Monitoring the functionalization of single-walled carbon nanotubes with chitosan and folic acid by two-dimensional diffusion-ordered nmr spectroscopy

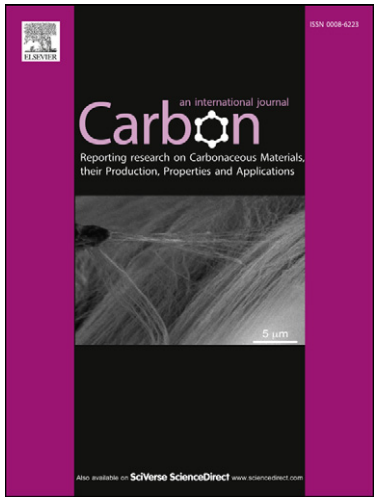
Castillo, John J.; Torres, Mary H.; Molina, Daniel R.; Castillo, Jaime; Svendsen, Winnie Edith; Escobar, Patricia; Martínez O, Fernando

Published in:
Carbon

Link to article, DOI:
10.1016/j.carbon.2012.02.010

Publication date:
2012

Accepted Manuscript



Monitoring the functionalization of single-walled carbon nanotubes with chitosan and folic acid by two-dimensional diffusion-ordered nmr spectroscopy

John J. Castillo, Mary H. Torres, Daniel R. Molina, Jaime Castillo-León, Winnie E. Svendsen, Patricia Escobar, Fernando Martínez O.

PII: S0008-6223(12)00131-5
DOI: 10.1016/j.carbon.2012.02.010
Reference: CARBON 7097

To appear in: *Carbon*

Received Date: 23 October 2011
Accepted Date: 2 February 2012

Please cite this article as: Castillo, J.J., Torres, M.H., Molina, D.R., Castillo-León, J., Svendsen, W.E., Escobar, P., Martínez O., F., Monitoring the functionalization of single-walled carbon nanotubes with chitosan and folic acid by two-dimensional diffusion-ordered nmr spectroscopy, *Carbon* (2012), doi: 10.1016/j.carbon.2012.02.010

Monitoring the functionalization of single-walled carbon nanotubes with chitosan and folic acid by two-dimensional diffusion-ordered nmr spectroscopy

John J. Castillo[1], Mary H. Torres[2], Daniel R. Molina [2], Jaime Castillo-León[3], Winnie E. Svendsen[3], Patricia Escobar[4], Fernando Martínez O.[1]*

[1]Centro de Investigaciones en Catálisis, Universidad Industrial de Santander, Sede Guatiguará UIS Km 2 vía Refugio, Piedecuesta, Colombia

[2]Laboratorio de Resonancia Magnética Nuclear, Universidad Industrial de Santander, Sede Guatiguará UIS Km 2 vía Refugio, Piedecuesta, Colombia

[3] Department of Micro and Nanotechnology, Technical University of Denmark, Lyngby, Denmark

[4] Centro de Investigaciones en Enfermedades Tropicales, Universidad Industrial de Santander, Sede Guatiguará UIS Km 2 vía Refugio, Piedecuesta, Colombia

ABSTRACT

A conjugate between single-walled carbon nanotubes, chitosan and folic acid has been prepared. It was characterized by diffusion ordered two-dimensional hydrogen-1 nuclear magnetic resonance and hydrogen-1 nuclear magnetic resonance spectroscopy which revealed the presence of a conjugate that was generated by the linkage between the carboxyl moiety of the folic acid and the amino group of the chitosan, which in turn was non-covalently bound to the single-walled carbon nanotubes. The obtained diffusion coefficient values demonstrated that free folic acid diffused more rapidly than the folic acid conjugated to single-walled carbon nanotubes-chitosan. The values of the proton

signal of hydrogen-1 nuclear magnetic resonance spectroscopy and two-dimensional hydrogen-1 nuclear magnetic resonance spectroscopy further confirmed that the folic acid was conjugated to the chitosan, wrapping the single-walled carbon nanotubes.

*Corresponding Author: Email: jojacale@gmail.com (J.J. Castillo)

1. Introduction

Over the past decade, the interest for using carbon nanotubes (CNTs) functionalized with different types of biomolecules has increased [1-3]. The nanometer size and unique physical properties of CNTs make them attractive for anticancer drug delivery [4-8], molecular transport [9-11], and new therapeutic mechanisms [12, 13]. CNTs have been activated to target various cancer cells using folic acid (FA). FA is an attractive ligand that is useful for targeting cell membranes and enhancing CNTs endocytosis by the folate receptor [14, 15]. FA receptors can be direct targets for drug delivery [16], which explains the diversity of strategies used for folate conjugation.

Conventionally, CNTs-FA has been characterized by UV-Vis (ultraviolet-visible spectroscopy), NIR spectroscopy (near-infrared) [17], AFM (atomic force microscopy) and TEM (transmission electronic microscopy) [9] but not with 2D NMR (two-dimensional nuclear magnetic resonance) experiments. The 2D NMR techniques have become popular since they efficiently map out 3D interactions within, or sometimes between, molecules [18]. The diffusion ordered spectroscopy (DOSY) technique is a non-invasive powerful technology that has been used for the analysis of a large variety of mixtures [19], as well as for the characterization of aggregates of varying sizes and hence different diffusion coefficients (DC) [20].

The DOSY technique has been referred to as “NMR chromatography” for its ability to “separate” the components of a complex mixture. In a recent application, Marega et al [21] used ^{1}H-2D DOSY spectroscopy to monitor the functionalization and purification of a carbon nanotube-polyethylene glycol (CNT-PEG) conjugate. There have been numerous publications on the characterization of various bioconjugates using

2D NMR techniques [22, 23]. However, studies related to the characterization of CNT-FA conjugates using 2D NMR spectroscopy have yet to be published.

The aim of this study was to characterize a conjugate formed by single-walled carbon nanotubes (SWCNTs), chitosan and FA by means of the DOSY 2D-NMR technique ^{1}H NMR spectroscopy. The values obtained for the diffusion coefficient confirmed the conjugation of FA to SWCNTs wrapped by the chitosan. The synthesis and utilization of these conjugate systems opens new possibilities for the treatment of infectious diseases and cancer cells [24].

2. Experimental

2.1. Materials

SWCNTs with diameters ranging from 2 to 5 nm and lengths between 500 and 2000 nm were purchased from Unydim, California. Chitosan with a molecular weight of 5000 Da, folic acid (FA) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were obtained from Sigma-Aldrich Corp. These and all other chemicals used in this work were of analytical grade.

2.2. Synthesis

2.2.1. Solubilization of single-walled carbon nanotubes

5 mg of SWCNTs were sonicated for 1 h in 5 mL of a 30% aqueous solution of chitosan. The solution was centrifuged at 2800 rpm for 20 min; the supernatant was collected and carefully separated from the solid. After aggregation, a large bundle of nanotubes was found to be present. The supernatant was washed with water several times to remove excess chitosan. Finally, the SWCNT solution was stored at 4°C. The

figure 1 shows the SWCNT-chitosan solution before and after the sonication and centrifugation. AFM images indicated that the majority of SWCNTs are present as separated single tubes coated with chitosan (see Supporting Information figure S1).

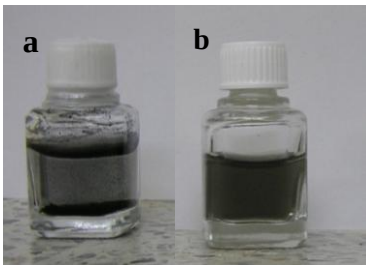


Figure 1. Single-walled carbon nanotubes-chitosan (SWCNTs) conjugates before (a) and after (b) sonication

2.2.2. Synthesis of the single-walled carbon nanotube-chitosan-folic acid conjugate

The conjugation of FA on SWCNT-chitosan was achieved using a similar methodology to that of Kang [17]. First, FA (2.5 mM) was added to a solution of SWCNT-chitosan (5 mL), to which EDC (3.5 mM) was subsequently charged. The mixture was protected from light and magnetically stirred overnight at room temperature. The solution was then dialyzed three times with PBS buffer of pH 7.0 (MCWO 10000) to ensure the complete removal of excess unconjugated chitosan, FA and EDC. In order to have an estimation of the amount of FA conjugated we made a calibration curve using UV-Vis with different solutions of FA at a wavelength of 364 nm. The absorbance value of the conjugated was used to calculate the concentration of FA (mM). The stability of the conjugated was evaluated using Zeta Potential measurements (DLS, Malvern Systems). The solution was refrigerated at 4°C awaiting NMR experiments.

2.3. Nuclear Magnetic Resonance characterization

2.3.1. Hydrogen-1 nuclear magnetic resonance spectroscopy of the single-walled carbon nanotube-chitosan-folic acid conjugate

NMR samples were prepared by dissolving 100 µL of the conjugate in 400 µL of D_2O (Merck, 99.8%). All the NMR analyses were performed on a Bruker Avance III, 400 MHz spectrometer.

2.3.2. DOSY 2D-NMR experiments of free folic acid single-walled carbon nanotube-chitosan-folic acid conjugate

2D-DOSY experiments are usually carried out by pulse field gradient spin-echo (PFGSE) decay in NMR-PFGSE-NMR- (pulse sequence ledbpgp2s, Bruker). The ledbpgp2s sequence was used for the measurements of diffusion coefficients, recording 32 scans for each gradient step, a linear gradient of 32 steps between 2% and 95%, a diffusion time (big delta) of 0.05 s, and the length of the square diffusion encoding gradient pulses (little delta) of 2.7 ms, unless otherwise stated. A total acquisition time of ca. 1 h 45 min was thus employed.

^{1}H NMR spectra (1D-DOSY) were acquired with the use of PFGSE; 4 scans, 95% gradient strength, big delta 0.05 s, and little delta 2.7 ms. All experiments were performed at 298 K.

For all spectra, the Bruker Presat program (ZGPR pulse sequence at water frequency, 4 scans and spectral widths of 10 ppm) was used for the suppression of water peaks.

The Topspin 2.1 software was used for processing all spectra.

3. Results and Discussion

The conjugate was prepared by a reaction of FA with SWCNT-chitosan in the presence of EDC (Figure 3). The EDC is the most popular carbodiimide for conjugating biological substances containing carboxylates and amines [23] and it was used as a crosslinking agent to mediate the formation of an amide between the carboxyl group of the folic acid and the amine group of the chitosan. Hydrolysis by water of the intermediate (EDC-FA) is the major competing reaction cleaving off the activated ester intermediate [26]. However we obtained the conjugate and not hydrolysis was observed (SI Figure S2 and S3). Similar results were obtained in other works [27-29]. The addition of *N*-hydroxysuccinimide (NHS) to EDC reaction is recommended to increase the stability of the intermediate and the yield of reaction [23]. The formation of a reactive intermediate, denoted O-acylisourea, allowed the reaction with a nucleophile such as a chitosan (primary amine), giving rise to an amide bond.

The absorbance values of free and conjugated FA (364 nm) were used to calculate the FA conjugated to SWCNT-chitosan (figure 2). The concentration of FA in the conjugate after dialysis was of 0.7 mM. This data indicates that approximately a 20% of FA was conjugated to SWCNT-chitosan.

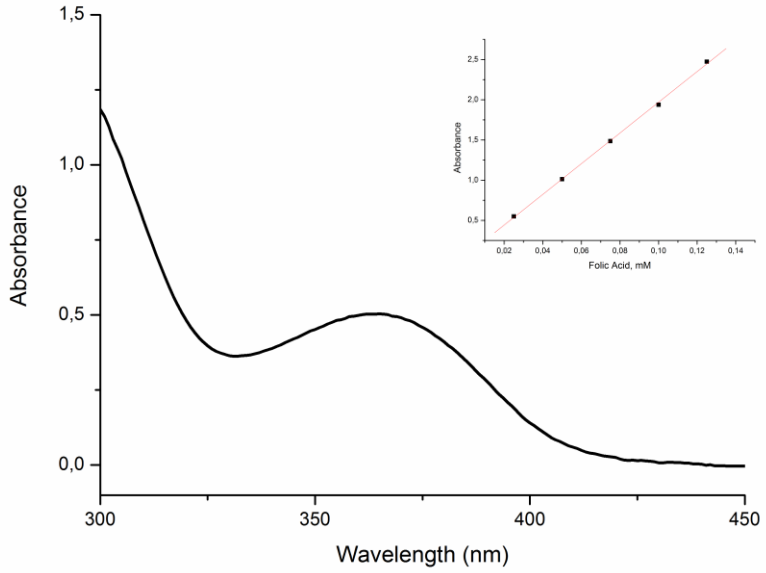


Figure 2. UV-Vis spectra of conjugated folic acid (FA), (inset) calibration curve of free FA

Zeta potential is a physical property that can be related to the stability of colloidal dispersions and indicate the degree of repulsion between adjacent, similarly charged particles in the dispersion [30]. Particles with values of zeta potential between +30 and -30 mV are considerable unstable and probably will precipitate, on the other hand particles with values more positive than +30mV and more negative than -30mV are normally considered stable. At pH value of 7.0 we obtained a zeta potential of -32.1 mW, this could be an indication that SWCNTs-chitosan-FA can form stable dispersion at physiological pH.

The ^{1}H NMR spectra of the EDC, free FA, chitosan, and conjugated FA are shown in the figures 4a-4d. ^{1}H NMR analysis confirmed the presence of the SWCNT-chitosan-FA conjugate (Figure 4d). It is apparent that the spectrum of the conjugate contains signals originating from chitosan and FA. The signals at 1.68, 2.64 and 2.95-3.6 ppm seen in the figure 4d were assigned to the resonance of the monosaccharide

protons; -$COCH_3$, -CH-NH-, and -CH_2-O-, respectively. These results are in concordance with previous investigations [27-29].

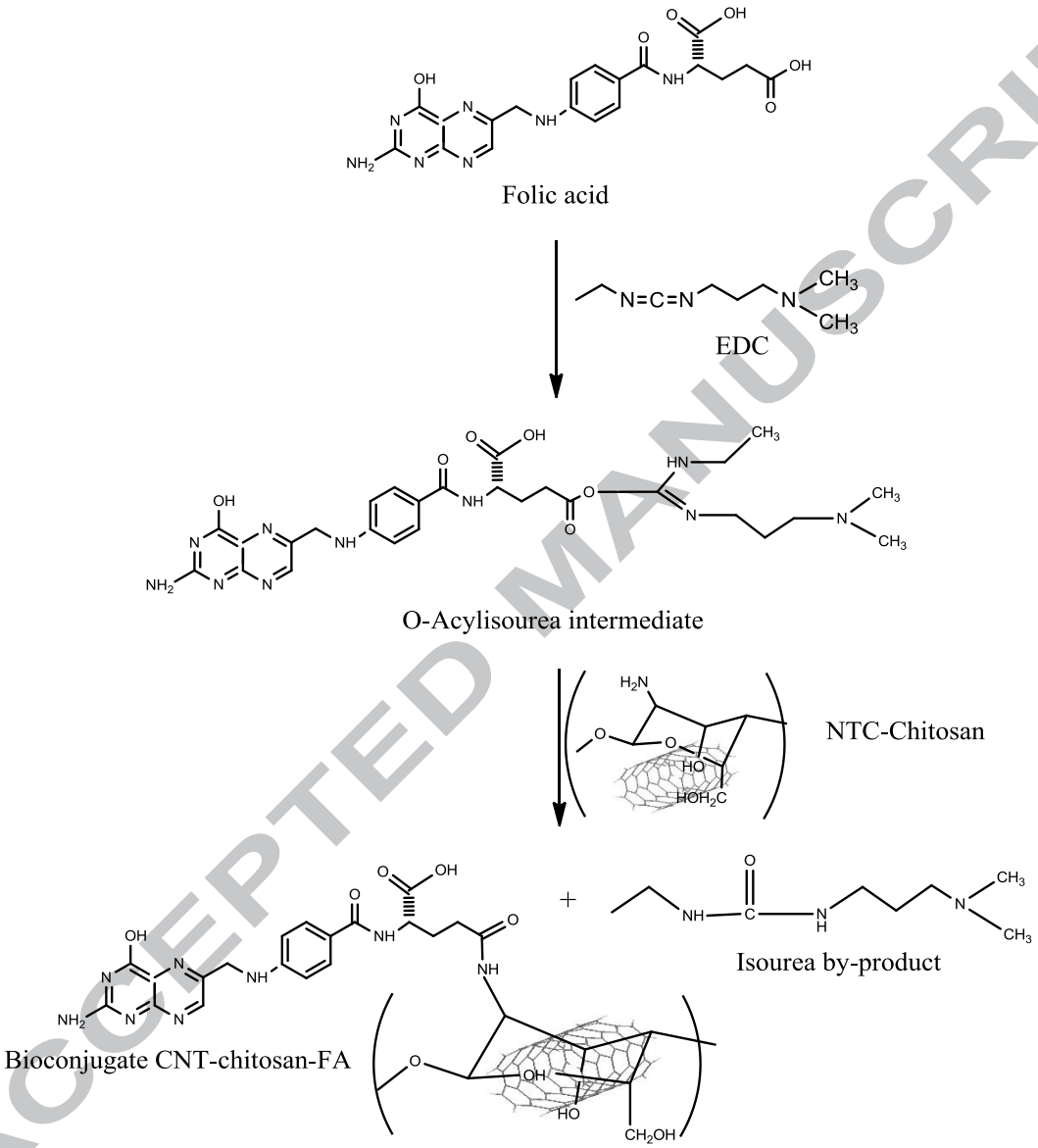


Figure 3. Conjugation of SWCNT-chitosan with FA.

The strong intensity of the signal at 1.90 ppm in the spectrum of Figure 4d was attributed to the protons of the methyl groups present in the isourea, which constitutes a secondary product of the reaction between chitosan and FA (Figure 3). The proton

signals appearing at 6.37 (d, *J* 8.7 Hz, 2H), 7.21 (d, *J* 1.7 Hz, 2H) correspond to the protons of para-aminobenzoic acid (PABA) from the folate and the signal at 8.16 (s, 1 H) corresponds to the pteridine moiety proton from the folate (Fig. 4d).

Figure 4d also shows a shift to high fields of the aromatic protons compared with the signals of free folic acid which were located at 6.12 (d, *J* 8.3 Hz, 2 H), 7.06 (d, *J* 8.3 Hz, 2 H), 7.93 (s, 1 H) (Fig. 4b). The shift could be due to the protective effect of the π electrons from SWCNT which produced an induced current and a magnetic field in the opposite direction to the applied field, thus generating a protection and moving the signals of FA to high fields. In addition, we observed a decrease in the signal intensity of aromatic protons of conjugate FA compared with the intensity of the free FA after the dialysis process thus confirming the removal of free FA. This proved that the couple of the folate residue to the SWCNT-chitosan could be achieved using the EDC-mediated reaction [27,28].

The resolution of the NMR signals was enhanced in all spectra using a ZGPR sequence pulse. This sequence was used in order to suppress water peaks.

The Stejskal-Tanner equation (1) shows the dependence between DC and the decay of the signal amplitude [31],

$$S = S_0 e^{-D\gamma^2\delta^2 g^2 \Delta} \quad (1)$$

Where *S* is the signal amplitude, *So* is the signal amplitude had there been not diffusion, *D* is the diffusion coefficient, γ is the gyromagnetic radio, δ is the duration of gradient pulse, g is the strength of the gradient, and Δ is the diffusion time corrected for the effects of finite gradient pulse width. The increasing gradient produced a progressive attenuation of each signal; the attenuation of the signal depends of the DC

as in shown in the equation 1. By least-squares fitting and equation (1), it was possible to calculate the DC of each proton signal belonging to free and conjugated FA (figure 5).

The DOSY experiment yields a pseudo 2D spectrum with NMR chemical shifts in one dimension (horizontal axis) and self-diffusion coefficients in the other (vertical axis). The data from DOSY experiments were presented in a 2D contour-mode plot where cross-peaks between the DC values and the chemical shifts were given for each proton signal of the conjugate.

The attenuation of the signals of the chemical shifts from free FA and that conjugated to SWCNT-chitosan was used to generate diffusion coefficients.

With the experimental parameters optimized, full signal attenuation was achieved after 32 steps of linearly increasing the gradient strength from 2% to 95%.

Figure 5a shows the bidimensional map of the DC of free FA in where all proton signals are characterized by the same DC, which means that they all are due to the free FA. All the proton signals of FA were around a DC value of 5.0×10^{-10} m^2/s. The DC value of approx. 3.5×10^{-10} m^2/s corresponds to the FA conjugated to SWCNT-chitosan (Fig. 5b). In this region, proton signals belonging to the chitosan and FA are included.

The values of DC presented above reveal that the free FA diffused more rapidly than the FA conjugated to SWCNT-chitosan, thus confirming the rapid diffusion of free FA compared with its conjugated counterpart. This in turn proved the formation of a conjugate between FA and chitosan wrapping SWCNTs.

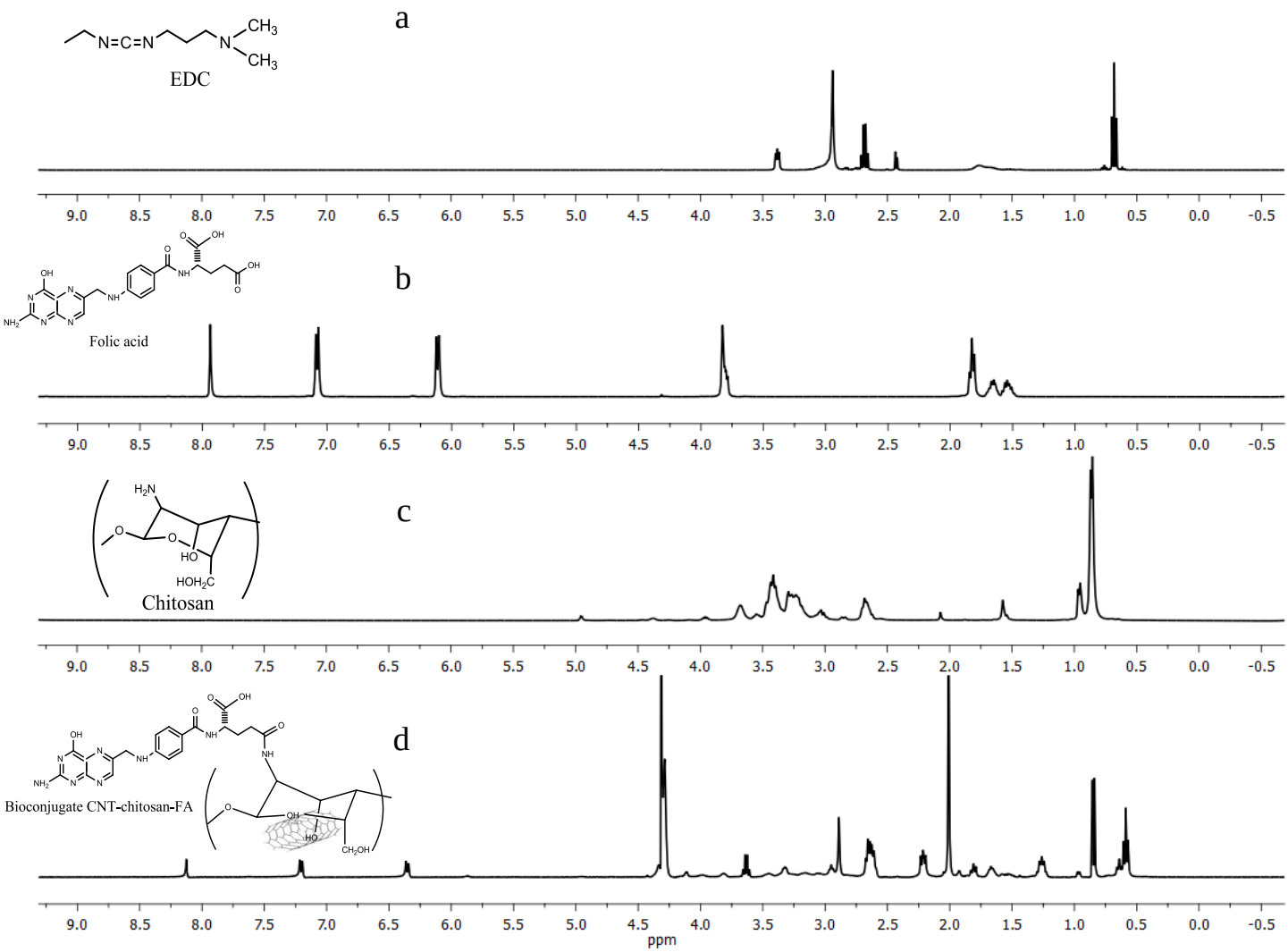


Figure 4. ^{1}H NMR spectra of: (a) EDC; (b) FA; (c) chitosan; (d) SWCNT-chitosan-FA conjugate

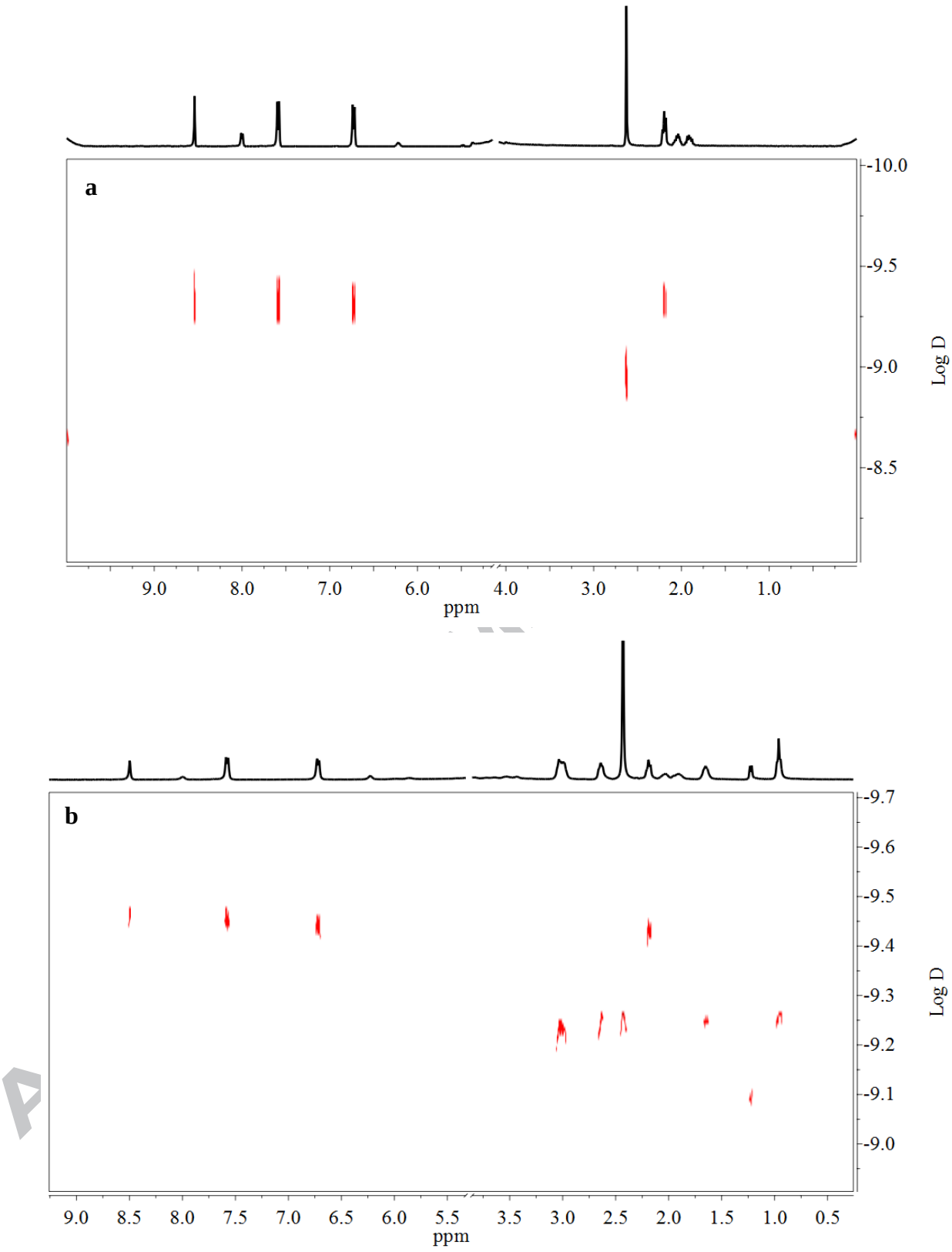


Figure 5. ^{1}H DOSY spectrum of (a) non-conjugated folic acid and (b) folic acid conjugated to SWCNT-chitosan

The presence of the isourea as a secondary product of the reaction was confirmed by the DC with a value of 5.9 x 10^{-10} m^2/s (Figure 5b) and the faster diffusion as opposed to free and conjugated folic acid. Table 1 shows a comparison of DC values of all the compounds.

Control DOSY experiments of EDC and the intermediate EDC-FA showed values of 7.08 x 10^{-10} m^2/s and 4.2 x 10^{-10} m^2/s respectively (see SI figure S4) thus showing the rapid diffusion of free EDC compared with FA conjugated to EDC.

Table 1 Diffusion coefficients determined by 2D DOSY

Compound	Log D	[a]Diffusion coefficient (10^{-10} m^2/s)
Isourea	-9.23	5.9
Free folic acid	-9.30	5.0
Conjugated SWCNT-chitosan-FA	-9.45	3.5

[a]Diffusion coefficients obtained by means of antilog D

Following the NMR and DOSY experiments black rings around the walls of NMR tube were observed. We believe that this sedimentation could be due to the catalyst iron particles which remain after the synthesis of SWCNTs, the iron particles provide magnetic properties to the bioconjugate SWCNT-chitosan-FA [32]. The magnetic field from NMR spectrometer could align the SWCNTs depositing thus on the walls of NMR tube. This phenomenon has been studied and used to induce the alignment of SWCNTs aqueous solution under the influence of magnetic field [32]. After sedimentation is possible to re-dissolve the bioconjugate from NMR tube by mean of sonication and thus obtain and useful NMR spectra, however to have a full explanation further experiments are necessary to clarify this situation.

4. Conclusions

According to the present study, a two-dimensional diffusion-ordered NMR spectroscopy with presaturation of the solvent (ZGPR) could be successfully employed to monitor the conjugation of folic acid to SWCNTs-chitosan. The obtained diffusion coefficient values revealed that free folic acid diffused more rapidly than its counterpart conjugated to SWCNT, thus proving the efficiency of the synthesis method when it comes to attaching the folic acid molecules around the SWCNT. This is an important requirement when developing conjugates aimed for drug-delivery systems or alternative treatments in nanomedicine.

2D-DOSY analysis is thus as a new tool for monitoring and characterizing the functionalization of different types of nanomaterials with biomolecules.

ACKNOWLEDGMENTS. The Colombian Administrative Department of Science, Technology and Innovation, COLCIENCIAS, (Project 110245921468) and the Danish Agency for Science Technology and Innovation (FTP 271-08-0968) are gratefully acknowledged for financial support.

REFERENCES

[1] Liu Z, Tabakman S, Welsher K, Dai HJ. Carbon Nanotubes in Biology and Medicine: In vitro and in vivo Detection, Imaging and Drug Delivery. Nano Research. 2009 Feb;2(2):85-120.

[2] Wang X, Liu LH, Ramstrom O, Yan MD. Engineering Nanomaterial Surfaces for Biomedical Applications. Experimental Biology and Medicine. 2009 Oct;234(10):1128-39.

[3] Xing ZC, Chang YM, Kang IK. Immobilization of biomolecules on the surface of inorganic nanoparticles for biomedical applications. Science and Technology of Advanced Materials. 2010 Feb;11(1).

[4] Adeli M, Hakimpoor F, Ashiri M, Kabiri R, Bavadi M. Anticancer drug delivery systems based on noncovalent interactions between carbon nanotubes and linear-dendritic copolymers. Soft Matter. 2011;7(8):4062-70.

[5] Bhirde AA, Patel V, Gavard J, Zhang GF, Sousa AA, Masedunskas A, et al. Targeted Killing of Cancer Cells in Vivo and in Vitro with EGF-Directed Carbon Nanotube-Based Drug Delivery. Acs Nano. 2009 Feb;3(2):307-16.

[6] Sahoo NG, Bao HQ, Pan YZ, Pal M, Kakran M, Cheng HKF, et al. Functionalized carbon nanomaterials as nanocarriers for loading and delivery of a poorly water-soluble anticancer drug: a comparative study. Chemical Communications. 2011;47(18):5235-7.

[7] Sobhani ZSZ, Dinarvand R, Atyabi F, Ghahremani M, Adeli M. Increased paclitaxel cytotoxicity against cancer cell lines using a novel functionalized carbon nanotube. International Journal of Nanomedicine. 2011;6:705-19.

[8] Thakare VS, Das M, Jain AK, Patil S, Jain S. Carbon nanotubes in cancer theragnosis. Nanomedicine. 2010 Oct;5(8):1277-301.

[9] Kam NWS, O'Connell M, Wisdom JA, Dai HJ. Carbon nanotubes as multifunctional biological transporters and near-infrared agents for selective cancer cell destruction. Proceedings of the National Academy of Sciences of the United States of America. 2005 Aug;102(33):11600-5.

[10] Liu QL, Chen B, Wang QL, Shi XL, Xiao ZY, Lin JX, et al. Carbon Nanotubes as Molecular Transporters for Walled Plant Cells. Nano Letters. 2009 Mar;9(3):1007-10.

[11] Weng XX, Wang MY, Ge J, Yu SN, Liu BH, Zhong J, et al. Carbon nanotubes as a protein toxin transporter for selective HER2-positive breast cancer cell destruction. Molecular Biosystems. 2009;5(10):1224-31.

[12] Lacerda L, Bianco A, Prato M, Kostarelos K. Carbon nanotubes as nanomedicines: From toxicology to pharmacology. Advanced Drug Delivery Reviews. 2006 Dec;58(14):1460-70.

[13] Yang WR, Thordarson P, Gooding JJ, Ringer SP, Braet F. Carbon nanotubes for biological and biomedical applications. Nanotechnology. 2007 Oct;18(41).

[14] Zhang XK, Meng LJ, Lu QH, Fei ZF, Dyson PJ. Targeted delivery and controlled release of doxorubicin to cancer cells using modified single wall carbon nanotubes. Biomaterials. 2009 Oct;30(30):6041-7.

[15] Yang XY, Zhang ZH, Liu ZF, Ma YF, Yang RC, Chen YS. Multi-functionalized single-walled carbon nanotubes as tumor cell targeting biological transporters. Journal of Nanoparticle Research. 2008 May;10(5):815-22.

[16] Xia W, Low PS. Folate-Targeted Therapies for Cancer. Journal of Medicinal Chemistry. 2010 Oct;53(19):6811-24.

[17] Kang B, Yu DC, Dai YD, Chang SQ, Chen D, Ding YT. Cancer-Cell Targeting and Photoacoustic Therapy Using Carbon Nanotubes as "Bomb" Agents. Small. 2009 Jun;5(11):1292-301.

[18] Johnson CS. Diffusion ordered nuclear magnetic resonance spectroscopy: principles and applications. Progress in Nuclear Magnetic Resonance Spectroscopy. 1999 May;34(3-4):203-56.

[19] Viel S, Capitani D, Mannina L, Segre A. Diffusion-ordered NMR spectroscopy: A versatile tool for the molecular weight determination of uncharged polysaccharides. Biomacromolecules. 2003 Nov-Dec;4(6):1843-7.

[20] Cohen Y, Avram L, Frish L. Diffusion NMR spectroscopy in supramolecular and combinatorial chemistry: An old parameter - New insights. Angewandte Chemie-International Edition. 2005;44(4):520-54.

[21] Marega R, Aroulmoji V, Bergamin M, Feruglio L, Dinon F, Bianco A, et al. Two-Dimensional Diffusion-Ordered NMR Spectroscopy as a Tool for Monitoring Functionalized Carbon Nanotube Purification and Composition. Acs Nano. 2010 Apr;4(4):2051-8.

[22] Clementi A, Aversa MC, Corsaro C, Spooren J, Stancanelli R, O'Connor C, et al. Synthesis and characterization of a colloidal novel folic acid-beta-cyclodextrin conjugate for targeted drug delivery. Journal of Inclusion Phenomena and Macrocyclic Chemistry. 2010 Apr;69(3-4):321-5.

[23] Hermanson GT. Bioconjugated techniques. 2nd Editorial Elsevier. 2008:219-224.

[24] Castillo JJ, Novoa LV, Martínez F, Escobar P. Carbon nanotubes-chitosan in HOS and THP-1 cells. Revista Salud UIS. 2011 Jun;43(1):21-26.

[25] Linde GA, Laverde A, de Faria EV, Colauto NB, de Moraes FF, Zanin GM. The use of 2D NMR to study beta-cyclodextrin complexation and debittering of amino acids and peptides. Food Research International. 2010 Jan;43(1):187-92.

[26] Gilles MA, Hudson AQ, Borders CL. Stability of water-solubles carbodiimide in aqueous solution. Analytical Biochemistry. 1990 Apr;184:244-248.

[27] Yang SJ, Lin FH, Tsai KC, Wei MF, Tsai HM, Wong JM, et al. Folic Acid-Conjugated Chitosan Nanoparticles Enhanced Protoporphyrin IX Accumulation in Colorectal Cancer Cells. Bioconjugate Chemistry. 2010 Apr;21(4):679-89.

[28] Wan A, Sun Y, Li HL. Characterization of folate-graft-chitosan as a scaffold for nitric oxide release. International Journal of Biological Macromolecules. 2008 Dec;43(5):415-21.

[29] Li P, Wang Y, Zeng F, Chen L, Peng Z, Kong L. Synthesis and characterization of folate conjugated chitosan and cellular uptake of its nanoparticles in HT-29 cells. Carbohydrate Research. 2011 Jan;346(6):801-806.

[30] Shieh YT, Wu HM, Twu YK, Chung, YC. An investigation on dispersion of carbon nanotubes in chitosan aqueous solution. Colloid Polymer Science. 2009 Oct;288(4):377-385.

[31] Stejskal EO. Use of spin echoes in a pulsed magnetic-field gradient to study anisotropic, restricted diffusion and flow. Journal of Chemical Physics. 1965 Jul;43(10):3597-3603.

[32] Kordás K, Mustonen T, Tóth G, Vähäkangas J, Uusimäki A, Jantunen H, Gupta A, Rao KV, Vajtai R, Ajayan P. Magnetic-Field induced efficient alignment of carbon nanotubes in aqueous solution. Chemistry of Materials. 2007 Nov;(19):787-791

Table captions

Table 1 Diffusion coefficients determined by 2D DOSY

Figure captions

Figure 1. Single walled carbon nanotubes-chitosan (SWCNT) conjugate before (a) and after (b) sonication

Figure 2. UV-Vis spectra of conjugated folic acid (FA), (inset) calibration curve of free FA

Figure 3. Conjugation of SWCNT-chitosan with FA.

Figure 4. ^{1}H NMR spectra of: (a) EDC; (b) FA; (c) chitosan; (d) SWCNT-chitosan-FA conjugate

Figure 5. ^{1}H DOSY spectrum of (a) non-conjugated folic acid and (b) folic acid conjugated to SWCNTs-chitosan