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Afr. J. Biomed. Res. Vol.19 (January, 2016); xx-xx

Full Length Research Paper

Interaction of Multiwalled Carbon Nanotube Produces Structural Alteration of DNA

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ABSTRACT

Multiwalled carbon nanotube (MWCNT) has been found to produce structural changes in Calf Thymus-DNA (CT-DNA). The interaction or binding of the multi-walled carbon nanotubes (MWCNT) was investigated in order to discover if it brings about any significant changes of the DNA double helix using CD spectra of the CT-DNA at two concentration levels of MWCNT representing an increasing MWCNT/DNA molar ratio. In addition, spectrophotometric titrations between MWCNT and CT-DNA were carried out in order to utilize spectral changes as a means of detecting specific binding modes of either intercalation or degradation of DNA. Interactions of MWCNT induced significant changes in the CD spectra of the B-form of natural DNA. The intensities of the positive CD band at 280 nm decreased significantly. This decrease was found to be concentration-dependent. Following spectrophotometric titrations; specific subtle conformational changes were observed with a molar ratio combination of 2:1 between MWCNT and CT-DNA and these were characterized by a formation constant of the order of 10^3 M^{-1} and a negative Gibbs free energy suggesting that MWCNT avidly binds to DNA. Thermodynamic considerations revealed that electrostatic interactions between the DNA base pairs and the MWCNT are taking place accounting for the negative free energy change, positive enthalpy change with a small entropy change. The results obtained in the study of the binding interactions of MWCNT with DNA confirm that a cytogenetic effect of MWCNT with DNA is a possibility in vivo.

Keywords: *MWCNT; CT-DNA; CD-spectra; Spectrophotometric studies; Thermodynamic considerations*

INTRODUCTION

Nanomaterials are defined as materials that have at least one dimension between 1 and 100 nanometers. At this size, materials begin to exhibit unique properties that affect physical, chemical, and biological behaviors. Engineered nanomaterials have found significant relevance in various industrial sectors. With their unique physico-chemical properties, carbon nanotubes (CNT) have gained immense application in industrial and biomedical sectors. The global production of CNT has increased remarkably over the past decade (Lux Research, 2004; Sun et al, 2014). This potentially increases the chance exposure and release into the environment. The consumers and most importantly the

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Received: May, 2015
Accepted: November, 2015

Abstracted by:
Bioline International, African Journals online (AJOL), Index Copernicus, African Index Medicus (WHO), Excerpta medica (EMBASE), CAB Abstracts, SCOPUS, Global Health Abstracts, Asian Science Index, Index Veterinarius

workers are primarily at risk of being exposed. Over the past decade studies have reported CNT induced toxicity in animal models, *in vitro* and *in vivo* (Shvedova et al, 2003; Manna et al, 2005; Magrez et al, 2006; Zhu et al, 2007; Muller et al, 2008; Wirnitzer et al, 2009; Ghosh et al, 2011). MWCNT-induced genotoxicity has also been reported in other test systems including plants (Lin et al, 2009; Tan et al, 2009; Serag et al, 2010; Ghosh et al, 2011; Khodakovskaya et al, 2013).

In one of our previous studies, we reported DNA damage induced by MWCNT in plant and animal models, *in vitro* and *in vivo* (Ghosh et al, 2011). Additionally, our results suggested formation of possible crosslink between MWCNT and DNA. In one of our more recent publications, it was found that MWCNT is capable of inducing both genetic and epigenetic changes in plant systems using *Allium cepa* as a model (Ghosh et al, 2015). While oxidative stress might play an important role in MWCNT-induced DNA damage, direct interaction between MWCNT and DNA cannot be ruled out. In this context, we deemed it necessary to investigate the extent and nature of MWCNT-DNA interactions with a view to appropriately delineating the mode of interaction through a mechanistic-based binding study of MWCNT and CT-DNA in vitro. Circular dichroism and UV–Vis spectrophotometry were used to study the interaction at different molar ratios. We believe that result of the present study would provide information regarding MWCNT-DNA interactions, which would be important in understanding the events leading to genetic and epigenetic changes reported. Additionally, the result of MWCNT-DNA interactions study might provide basis for the designing of safer nanomaterials and at the same time providing a background for newer applications.

MATERIALS AND METHODS

Multiwalled carbon nanotubes (MWCNT)

The MWCNT powder as obtained from Sigma–Aldrich, USA (Product code: 694185-1G), was characterized using Transmission Electron Microscope (Jeol JEM-2100 LaB6, 200 kV) and scanning electron microscopy (Hitachi S-415A electron microscope at 25 kV). The complete characterization of the material has been previously published (Ghosh et al, 2015).

Circular dichroism (CD) spectra and UV-Vis spectrophotometric analysis

The high polymerised double-stranded sodium salt of calf thymus DNA (activated) used in this study was purchased from Sigma Chemical Company, St. Louis, USA. The stock solution of DNA was prepared by dissolving DNA in 10 mM of the Tris–HCl buffer at pH 7.4. The reaction of MWCNT and DNA was initiated by incubating at 37 °C for 30 min. Following incubation period the absorption spectrum for each reaction was recorded using a Beckman Coulter DU® 730 life Science UV–VIS spectrophotometer. The circular dichroism (CD) spectra of the solutions were recorded using a spectro-polarimeter (JASCO, model J-815) in a 1 cm quartz cuvette, following incubation period.

RESULTS AND DISCUSSION

Circular Dichroism spectra

To establish whether the interaction or binding of the multi-walled carbon nanotubes (MWCNT) brings about any significant changes of the DNA double helix, CD spectra of the CT-DNA were recorded using two concentration levels of MWCNT representing an increasing MWCNT/DNA molar ratio. The observed CD spectra are presented in Figure 1.

The observed CD spectrum of natural CT-DNA consists of a positive band at 280 nm due to base stacking and a negative band at 250 nm due to helicity, which is characteristic of DNA in right-handed B-form (Kashanian et al, 2008). The interactions of MWCNT induced significant changes in the CD spectra of DNA. The intensities of the positive CD band at 280 nm decreased significantly. This decrease is concentration-dependent. On incubation with 5 µg/mL MWCNT, a 3.95 % decrease in intensity of this band was observed. However, on increasing the concentration of the nanoparticle five times (25 µg/mL), the decrease became more intense as it was up to 16.22 % showing a positive correlation between the increase in concentration of MWCNT and the changes in the intensity of the dichroic band of CT-DNA at 280 nm. A minute positive CD band at 310 nm was also observed in the natural CT-DNA which became negative on interaction with MWCNT at the two concentration levels studied.

Likewise, the negative CD bands of CT-DNA between 230 and 250 nm were drastically altered in the presence of MWCNT, though a not too perfect correlation was observed here. Increasing the concentration of MWCNT to 25 µg/mL completely eliminated the characteristic negative dichroic band at 340 nm. An induced negative band at 350 nm for the CT DNA–MWCNT interaction at 5 µg/mL concentration of the nanomaterial was also observed. These findings are indicative of deep conformational changes of the DNA double helix following the interactions of DNA macromolecule with MWCNT. The presence of a new

band at 330 nm with 5 µg/mL MWCNT suggests that binding produces a new chromophore. However, the binding leading to this new chromophore does not appear to have been generated by a permanent avidly binding mechanism such as covalent bond which would have resulted in an intercalative binding. This CD band disappeared in the presence of higher concentration of MWCNT suggesting that a saturation effect of MWCNT on DNA may have been attained and the presence of more MWCNT molecules cannot effect grossly significant conformational changes. In addition, the complete near zero CD band at 340 nm suggests a tight binding with possible drastic conformational changes taking place in the DNA macromolecule.

Spectrophotometric titrations

Binding or interaction of a compound with DNA causes absorption spectral changes that can be used to detect the possible mechanism of binding of the compound leading to its DNA intercalation or degradation. Two approaches have been widely adopted; the study of fluorescence spectral changes of the complex or the absorption spectral changes. The latter was adopted in this study since the absorption spectral behaviour of the natural DNA are well characterised for which minor changes in spectral data can be used to decipher the presence or absence of significant interactions.

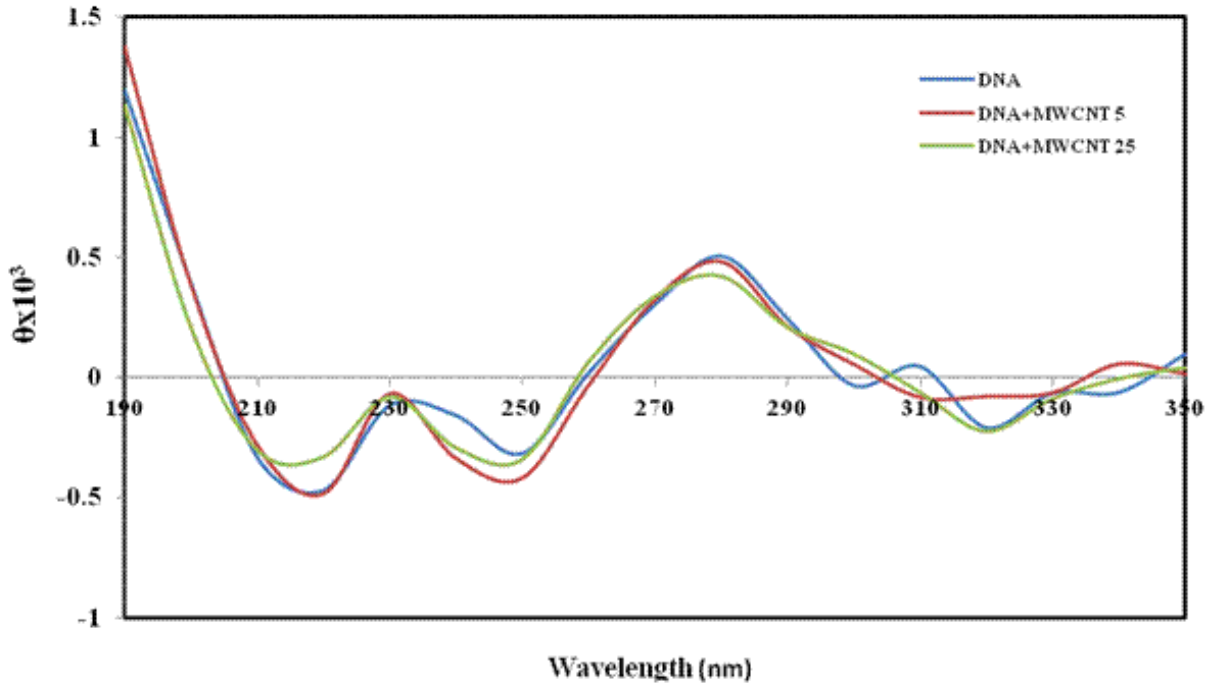

Figure 1:
Circular dichroism spectra of CT DNA in the presence of increasing amounts of MWCNT

Table1:
Electronic absorption spectral data of the complexes formed by MWCNT with varying ratios of ds-DNA

	λ_{max}, nm (log ε_{max})				
	DNA mole ratios				
	1.0	0.3	0.15	0.075	0.0375
Peaks	225 (3.564)	225 (3.564)	225 (3.564)	225 (3.564)	225 (3.564)
	250 (3.580)	245 (3.580)	245 (3.580)	250 (2.582)	250 (2.582)
	270 (3.543)	265 (3.551)	265 (3.563)	265 (3.555)	265 (3.561)
			275 (3.541)	275 (3.550)	275 (3.558)
Valleys (nm)	235	235	235	235	235
	-	260	260	260	260

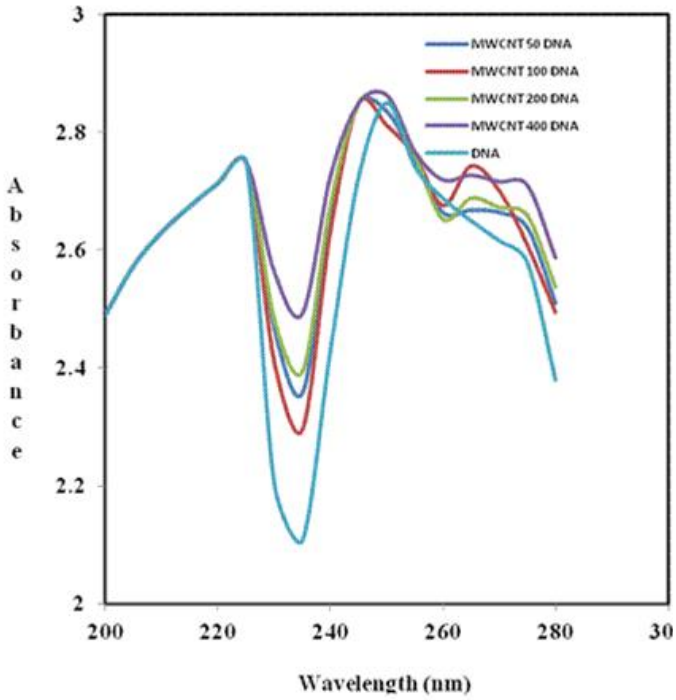

Figure 2:
Absorption spectra (UV region) of DNA alone and in combination with varying concentrations of MWCNT (μg/mL)

The interaction of small molecules with DNA can be by either covalent or non-covalent bonding. In a covalent binding the reactive part of the compounds interacts with a nitrogen base of DNA such as guanine N7 e.g., cisplastin (Mpountoukas et al, 2010). In the non-covalent DNA interactions, forces such as intercalative, electrostatic and groove binding of cationic metal complexes along outside of DNA helix, the major or minor groove have been recognised. Intercalation involves the partial insertion of aromatic heterocyclic rings between the DNA base pairs (Polyanichko et al, 2004) and it is a general observation that the binding of an intercalative molecule to DNA is accompanied by a large hypochromic and signi□cant bathochromic shifts due to strong stacking interaction between the aromatic chromophore of the ligand and the DNA base pairs. The extent of spectral changes is de□nitely related to the strength of binding and the spectra for intercalators are more perturbed than those for groove binders (Kelly et al, 1985; Chow et al, 1992). The spectra of CT-DNA with varying concentrations of MWCNT are presented in Figure 2. There are some subtle changes in the spectra of DNA relative to the presence of different concentrations of MWCNT. As presented in Table 1, there are three clearly identifiable peaks for CT-DNA (mole ratio 1.0); these are found at 225, 250 and 270 nm. Increasing the mole ratio of MWCNT relative to that of DNA leads to some observable changes.

The peak at 225 nm did not show any changes in terms of the peak and the absorptivity. This suggests that the intact structures of the nucleobases are not affected by the interaction of the MWCNT with DNA. This appears to be in conformity with the results obtained for the CD spectra where conformational changes are suggested. The two other peaks for the CT-DNA suffered some alterations on interacting with MWCNT. There was a slight hypsochromic shift ($\Delta\lambda$ of -5 nm) with lower concentrations of MWCNT (DNA ratio of 0.3 and 0.15) with no change in absorptivity

However, increasing the MWNCT molar ratio relative to DNA beyond 0.15 led to a reversal of the absorption peak back to 250 nm with highly significant hypochromic shifts at both molar ratios of 0.075 and 0.0375. These observations suggest that there is a perturbation of the intact structure of the DNA structure following an increase in the relative amount of the nanomaterial. Similar results were obtained with the peak at 270 nm. Increasing MWCNT concentrations led to hypsochromic (or blue) shift of the spectra with a $\Delta\lambda$ of -5 nm. This shift is accompanied by increasing slight hyperchromic shift up to 0.15 molar ratio of CT-DNA. Of the greatest significance is the appearance of new minor peaks at 275 nm for the higher molar concentrations of MWCNT (0.85 to 0.9625) with increasing hyperchromicity. Significant changes were also observed for the main valley in the DNA spectrum (235 nm). Pronounced hyperchromic shifts were observed as the concentration of MWCNT increased. Noteworthy is the appearance of a new valley at 260 nm (found to be also concentration-dependent) which is not present in the natural DNA spectra. All these suggest specific molecular perturbations of intact DNA by the presence of the MWCNT

Determination of stoichiometric ratio

The Job's method of continuous variation (Rose, 1964) was utilised to study the stoichiometric ratio at which each of the dyes combined with DNA. Generally, the binding of substrates to DNA helix have been characterised classically through spectrophotometric titrations, by following the changes in absorbance and red or blue shifts of the spectrum as a function of mole ratio of the interacting pair (Kashanian et al, 2008; Ahmadi & Bakhshandeh, 2009). The mole ratio plot for the interaction of MWCNT with DNA is presented in Fig. 3.

The mole ratio plot was constructed from the absorbance of the peak at 275 nm for the complex

formed between DNA and MWCNT. As can be seen from Fig 3, two points of inflection was obtained suggesting that two moles of MWCNT is combining with one mole of DNA molecule. This 2:1 molar interaction may explain why the previously suggested perturbation of the molecular structure of DNA seem to occur as observed from the results of CD and UV spectroscopic measurements.

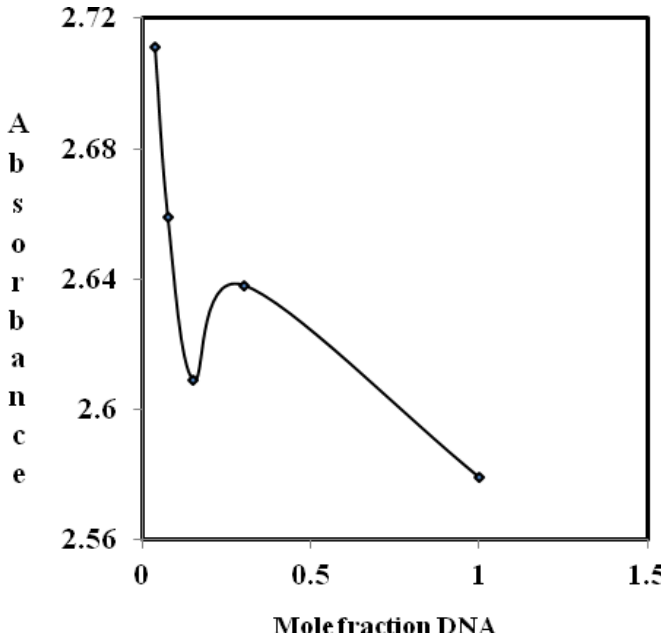

Figure 3:
Determination of stoichiometric ratio for the interaction between DNA and MWCNT at 275 nm

Estimation of formation constant and Thermodynamic parameters

The DNA is hydrated by water molecules and on interaction with a substrate several forces such as hydrogen bond, van der Waals interaction, ion-induced dipole interactions or hydrophobic bonding can make a substrate approach the DNA reactive sites for binding. Thus, any factor that will favour removal of the expunged water molecule will allow for the stability of the DNA-MWCNT complex. The energy required to drive such bond formation will be provided by the magnitude of the formation constant. From the equilibrium established, the formation constant can be estimated by the knowledge of molar absorptivities of the free and bound MWCNT molecules. For small molecules binding to active sites on a macromolecule, the formation or binding constant can be estimated from the expression in equation 1 (Benesi & Hildebrand, 1949).

$$\frac{Ao}{A-Ao} = \frac{\varepsilon_G}{\varepsilon_{H-G}-\varepsilon_G} + \frac{\varepsilon_G}{\varepsilon_{H-G}-\varepsilon_G}\frac{1}{K_f\,[DNA]} \quad (1)$$

Where A_0 and A are the absorbances of free and bound MWCNT
ε_G and $\varepsilon_{H\text{-}G}$ are their molar absorptivities, respectively.
K_f is formation or binding constant

The formation constant was obtained as $1.3332 \times 10^3\ M^{-1}$. The value of the formation constant suggests that MWCNT avidly interacted with CT-DNA.

The standard free energy change for the formation of the molecular complex between MWCNT and DNA was estimated using equation 2, where R is the gas constant, T is the temperature in Kelvin and K_f is the formation constant ($mol^{-1}dm^3$).

$$-\Delta G = RT\ln K_f \quad \text{----------} \quad 2$$

The Gibbs free energy was found to be -17.528 $KJmol^{-1}$. The negative values of ΔG reveal that the interaction process is spontaneous and the support force is provided by the large formation constant. The other thermodynamic parameters estimated were the enthalpy change (ΔH) and the entropy change (ΔS). The estimated values are respectively 17.531 $KJmol^{-1}$ and 1.024×10^{-5} $KJmol^{-1}$.

The binding thermodynamics as observed by Ahmadi et al (2011) reflect a subtle balance between the hydrogen, Van der Waals or multiple bonds and entropic effects. The change in entropy is governed by the release of counter ions and water from DNA and the bounded molecules. For transfer of small molecules from polar to non-polar environments, hydrophobic interactions usually give $\Delta H>0$ and $\Delta S>0$ with negative ΔG (Hans & Bekker, 1997). In general, the binding of an intercalator to DNA is driven entirely by a large favorable enthalpy reduction but with an unfavorable entropy decrease, and the binding of a major groove binder to DNA is driven by a large favorable increase in entropy. Electrostatic interactions, on the other hand, exhibit small enthalpy and positive entropy changes. Hydrogen bonding and van der Waals interaction are usually characterized by negative standard enthalpies of interaction. Thus from the results obtained it can be inferred that electrostatic interactions between the DNA base pairs and the MWCNT are taking place accounting for the negative free energy change, positive enthalpy change with a small entropy change. This might explain why concentration-dependent changes in the CD spectra

seem to be limited for higher molar ratios of MWCNT. Persistent exposure of DNA to the presence of MWCNT may in the long run lead to profound structural changes as observed in our recent study (Ghosh et al, 2015).

In conclusion, the binding interactions of Multiwalled carbon nanotubes led to subtle but significant changes in the conformation of double-helix DNA. Specific perturbations of the B-form of the right-handed double helix of the DNA have been linked to electrostatic interactions. These suggest that the previous *in vivo* cytogenetic effects of MWCNT on DNA are proven *in vitro* to be driven by specific intermolecular binding forces of attraction and not permanent bond formation.

Acknowledgement

The authors would like to acknowledge the CAS, Department of Botany, University of Calcutta for the research facility.

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