The different N concentrations induced cytocompatibility and hemocompatibility of MWCNTs with CNx coatings


A R T I C L E  I N F O

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- Multiwalled carbon nanotubes
- CNx
- N/C atomic composition ratio
- Cytocompatibility
- Hemocompatibility

A B S T R A C T

Carbon nitride coatings were deposited on the multiwalled carbon nanotubes (MWCNTs) which were prepared by chemical vapor deposition (CVD) using ion beam assisted deposition (IBAD) system. The morphology of the MWCNTs as well as variation in composition and chemical bonds between atoms in the coatings were measured using transmission electron microscopy (TEM) and X-ray photoelectron spectroscopy (XPS). The relationship between N/C atomic composition ratio and chemical bonding states and cytocompatibility and hemocompatibility of the MWCNTs with CNx coatings was investigated using fibroblast cell (L929) adhesion and blood clotting assays. The results revealed that the higher N/C or sp2C–N/sp3C/N ratio stimulated the cell growth, proliferation, and spreading and prolonged the kinetic blood-clotting time and recalcification time.

1. Introduction

Since the discovery of carbon nanotubes (CNTs) in 1991 [1], a wide range of current and/or future applications of CNTs has been proposed owing to their amazing electronic, mechanical, and structural properties [2,3]. The advent of engineered CNTs in commercial and industrial applications raised concerns about the potential impacts of CNTs on human health and environmental safety [4–7]. The biological compatibility is determined mainly by the chemical composition of the implant material. Properties of the biomaterial surface, such as its energy, relief and roughness, are important for the hemocompatibility and the deposition of cells [8,9]. So, surface modification of CNTs will cause an important impact on its clinical applications.

In considering surface modification of biomaterials, carbon nitride (CNx) appeared to have an advantage in that they show better biocompatibility. CNx coating used to modify the properties of the CNTs surface may change the ratio of proteins absorbed on the surface and, hence, improve adhesion and growth of cells [9–12]. In this work, we married multiwalled carbon nanotubes (MWCNTs) with CNx coatings to study the effects of N/C atomic composition ratio or sp²C and sp³C bonds on biocompatibility. Recently, there have been few studies on the biocompatibility of CNx coating which deposited on the surface of the MWCNTs, especially for the mechanism of biocompatibility from sp²C or sp³C bond. The aim of this work was to study the biocompatibility of the MWCNTs with CNx coatings by investigating blood-clotting time and cell adhesion in cultures, and obtain the insight into the function of N/C atomic composition ratio or sp²C and sp³C bonds in the contact between the tissue and material surface.

2. Experimental details

2.1. Preparation and measurement of the MWCNTs with CNx coatings

The synthesis of carbon film coated on a SiO2 substrate was carried out using a chemical vapor deposition (CVD) system at 750 °C for half an hour. The gas flow rates of argon and ethylene were 250 sccm and 100 sccm respectively. The carbon film should be responsible for the adhesion strength enhancement between the SiO2 substrate and MWCNTs. After that, the MWCNTs and SDS were dissolved in deionized water with ultrasonic dispersion for 5 min. After centrifugation of 10 min, the upper supernatant was directly sprayed onto the SiO2 substrates coated with carbon film with air brush pistol at 100 °C to form MWCNTs. Then the MWCNTs were immersed into deionized water for 10 min and then dried in a dry oven at 80 °C for 30 min to remove the SDS [13,14]. CNx coatings were deposited on the surfaces of the MWCNTs by ion beam assisted deposition (IBAD) system, as shown in Fig. 1. In this process, the chamber was evacuated to a base pressure lower than 3.0×10⁻⁷ Pa prior to the deposition. Graphite target (70×70 mm²) was sputtered by Ar⁺ from Kaufman...
gun at 1.05 keV/25 mA to deposit C coating at 2.0×10⁻² Pa, and the resulting coating was simultaneously bombarded at a N⁺ beam current of 200 eV/5–25 mA from Kaufman gun to prepare CNₓ coatings with different N/C atomic composition ratios on the MWCNTs for 30 min. The current densities of N⁺ are 0.25–1.27 mA/cm².

X-ray photoelectron spectroscopy (XPS) was performed to investigate N/C atomic composition ratio and chemical bonding states of the surfaces using a PHI5000 versa probe system (Al (Kα) X-ray source 1486.6 eV). The detailed morphologies of the MWCNTs before and after CNₓ coating were characterized using a JOEL JEM2100 high-resolution transmission electron microscope (HRTEM) and a field-emission scanning electron microscope (FESEM, Hitachi S-3000N). A CAM KSV021733 optical contact-angle inclinometer (Nunc, Finland) was also used to measure the contact angle of the samples.

2.2. Cell-adhesion and blood-clotting assays

Mouse fibroblast cells (L929) were used to investigate the cytocompatibilities of the four MWCNTs with CNₓ coatings. L929 cells were cultured in Earle’s salt supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were plated onto 75 mm² culture flasks and maintained at 37 °C in a humidified atmosphere with 5% CO₂ in air. They were released from culture flasks using a trypsin and phosphate buffer solution pH 7.4 (PBS). The inoculum density of the fibroblast cells is 2.5×10⁵ cells/ml. After 1 to 7 days in the incubator (culture intervals of 0.5, 1, 2, 3, 5, and 7 days), the medium was removed and the cell monolayer was washed several times with PBS and then isolated by trypsin for enumeration. Confocal scanning laser microscope (CSLM) (Nikon eclipse 90) and SEM were employed to observe cell morphology and stretching on the four samples.

Kinetic blood-clotting times were measured by the kinetic method. In this method, 0.1 ml of blood from a healthy adult rabbit was immediately dropped onto the surfaces of the samples. After a predetermined time, the samples were transferred into a beaker containing 50 ml of distilled water. The red blood cells which had not been trapped in a thrombus were hemolyzed, and the free hemoglobin was dispersed in the solution. The concentration of free hemoglobin in the solution was colorimetrically measured at 540 nm with a spectrophotometer. The optical density at 540 nm (O.D.₅₄₀nm) of the solution vs. time was plotted. In general, the O.D.₅₄₀nm value decreases with blood clotting.

The recalcification times of the materials were measured by recording the interval time from introducing 0.1 ml of CaCl₂ solution into a test-tube containing the samples and 0.1 ml of rabbit plasma to find white particles in the plasma.

3. Results and discussion

Fig. 2 shows SEM images of the MWCNTs, MWCNTs with CN₀₂.₂₁ coatings (N⁺ beam current of 25 mA) and MWCNTs with CN₀₂.₇ coatings (N⁺ beam current of 10 mA), respectively. The surface of the MWCNTs which has porous structure is rough, as shown in Fig. 2(a). And the water contact angle measured on the pristine MWCNTs is 124.86°. Generally speaking, a surface with water contact angle larger than 65° is defined as hydrophobic. So, the MWCNT without CNₓ coating reveals hydrophobicity. Apparent thin film covering the MWCNTs can be observed after coated CNₓ coating on them (Fig. 2(b, c)). Samples’ surfaces are flatter and there are few voids as shown in Fig. 2(b, c). The contact angles of the MWCNTs with CNₓ coatings drop to 37.6₃° and 47.0₁°, indicating hydrophilic surfaces due to CNₓ coatings.

To investigate detail morphologies of the MWCNTs with CNₓ coating, HRTEM characterizations are performed, as shown in Fig. 3. The tubular structures of these two samples are seen in these figures. MWCNTs’ outer diameters are approximately 30–50 nm, and the wall thicknesses are approximately 10 nm, as shown in Fig. 3(a, b). It can be seen that the graphite layers of the MWCNTs are generally parallel to each other with small curvature. Before depositing the CNₓ coating on the MWCNTs, the deposition rates of the CNₓ films...
on the Si(100) substrates with variation of Nitrogen beam current have been examined. The film thicknesses for the samples prepared with variation of Nitrogen ion beam are all 15 nm, which means that deposition rates were 0.5 nm/min. Fig. 3(c–f) shows HRTEM images of the MWCNTs with CN0.21 coating at different magnification times. The structures of the CNx coated on the MWCNTs are clear (shown by white rulers). Because the CNx coatings were deposited by the IBAD method on the MWCNTs layered on the substrates, it results in selective deposition on the top surface of the MWCNTs. The underside of the MWCNTs in the top layer and the other MWCNTs underneath cannot be coated in the same way, as shown in Fig. 3(d). And for some of the MWCNTs with CNx coating on top, the CNx layer is thick (Fig. 3(f)), for others beneath it's thinner (Fig. 3(e)). Because the cells only adhere to the top surface of the MWCNTs with CNx coating when they were seeded on the samples, the underside of the MWCNTs in the top layer and the other MWCNTs underneath which cannot be coated with CNx do very little affecting the results of the experiment.

XPS is used to characterize the differences in surface chemical bonding states and nitrogen concentration of the MWCNTs with CNx coatings deposited by different Nn+ beam current densities, which would cause obvious changes in the hemocompatibility and the cell adhesion. The chemical bonding states are obtained by subtracting the background with the Shirley's method and deconvoluting the spectra by a curve-fitting method using a non-linear least squares fitting to a mixed Gaussian–Lorentzian product function. From the XPS analysis, the N/C atomic composition ratios are 0.21, 0.27, 0.26, 0.21 corresponding to the Nn+ beam currents of 5, 10, 15, 25 mA. We mark these samples successively by CN-1, CN-2, CN-3, CN-4 and divide them into two groups according to the similar N/C ratio.

Fig. 4 shows the high-resolution XPS core-level spectra of C1s and N1s of the four samples. Tables 2 and 3 indicate their C1s and N1s peak positions and areas. The C1s peak is decomposed into five Gaussian components, referring to the bonds: sp²C–C (~284.7 eV), sp³C–C (~285.6 eV), sp³C–N (~286.4 eV), sp³C–N (~287.9 eV), and sp³C–O (~288.6 eV) [15–18]. The N1s peak has also revealed sp² and sp³C–N bonds at 400.1 and 398.7 eV respectively [17,18]. From the data, it is indicated clearly that with the beam current increasing, the ratio of the tetrahedral sp³C–C bonds decreases and the ratio of the sp³C–C bonds increases, while the diamond-like sp³C–N and sp³C–N bonds have the opposite changes. This difference reveals a strong change in the surface chemical bonding states.

The relationship between the sp³C/sp³C and the cell adhesion is shown in Fig. 5. For group 1, the cell adhesion numbers on CN-1 are more than CN-4 from days 1 to 7, despite that the cell numbers reduce gradually after 5 days. In addition, the cell concentrations on CN-2 are larger than CN-3, which sustains an increase throughout the incubation period. As shown in SEM images of CN-1 and CN-3, the adhered cells spread flat with rich pseudopod which suggests that the MWCNTs with CNx coating provide better conditions for the cellular spreading and the pseudopod spreading. Maximum numbers of L929 cells adhered to CN-1, CN-2 and CN-3 are almost the same. The cell adhesion numbers also increase with the sp³C–N/sp³C–N and sp³C–C/sp³C–C increasing in the same N/C ratio. These results indicate that the major factor causing cell adhesion is not only higher nitrogen content, but also the higher sp³C–N/sp³C–N or sp³C–C/sp³C–C ratio.

Table 1

<table>
<thead>
<tr>
<th>Samples</th>
<th>CN-1</th>
<th>CN-2</th>
<th>CN-3</th>
<th>CN-4</th>
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<tbody>
<tr>
<td>Beam current (mA)</td>
<td>5</td>
<td>10</td>
<td>15</td>
<td>25</td>
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<tr>
<td>N (%)</td>
<td>17.63</td>
<td>21.34</td>
<td>20.93</td>
<td>17.54</td>
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<tr>
<td>N/C (atm)</td>
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<td>0.27</td>
<td>0.26</td>
<td>0.21</td>
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</table>
Fig. 4. C1s XPS spectra of CN-1 (a), CN-2 (b), CN-3 (c), CN-4 (d), and N1s XPS spectra of CN-1 (e), CN-2 (f), CN-3 (g), CN-4 (h).
In addition, using immunofluorescence techniques, microfilaments and microtubules are stained, which are the main components of the cytoskeleton. Meanwhile the nuclear DNA stains with a different fluorescent dye and then combines the three photographs taken by confocal scanning laser microscopy (CSLM) in the same viewing field, with same exposure times, as shown in Fig. 6. The CSLM images show the morphology of mouse-fibroblast cells fixed on the surface of four samples after the incubation of one day. It can be seen from Fig. 6 that typical triangular cells adhere to the surface of all the samples. With the \( sp^2C-N/sp^3C-N \) and \( sp^3C-C/sp^2C-C \) increasing, the cell numbers on the materials in the same N/C ratio are larger. Cells spread more flat and there were more pseudopodia and microvilli on the cell surface. Fig. 6 also suggests that the samples with higher N/C ratio provide better conditions for the fibroblast growth, because the cell numbers on CN-2 and CN-4 are larger than the two other samples.

From these results, we consider the mechanism for improving cell adhesion. Protein absorption is an early in vivo event in the interaction between implanted biomaterial and living tissue. All proteins have NH\(_2\) and COOH groups at the ends, where, according to Takashima et al. [19], the NH tends to be positively charged and the COOH negatively charged. Thus, a surface with an organized arrangement of functional groups can act as a site for the cell growth [20]. The MWCNTs with CN\(_x\) coatings include \( sp^2C-N \) and \( sp^3C-N \) bonds at the surface and polarize at the surface due to the difference in electronegativity between carbon and nitrogen [21]. So the higher N/C ratio means more sites for the cell growth, which explains why CN-2 and CN-3 have more cells on their surfaces. With \( sp^2C-N/sp^3C-N \) ratio increasing, there are more unsaturated bonds in the samples, which causes the cell adhesion numbers to increase with the number of protein attached the material surface. Thus, the cell adhesion numbers increase with the \( sp^2C-N/sp^3C-N \) increasing in the same N/C ratio. For group 1, the cell adhesion numbers on CN-1 are more than CN-4. And the cell concentrations on CN-2 are larger than CN-3 throughout the incubation period for group 2.

Thrombus formation occurs when a biomaterial surface comes into contact with blood, prompted by rapid adsorption of plasma proteins onto the surface within the first few seconds of contact. This adsorption is regarded as the first major event in the coagulation process [22]. Among the plasma proteins, fibrinogen is regarded as the key protein that triggers platelet adhesion, activation and aggregation. Subsequently, coagulation factors are released, initiating the coagulation cascade and the eventual formation of a thrombus [23]. The kinetic blood-clotting time of all the samples is shown in Fig. 7. The higher optical density of the hemolyzed hemoglobin solution which changes with time means the better thromboresistance. The time when the optical density is 0.1 is defined as the kinetic blood-clotting time [17]. The result shows that the blood-clotting times of CN-3 (50 min) and CN-4 (41 min) are longer.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>( C_{1s} ) peaks of the MWCNTs with CN(_x) coatings.</th>
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<td>Samples</td>
<td>Peak position (eV)</td>
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<tr>
<td>CN-1</td>
<td>CN-2</td>
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<tr>
<td>sp(^2)C–C</td>
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<td>sp(^3)C–C</td>
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<th>Table 3</th>
<th>( N_{1s} ) peaks of the MWCNTs with CN(_x) coatings.</th>
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<td>CN-2</td>
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<tr>
<td>sp(^3)C–N</td>
<td>398.7</td>
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<tr>
<td>sp(^2)C–N</td>
<td>400.0</td>
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Fig. 5. L929 mouse fibroblast cell numbers on the MWCNTs with CN\(_x\) coatings vs. incubation time. The insets are their SEM images.
than CN-2 (31 min) and CN-1 (40 min) respectively, which means the better thromboresistance of CN-3 and CN-4.

Recalcification time is also obtained using venous blood of rabbit. The longer recalcification time of the materials suggested the better anti-coagulability. Fig. 8 shows the recalcification time of the four samples. The same results among CN-1, CN-2, CN-3 and CN-4 can be observed. Their change tendency implies that higher sp$^2$C–N/sp$^3$C–N ratios in the same N/C ratio can prolong recalcification time.

Factors influencing the hemocompatibility of biomaterials lie in several aspects: surface roughness, hydrophilicity or hydrophobicity, surface charge density and so on [24]. Many studies have demonstrated that higher hydrophilicity and superficial energy appeared to be the primary factor improving the surface blood-contacting properties [25,26]. Materials with high hydrophilicity and superficial energy are likely to be covered by a protein-dominating ‘conditioning film’ that may yield good hemocompatibility [27,28]. As shown in Fig. 9, the contact angles of CN-1, CN-2, CN-3 and CN-4 are about 40.08°, 47.01°, 30.77° and 37.63°, respectively. This explains why CN-3 and CN-4 exhibit better thromboresistance.

In addition, the hemoglobin, platelet and a few of plasma protein in blood tend to be negatively charged. Usually the material surface with more unsaturated bonds in electronegativity has better thromboresistance according to principle of same electric charge mutual repulsion. However, many factors, such as protein adsorption on the material surface and positive charge in blood are all assumed to result in positive effects on the hemocompatibility of the materials. Thus, the suitable density of charge will promote the hemocompatibility [29]. A suitable ratio of sp$^3$C–N to sp$^2$C–N can provide the optimum density of charge to promote the hemocompatibility, which is the possible reason for better thromboresistance for the CN-3 and CN-4 samples.

Fig. 6. CLSM images of mouse fibroblast cells fixed on CN-1 (a), CN-2 (b), CN-3(c), CN-4 (d).

Fig. 7. The O.D.540 nm values of the MWCNTs with CNx coatings vs. blood-clotting time.

Fig. 8. Recalcification times of the MWCNTs with CN-1, CN-2, CN-3, and CN-4.
4. Conclusions

In this work, we deposited about 15 nm-thick CNx coatings with different N/C atomic composition ratios on the MWCNTs. From the XPS analyses we found that the high N/C atomic composition ratio can increase the contents of the sp$^2$C–C bonds and the unsaturated degree of the N-groups of the coatings. The comparison of the cytocompatibility and hemocompatibility between the MWCNTs with different N/C ratio CNx coatings displayed that the high N/C ratio and sp$^2$C/N/sp$^3$C/N ratios might accelerate L929 cell adhesion and proliferation, while shortened the kinetic blood-clotting time and recalcification time. The mechanism of N/C and sp$^2$C/N/sp$^3$C/N ratios affecting biocompatibility is very complicated. Long-term effects need to be studied with further in vivo assays.

Acknowledgments

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References