and a control monoclonal antibody of the same isotype (IgG2a) were allowed to adsorb for 5 min at a flow rate of 5 μL/min, to prepare the experimental channel and the control channel on the chip, respectively. The adsorption step was followed by the injection of the analytes [solvent, control NT, peptide−carbon NT, and peptide in HBS (NaCl 150 mM, Hepes 10 mM pH = 7.4, NP20 at 0.005%)] at a flow rate of 30 μL/min for 4 min, followed by a dissociation phase of 5 min. The anti-mouse Fcγ ligand was regenerated by a 10 mM HCl solution passing over the two channels for 30 s. The results were corrected by subtracting from the experimental sensorgram the sensorgram obtained with the control antibody, to take into account nonspecific interactions, and by subtracting the experimental sensorgram obtained with the solvent, to take into account the differential dissociation rate of the two monoclonal antibodies from the anti-mouse Fcγ IgG.

NMR Spectroscopy. The identification of amino acid spin systems and sequential assignment were made using a combination of TOCSY (using MLEV-17 or DIPSI2[27] spin-lock sequences), NOESY, ROESY,[28] and HMQC experiments (see Supporting Information for acquisition and processing parameters). One- and two-dimensional (1D and 2D, respectively) NMR spectra were recorded on a Bruker model ARX 500 MHz spectrometer. The sample was dissolved in CD_3CN or H_2O/*t*-BuOH-d_9 (9:1). The spectra were acquired at a temperature of 300 K and referenced to the peak of the solvent. WATERGATE pulse sequence was applied for the suppression of the water signal.[29]

Transmission Electron Microscopy. Peptide−NT **3** was suspended in diethyl ether, deposited on the grid (Formvar support film on copper 400 mesh-grid), and, after evaporation of the solvent, analyzed on a Hitachi model 600 HS TEM microscope at 110 kV. In contrast, peptide−NT **6** was solubilized in methanol and analyzed as described for **3**. In both cases, a 3% uranyl acetate−water solution was added on the grid after the sample, to increase the contrast of the image, followed by washings.

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Supporting Information Available: RP-HPLC, 2D NMR including details on acquisition and processing parameters, and ELISA for peptide−carbon nanotubes **3** and **6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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