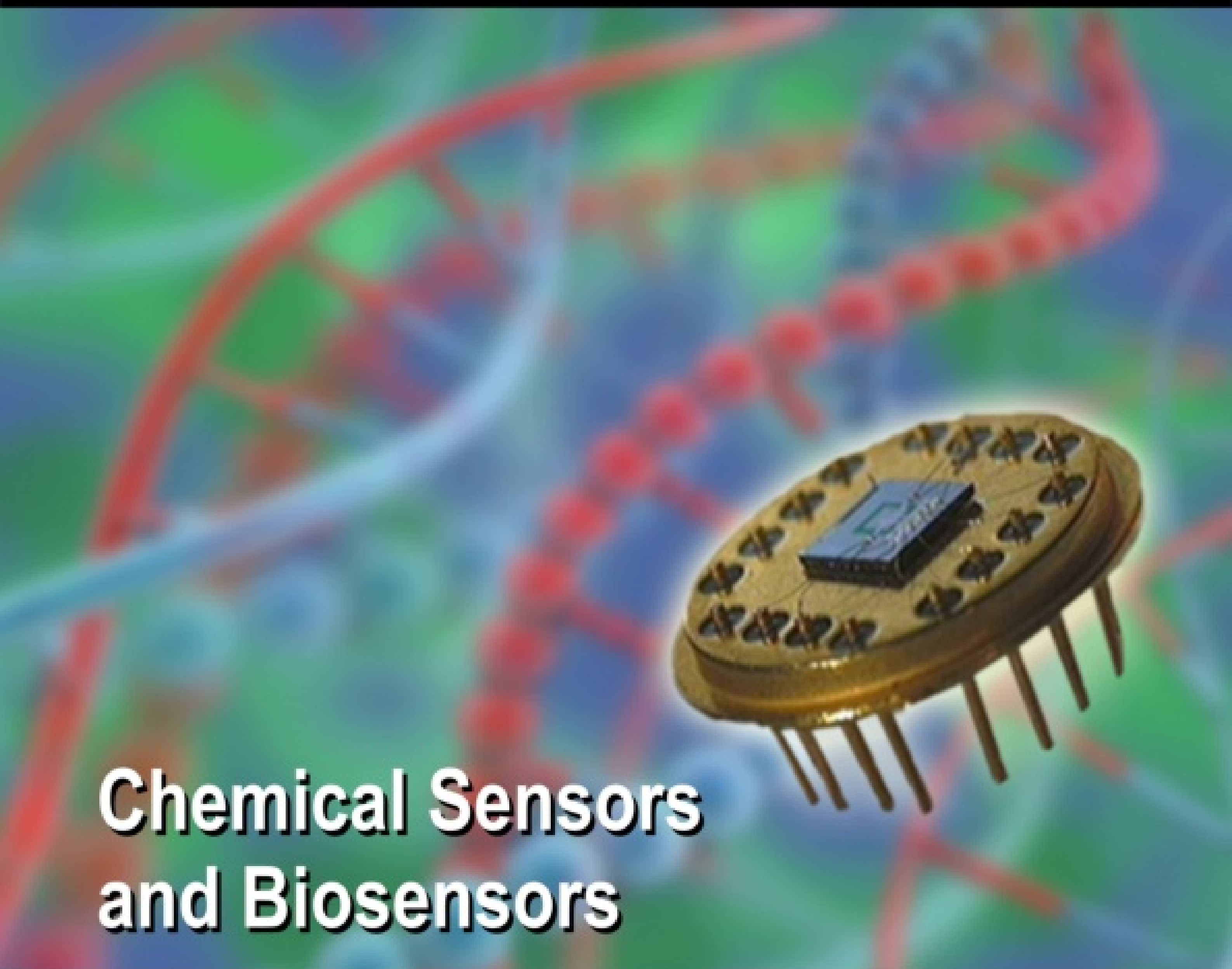
ISSN 1726-5749

SENSORS & TRANSDUCERS

vol. 88
2/08



Chemical Sensors and Biosensors

International Frequency Sensor Association Publishing

IFSA

Sensors & Transducers

Volume 88
Issue 2
February 2008

www.sensorsportal.com

ISSN 1726-5479

Sensors & Transducers Journal (ISSN 1726-5479) is a peer review international journal published monthly online by International Frequency Sensor Association (IFSA). Available in electronic and CD-ROM.

Sensors & Transducers Journal

Contents

Volume 88
Issue 2
February 2008

www.sensorsportal.com

ISSN 1726-5479

Research Articles

Authors are encouraged to submit article in MS Word (doc) and Acrobat (pdf) formats by e-mail: editor@sensorsportal.com
Please visit journal's webpage with preparation instructions: http://www.sensorsportal.com/HTML/DIGEST/Submition.htm

Sensors & Transducers Journal, Vol. 88, Issue 2, February 2008, pp. 1-8

Sensors & Transducers

ISSN 1726-5479

http://www.sensorsportal.com

Confocal Microscopy of Bioconjugated Carbon Nanotubes for Biosensor Applications

Kasif TEKER
Department of Physics & Engineering,
Frostburg State University, Frostburg, MD, USA
Tel.: 301-687-4339
E-mail: kteker@frostburg.edu

Received: 29 January 2008 /Accepted: 22 February 2008 /Published: 26 February 2008

Abstract: Carbon nanotubes (CNTs) have many unique properties such as high surface area, hollow cavities, and excellent mechanical and electrical properties. Solubilization and biological functionalization of carbon nanotubes have greatly increased the usage of carbon nanotubes in biomedical applications such as biosensors and nanoprobes. This paper presents biofunctionalization of single wall carbon nanotubes (SWNT) with antibodies, which are specific to insulin-like growth factor 1 receptor (IGF1R) in breast cancer cells, for their potential applications as carriers in drug or gene delivery systems. A high degree of binding (~ 80%) between antibodies and SWNTs has been successfully demonstrated via confocal microscopy and further verified by transmission electron microscopy (TEM). Control experiments revealed that non-specific binding of antibodies onto SWNTs could be prevented through pre-treatment with a biocompatible polymer, polyethylene glycol (PEG) which could open up new opportunities for future generation biosensors with superior specificity and selectivity. *Copyright © 2008 IFSA.*

Keywords: Confocal Microscopy, Drug Delivery, Antibody Functionalization, Breast Cancer, IGF1 receptor

1. Introduction

Carbon nanotubes (CNTs) have attracted interest in many fields and applications due to their 1D nature [1], superior mechanical [2] and electronic properties [3-6]. CNTs are analogous to a 2D graphite sheet rolled into tubes of diameter 1-10 nm, and hence form hollow tubules of a single layer of carbon atoms. Nanotubes have very high surface-to-volume ratios and, therefore, promise very high sensitivities [7]. Furthermore, novel biological devices, fabricated by integrating nanotubes with organic molecules, would enable new research fields and applications such as *in situ* modification of living cells or their physiological activities.

Carbon has been the most commonly used support material for the immobilization of biomolecules due to its strength, stability, and electrical conductivity [8]. Erlanger and co-workers have produced antibodies specific for fullerenes [9, 10]. Carbon nanotubes with higher surface area, hollow cavities, and excellent mechanical and electrical properties would make nanotubes more suitable material for bioapplications compared to fullerenes. In a recent study, Erlanger et al. [11] demonstrated that a monoclonal antibody specific for fullerenes recognized and bound specifically to single wall carbon nanotubes. They found that the binding cavity was formed by clustering of hydrophobic amino acids, and the hydrophobic binding site of the antibody is sufficiently flexible to recognize SWNTs as well. In recent studies, nanotubes have been functionalized to investigate their biocompatibility and protein recognition capability. Balavoine et al. [12] reported direct crystallization of proteins such as streptavidin and HupR on the surface of the carbon nanotube. The proteins were able to crystallize in a helical conformation around the multi wall carbon nanotubes (MWNTs). The strong interaction between the proteins and carbon nanotubes is due to hydrophobic domains within the protein structure. They demonstrated that a single protein layer coats the nanotubes. Davis et al. [13] reported the immobilization of proteins and enzymes in carbon nanotubes. They demonstrated that small proteins and enzymes could be readily placed within the interior cavity of opened nanotubes. In another study, the immobilization of small proteins such as Zn-Cd Metallothionein on carbon nanotubes has been studied by high-resolution transmission electron microscopy [14]. It was postulated that the electron conductivity of carbon nanotubes could shield biomolecules from electron beam damage. In another recent study, specific binding of streptavidin onto SWNTs by co-functionalization of nanotubes with biotin and protein resistant polymers has been demonstrated [15].

Biofunctionalization of carbon nanotubes with antibodies can be utilized to develop sensors or arrays of sensors for diagnostic screening that may be cost-effective, simple, fast, and can potentially miniaturize the sample size down to a single-cell level that may enable early detection, diagnosis and treatment of cancer. In order to pursue these goals, the interaction of nanotubes with antibodies, and the change in material properties of nanotubes due to antibody adsorption need to be investigated. Antibodies are large protein molecules called immunoglobins that are produced in organisms upon exposure to foreign substances called antigens [16]. Immunoglobins of type G (IgG) are immunoglobins with an average molecular weight of ~150 kDa. They consist of four chains, 2 heavy and 2 light chains (see Fig. 1) linked by disulphide (*S-S)* bonds. The identity of antibodies is established by minor variations in the light or variable chain. If the antigen that the antibody binds to is very large, a set of varying antibodies is generated, called polyclonal antibodies. Monoclonal antibodies are formed when only one class of antibody is structured by the cells, instead of a host of variants. Furthermore, the antibodies can be used to protect against disease as well as helping to diagnose various illnesses, and to detect drugs and abnormal substances in blood.

In this paper the following studies will be presented: i) confocal microscopy of antibody functionalization of SWNTs, ii) control experiments through PEG pre-treatment for blocking non-specific antibody binding onto nanotube surfaces, and iii) binding studies of gold nanoparticle-conjugated antibodies with SWNTs by transmission electron microscopy. The advantage of using confocal microscopy is that it provides statistical evaluation of the co-localization of the labeled nanotubes with antibodies by combining high-resolution sampling with observation over extended areas without any damage to the antibodies. A non-covalent biofunctionalization scheme was followed due to several reasons such as simplicity of the steps involved, preservation of the bioactivity of the molecule, non interference on the sensor properties from host of molecules used to tag in covalent functionalization schemes, and tailorable surface properties of the nanotube. It should also be pointed out that the antibody-coated nanotubes could be used as probes of cell or membrane function due to their smaller size compared to present capillary intracellular probes.

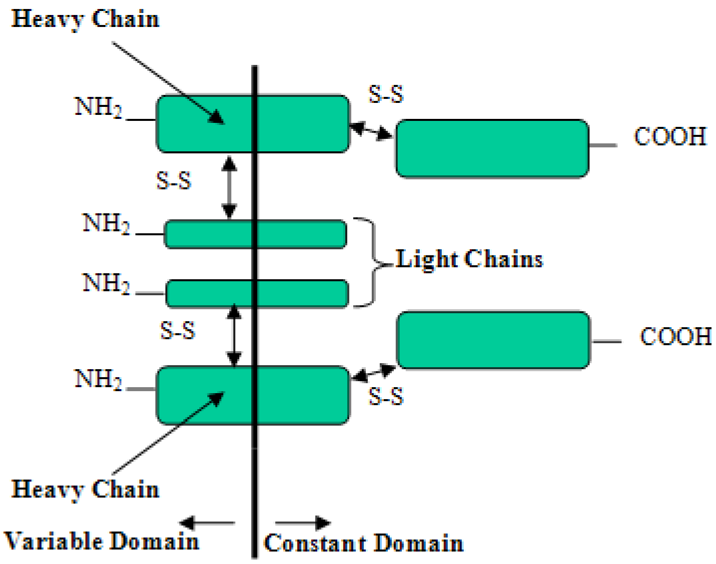


Fig. 1. A schematic of an antibody structure.

2. Experimental Details

The CNT solution was prepared in DI water with a surfactant, sodium dodecyl benzene sulfonate (NaDDBS: ICN Biomedicals, Inc.) [17], that ensured their separation in an aqueous environment. The entire mixture was gently agitated for 24 hr in a sonicator (Fisher Scientific Ultrasonic Cleaner, 60 Hz frequency, FS60H) at room temperature that resulted in separation of the SWNT bundles into individual SWNT. The surfactant to CNT ratio is 20 by weight. The nanotubes were then labeled with dihexyloxacarbocy anine iodide ($DiOC_6$: Molecular Probes, Inc.) [18], a dye that fluoresces in green with Argon laser (25 mW) at 488 nm excitation line. The $DiOC_6$ was prepared at 2 mg/ml in methanol and diluted in distilled water just prior to use with the CNT solution. The dye and nanotube solution were mixed at a 1:1 ratio, and allowed to incubate for 1 hour prior to confocal microscopy observation (Zeiss LSM 510 Multiphoton Confocal Microscope).

The antibodies used were a secondary polyclonal goat anti-mouse IgG (Molecular Probes, Inc.) and primary mouse monoclonal IgG (EMD Biosciences). They were prepared in Phosphate Buffered Saline (PBS) solution (0.138M NaCl, 0.0027M KCl, pH 7.4), by diluting a 2 mg/ml antibody solution with PBS to a ratio of 1:10 (antibody : PBS), just prior to use. The secondary antibody was pre-labeled with Alexa 546 (Molecular Probes, Inc.), a dye that fluoresces in red with HeNe laser (1 mW) at 543 nm excitation line. The CNT solution and secondary antibody solution were then mixed in a microfuge tube and allowed to interact for up to 2 hours. Centrifuging was done to the antibody solution when necessary to eliminate unnecessary fluorescence. The un-labeled primary was first tagged with the fluorescently labeled secondary antibody and then introduced to the CNT solution in the same way as the secondary. In addition to the confocal microscopy analysis, transmission electron microscopy (TEM) has been used (Zeiss CEM 902). For TEM analysis, the CNTs were incubated with the secondary goat anti-mouse IgG conjugated to 12 nm colloidal gold particles (Jackson Immunolabs).

3. Results and Discussions

Confocal microscopy is a widely used tool for fluorescent imaging of biological objects [19]. Confocal microscopy provides observation of spatially extended areas as well as high-resolution analysis. The advantage of fluorescence for microscopy is the possibility to analyze location and expression of many target molecules at the same time. Furthermore, a full three-dimensional view of the sample can be obtained by integrating the optically sectioned thin slices almost in real time. Therefore, confocal microscopy has been used to view fluorescently tagged CNTs in a method similar to that of viewing biological materials such as antibodies to accurately analyze and quantify their interaction. In order to evaluate the degree of binding over extended sampling areas, weighted co-localization coefficients (WCC) for both the CNTs and antibodies were calculated. WCC can be defined as the ratio of the intensity of co-localized area of a particular channel (color) to the intensity of total area above threshold intensity of that channel (color). It has been established that the co-localization coefficients can provide quantitative information in dual-color images [20], and the same procedure was followed here for antibody functionalization.

First, surfactant-mediated aqueous solubility of CNTs has been demonstrated. Aqueous solubility of carbon nanotubes has significant implications in biochemistry and biomedical engineering, in which organic solvents cannot be used due to their incompatibilities with living cells and organisms. Further, it is very critical to preserve inherent material properties of carbon nanotubes following to the solubility treatments. Therefore, non-covalent stabilization of carbon nanotubes using surfactants has become an attractive approach for nanotube dispersion and separation. The NaDDBS has the ability to break up the CNT bundles into individual nanotubes without forming chemical bonds. Following separation, the nanotubes were labeled using a flourophore before antibody functionalization for studying the extent of antibody interaction with nanotubes using confocal microscopy. Thus, the well-separated nanotubes were labeled with the dye $DiOC_6$. Labeling CNTs with conventional flourophores offers several advantages for studying the interaction of biological molecules on carbon nanotubes. First, it allows the visualization of smaller carbon nanotubes approaching individual nanotubes using confocal microscopy without aid from electron microscopic techniques. It doesn’t damage the nanotube lattice, thereby preserving the inherent material properties of the nanotubes [18]. When antibodies are functionalized on the nanotube, the resultant change in the material properties of the nanotube stems from the antibodies, as the flourophores and surfactant do not change the sp^2 bonded graphene sidewall. Further, nanotubes coated with flourophores can also be used as contrast agents for high contrast imaging of cells and tissues for biomedical imaging applications. Furthermore, the labeling allowed imaging the SWNT inside CHO cells using streptavidin-biotin SWNT conjugates that showed the effectiveness of the SWNT conjugates for penetrating cell membranes [21]. The observations of the cell membrane permeability for carbon nanotubes have offered great opportunities for medical applications in delivery of drugs, proteins, or genes into living cells. For successful imaging and for higher contrast images, however, it is essential that the nanotubes are well-separated and labeled using flourophores. Fig. 2 shows the well-separated and labeled nanotubes. A more detailed discussion about non-covalent separation and labeling of carbon nanotubes can be found in another report [21]. After successful non-covalent separation and labeling of carbon nanotubes, the antibody functionalization of the CNTs has been conducted.

Fig. 3 (a) is an overlap confocal image of CNTs (green) and antibodies (red). To evaluate the degree of binding over extended areas, weighted co-localization coefficients (WCC) for both the CNTs and antibodies were calculated. Fig. 3(b) is a scattergram, which provides quantitative information about the image. The WCCs were found to be about 0.80 for both the CNTs and antibodies. It was also found that separation of the SWNT using sonication introduced defects that enhance antibody binding directly to the surface of the SWNT [21]. Furthermore, it is believed that the observed high degree of antibody binding could be associated with the amino affinity of carbon nanotubes despite the fact that the mechanism of non-specific adsorption of proteins onto the nanotube surfaces is not known.

However, hydrophobic interaction has been the most widely accepted mechanism for non-specific adsorption of proteins onto the nanotube surfaces.

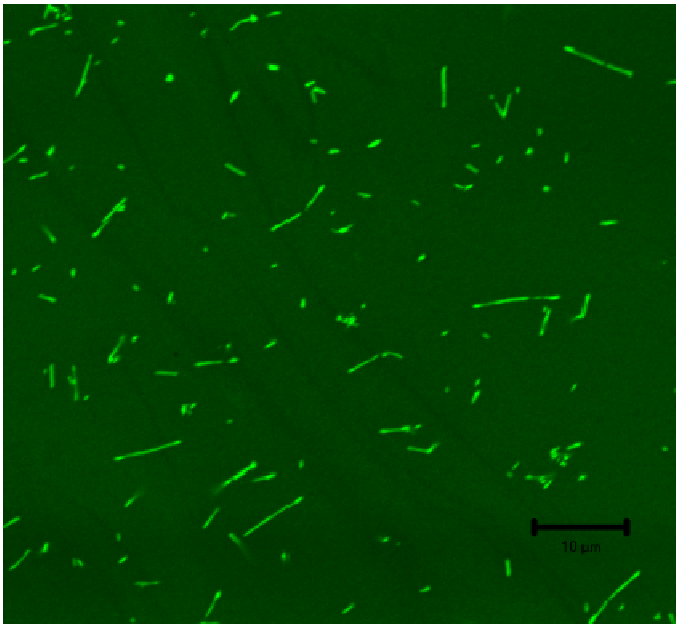
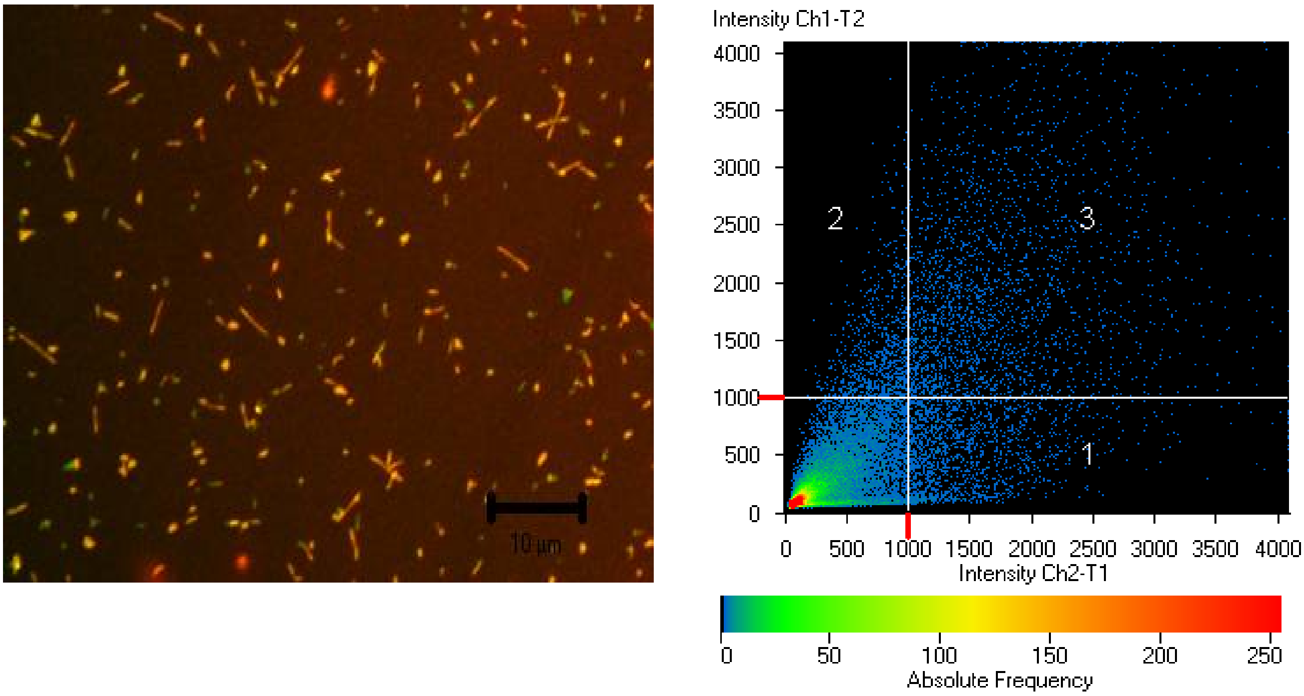


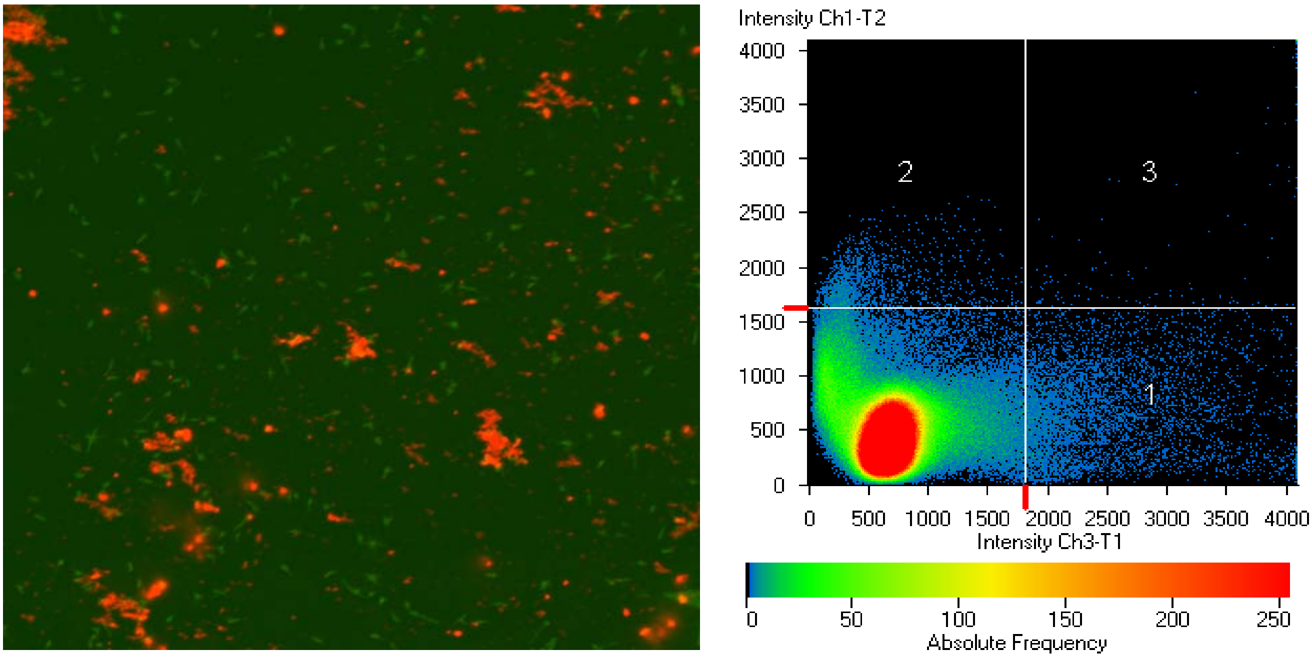
Fig. 2. Confocal microscopy image of SWNTs coated with $DiOC_6$ (green). The SWNTs are very well-separated and labeled.



(a) (b)

Fig. 3. (a) Confocal microscopy image of a green-dye-labeled CNTs and subsequently coated with the red-dye-conjugated antibodies. A high degree of selective attachment of the antibodies to the CNTs appears; (b) Scattergram to measure the co-localization between antibodies and CNTs via calculation of the WCC. Region 1 represents pixels in red channel, region 2 represents pixels in green channel and region 3 represents co-localizing pixels. The WCC is about 0.80 (from the region 3 of Fig. 3b).

Having successfully demonstrated non-specific adsorption of antibodies onto the nanotube surfaces, the control experiments have been carried out by polyethyleneglycol (PEG), a bio-compatible polymer, to (i) verify the confocal microscopy analysis and (ii) develop a new functionalization scheme in preventing non-specific antibody binding to SWNTs. To perform the control experiments, the surfactant-treated nanotubes in aqueous solution were coated with PEG solution (5 mM in water). Following that, the PEG-treated nanotubes were incubated with antibodies prior to confocal microscopy analysis. Fig. 4(a) is the overlap confocal microscope image of the CNTs (green), coated with PEG, and then incubated with antibodies (red) for ~2 hours. It can be seen that co-localization between antibodies and nanotubes is very minimal (clearly distinct green and red pixels without significant overlap) as compared to the Fig. 3(a). In fact, the WCC was found to be less than 0.05 through the corresponding scattergram for the same image (see Fig. 4b). These control experiments indicate that PEG along with the surfactant (NaDDBS) is a very effective method in preventing non-specific antibody binding onto nanotubes. Further, the high degree of binding between nanotubes and antibodies in the absence of PEG is verified. This study demonstrates a scheme to control antibody affinity of CNTs in aqueous solutions, which is very valuable to their potential biological and biomedical applications.



(a) (b)

Fig. 4. (a) Confocal microscopy image of $DiOC_6$ coated CNTs (green) treated with PEG, and subsequently incubated with antibodies (red). No significant antibody binding to SWNTs was observed (clearly distinct green and red pixels without significant overlap); (b) Scattergram to measure the co-localization between antibodies and CNTs via calculation of WCC; (region 1 represents the red channel, region 2 the green channel and region 3 represents the co-localized area). The WCC is less than 0.05 (from the region 3 of the Fig. 4b).

To further verify antibody binding onto the nanotubes, TEM analysis was performed. Biological specimens are generally difficult to view using conventional TEM methods due to the low contrast of the specimen. To overcome this difficulty, the CNTs were incubated with gold nanoparticle-antibody conjugates. The size of these conjugates ranges about 30 nm. The TEM grid was treated with phosphotungstic acid for negative staining before the TEM observation. Negative staining provides high contrast over the entire surface of the grid containing the samples. Fig. 5 exhibits selective attachment of the antibodies, which are conjugated to the gold nanoparticles, onto the SWNT bundle. Gold nanoparticle-antibody conjugates appear as black spots against the SWNT bundle (see Fig. 5). Although TEM involves many sample preparation steps, the technique was very useful as a verification tool particularly at the initial stages of the confocal microscopy analysis.

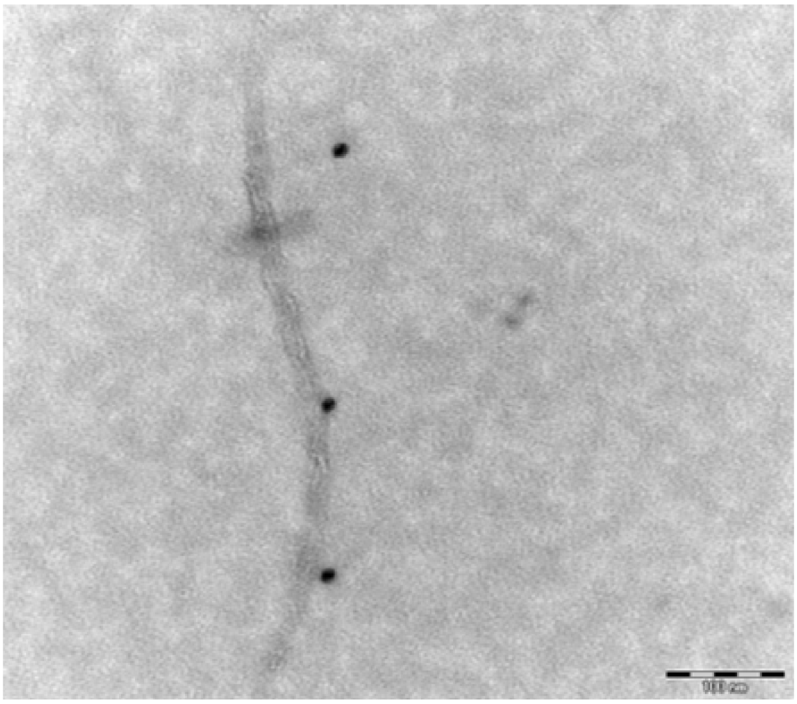


Fig. 5. TEM image of SWNT bundle incubated with antibodies that were conjugated with 12 nm gold nanoparticles (visible as black dots). The scale bar is 100 nm.

4. Conclusions

Biofunctionalization of carbon nanotubes with polyclonal and monoclonal antibodies through confocal microscopy and TEM have been successfully demonstrated. A high degree of co-localization (about 0.80) was observed and quantified by confocal microscopy analysis. Control experiments have shown that PEG along with the surfactant (NaDDBS) is a very effective biofunctionalization scheme in preventing non-specific antibody binding to the nanotube surfaces. Analysis of the images taken by confocal microscope revealed a minimal co-localization (less than 0.05) between nanotubes and antibodies with the presence of a blocking agent, PEG. These findings indicate that bioconjugated carbon nanotubes can be used as high efficiency drug, protein and gene delivery systems in various therapeutic applications. Furthermore, these studies demonstrate the effectiveness of confocal microscopy in studying the interactions between carbon nanotubes and biomolecules.

5. Acknowledgements

The author thanks Dr. K. Czymmek (Delaware Biotechnology Institute, Bioimaging facility) and Dr. B. Panchapakesan (University of Delaware) for their valuable discussions.

References

[1]. S. J. Tans, M. H. Devoret, H. Dai, A. Thess, R. E. Smalley, L. J. Geerligs, C. Dekker, Individual Single-Wall Carbon Nanotubes as Quantum Wires, *Nature*, Vol. 386, 1997, p. 474.
[2]. R. S. Ruoff, D. C. Lorents, Mechanical and Thermal Properties of Carbon Nanotubes, *Carbon,* Vol. 33, 1995, p. 925.
[3]. R. Saito, G. Dresselhaus, M. S. Dresselhaus, Physical Properties of Carbon Nanotubes, *Imperial College Press*, 1998.
[4]. M. Bockrath, D. H. Cobden, P. L. McEuen, N. G. Chopra, A. Zettl, A. Thess, R. E. Smalley, Single-Electron Transport in Ropes of Carbon Nanotubes, *Science*, Vol. 275, 1997, p. 1922.
[5]. Z. Yao, C. L. Kane, C. Dekker, High-Field Electrical Transport in Single-Wall Carbon Nanotubes, *Phys. Rev. Lett.* , Vol. 84, 2000, pp. 2941-2944.
[6]. L. Langer et al, Electrical Resistance of a Carbon Nanotube Bundle, *Journal of Materials Research*, Vol. 9, 1994, pp. 927-931.
[7]. C. Kiang, W. A. Goddard, R. Beyers, D. S. Bethune, Carbon Nanotubes with Single-Layer Walls, *Carbon*, Vol. 33, 1995, pp. 903-914.
[8]. T. D. Burchell, Carbon Materials for Advanced Technologies, *Pergamon*, New York, 1999.
[9]. B. Chen, S. R. Wilson, M. Das, D. J. Coughlin, and B. F. Erlanger, Antigenicity of Fullerenes: Antibodies Specific for Fullerenes and their Characteristics, *Proc. Natl. Acad. Sci. USA*, Vol. 95, 1998, p. 10809.
[10].B. C. Braden et al., X-ray Crystal Structure of an anti-Buckminsterfullerene Antibody Fab Fragment: Biomolecular Recognition of C60, *Proc. Natl. Acad. Sci. USA*, Vol. 97, 2000, p. 12193.
[11].B. F. Erlanger, B. Chen, M. Zhu, and L. Brus, Binding of an Anti-Fullerene IgG Monoclonal Antibody to Single Wall Carbon Nanotubes, *Nano Lett*., Vol. 1, 2001, p. 465.
[12].F. Balavoine, P. Schultz, C. Richard, V. Mallouh, T. W. Ebbesen, and C. Mioskowski, Helical Crystallization of Proteins on Carbon Nanotubes: A First Step towards the Development of New Biosensors, *Angew. Chem. Int. Ed.*, Vol. 38, 1999, p. 1912.
[13].J. J. Davis et al, The Immobilization of Proteins in Carbon Nanotubes, *Inorganica Chimea Acta*, Vol. 272, 1998, pp. 261-266.
[14].Z. Guo, P. J. Sadler, S. C. Tsang, Immobilization and Visualization of DNA and Proteins on Carbon Nanotubes, *Advanced Materials*, Vol. 10, 1998, pp. 701-703.
[15].M. Shim, N. W. S. Kam, R. J. Chen, Y. Li, H. Dai, Functionalization of Carbon Nanotubes for Biocompatibility and Biomolecular Recognition, *Nano Lett.* , Vol. 2, 2002, pp. 285-288.
[16].Editor: E. Kress-Rogers, *Handbook of Biosensors and Electronic Noses: Medicine, Food and the Environment*, CRC press, 1997.
[17].M. F. Islam, E. Rojas, D. M. Bergey, A. T. Johnson, A. G. Yodh, High Weight Fraction Surfactant Solubilization of Single-Wall Carbon Nanotubes in Water, *Nano Lett*., Vol. 3, 2003, pp. 269-273.
[18].R. Prakash, S. Washburn, R. Superfine, R. E. Cheney, M. R. Falvo, Visualization of Individual Carbon Nanotubes with Fluorescence Microscopy Using Conventional Fluorophores, *Appl. Phys. Lett.*, Vol. 83, 2003, pp. 1219-1221.
[19].P. C. Cheng, T. H. Lin, W. L. Wu, and J. L. Wu, Multidimensional Microscopy, *Springer Verlag NY Inc*., 1994.
[20].E. M. M. Manders, F. J. Verbeek, J. A. Aten, Measurement of Colocalization of Objects in Dual Color Confocal Images, *Journal of Microscopy*, Vol. 169, 1993, pp. 375-382.
[21].K. Teker et al., Applications of Carbon Nanotubes for Cancer Research, *NanoBiotechnology*, Vol. 1, 2005, pp. 171-182.

Guide for Contributors

Aims and Scope

Sensors & Transducers Journal (ISSN 1726-5479) provides an advanced forum for the science and technology of physical, chemical sensors and biosensors. It publishes state-of-the-art reviews, regular research and application specific papers, short notes, letters to Editor and sensors related books reviews as well as academic, practical and commercial information of interest to its readership. Because it is an open access, peer review international journal, papers rapidly published in *Sensors & Transducers Journal* will receive a very high publicity. The journal is published monthly as twelve issues per annual by International Frequency Association (IFSA). In additional, some special sponsored and conference issues published annually.

Topics Covered

Contributions are invited on all aspects of research, development and application of the science and technology of sensors, transducers and sensor instrumentations. Topics include, but are not restricted to:

- Physical, chemical and biosensors;
- Digital, frequency, period, duty-cycle, time interval, PWM, pulse number output sensors and transducers;
- Theory, principles, effects, design, standardization and modeling;
- Smart sensors and systems;
- Sensor instrumentation;
- Virtual instruments;
- Sensors interfaces, buses and networks;
- Signal processing;
- Frequency (period, duty-cycle)-to-digital converters, ADC;
- Technologies and materials;
- Nanosensors;
- Microsystems;
- Applications.

Submission of papers

Articles should be written in English. Authors are invited to submit by e-mail editor@sensorsportal.com 6-14 pages article (including abstract, illustrations (color or grayscale), photos and references) in both: MS Word (doc) and Acrobat (pdf) formats. Detailed preparation instructions, paper example and template of manuscript are available from the journal's webpage: http://www.sensorsportal.com/HTML/DIGEST/Submition.htm Authors must follow the instructions strictly when submitting their manuscripts.

Advertising Information