Effects of Covalent Functionalisation on the Biocompatibility Characteristics of Multi-Walled Carbon Nanotubes

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We report the effect of chemical modification of multi-walled carbon nanotubes (MWNTs) on their activation of the human serum complement system, as well as the adsorption of human plasma proteins on MWNTs. Four different types of chemically-modified MWNTs were tested for complement activation via the classical and alternative pathways using haemolytic assays. Human plasma protein binding was also tested using an affinity chromatography technique based on carbon nanotube-Sepharose matrix. Covalent functionalisation of MWNTs greatly altered the level of activation of the complement system via the classical pathway. For example, MWNTs functionalised with ε-caprolactam or L-alanine showed respectively >90% and >75% reduction in classical pathway activation compared with unmodified MWNTs. These results demonstrate for the first time that these types of chemical modification are able to alter considerably the levels of specific complement proteins bound by pristine MWNTs (used as a control experiment). The reduced levels of complement activation via the classical pathway, that are likely to increase biocompatibility, were directly correlated with the amount of C1q protein bound to chemically modified carbon nanotubes. An inverse correlation was also observed between the amount of complement factor H bound to chemically modified MWNTs and the level of complement consumption via the alternative pathway.

Binding of human plasma and serum proteins to pristine and modified MWNTs was highly selective. The chemical modifications studied generally increased nanotube dispersibility in aqueous media, but diminished protein adsorption.

Keywords: Carbon Nanotubes, Covalent Functionalisation, Complement, Biocompatibility, Human Plasma.

1. INTRODUCTION

Carbon nanotubes are single, or multi-cylindrical graphene structures that have remarkable structural, electronic¹–³ and mechanical properties.⁴ Because of these characteristics, uses have been envisaged for carbon nanotubes in several types of medical applications including protein biosensors,⁵ drug delivery vehicles⁶,⁷ and vaccines.⁸ To make these potential applications reality, biocompatibility and toxicity¹⁰ issues must be addressed first. The dispersion of carbon nanotubes in aqueous solutions has been considered as a key prerequisite for improving the biocompatibility performance of carbon nanotubes. In recent years ready dispersion of carbon nanotubes in water has been achieved.¹¹ However, for medical applications of carbon nanotubes such as vaccines and drug delivery, nanotubes should be dispersible not in pure water but in buffered physiological conditions,
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which has not frequently been examined. The selection of the most appropriate type of functionalisation to disperse carbon nanotubes in buffers will depend on the type of application that is intended for these nanomaterials. In this respect, although to date several methods including acid treatment and PEGylation (polyethylene glycol attachment) have been used to disperse carbon nanotubes and make them biocompatible, their efficacy is still questionable.

For example, the negative charge of carbon nanotube surfaces induced by the acid treatment can potentially interact with proteins of immune system, particularly with the ones of complement system. PEGylation has been used for two purposes, namely to disperse carbon nanotubes and to prevent protein adsorption; nevertheless it is not a well established fact that PEG assures the suppression of complement activation. As a matter of fact, it has been shown that complement activation causes opsonization of PEGylated liposomes due to covalent deposition of the C3b factor.

Extensive work has been reported on covalent functionalisation of both single-walled and multi-walled carbon nanotubes (SWNTs and MWNTs, respectively) using acid treatments. In contrast, to date much less effort has been made on covalent modification of pristine MWNTs, taking advantage only of the defects on the nanotube sidewalls. In this study we report the effects of the latter and related types of chemical modification of MWNTs, on their activation of the human serum complement system and on their adsorption of human plasma proteins. To make MWNTs biocompatible, the nanotubes were covalently functionalised with 1,8-diaminooctane, amino acids L-alanine and $\varepsilon$-aminocaproic acid (in the form of its polymer Nylon 6) (Fig. 1(A)), 1-octadecylamine and 1,6-hexanedithiol (Fig. 1(B)). The resulting modified MWNT are referred to as MWNT18, MWNT18A, MWNT18C, MWNTODA and MWNT16DT, respectively. To obtain MWNT18C and MWNT18A samples, the amino acid moieties were covalently attached to diaminoctane groups of MWNT18, which are bonded to MWNTs via their wall and end defects. 1-Octadecylamine and 1,6-hexanediol were also directly covalently attached to the defects of MWNT walls and ends. For all the chemical functionalisations, the gas phase technique was employed. In general, this procedure involves treatment of carbon nanotubes with organic reagents (amino acids, amines, thiols, etc.) as vapours under reduced pressure and a temperature of >150 °C.

The study of the activation of the complement system by new nanomaterials such as carbon nanotubes is one of many aspects of investigation of the biocompatibility properties of these nanomaterials. The complement system in human blood plasma is considered as the chief recognition and effector component of innate immunity; its major

Fig. 1. (A) Chemical attachment of 1,8-diaminooctane to MWNTs and further derivatisation reactions with L-alanine and $\varepsilon$-caprolactam. The chemical modification starts with the binding of 1,8-diamиноoctane to MWNTs, the reaction product is referred to as MWNT18. Subsequently, MWNT18 were treated with L-alanine, or $\varepsilon$-caprolactam vapours to give MWNT18A or MWNT18C products. (B) Chemical attachment of 1-octadecylamine and 1,6-hexanediol on MWNTs. The final products are referred to as MWNTODA and MWNT16DT.
role is to recognise and promote removal or destruction of non-self or altered-self materials (e.g., microbes and damaged host cells). It is also involved in inflammation and in the adaptive immune response. The complement system can be activated via three different pathways termed the classical, alternative and lectin pathways. The classical pathway is activated when C1q, the crucial recognition protein of this pathway, binds to the target surface. C1q is a 460 kDa protein, found in blood plasma at 80 μg/ml. The alternative pathway does not depend on recognition of foreign targets by a single recognition protein, such as C1q. Instead it relies on the spontaneous hydrolysis of the internal thiolester bond in C3. The resulting C3(H2O) forms a complex protease, C3(H2O)Bb, which cleaves C3 to C3b, which can bind covalently, via its exposed internal thiolester bond, to any surface, within close proximity which has exposed nucleophilic groups such as SH, NH2 and OH. Furthermore, the alternative pathway is regulated by the presence or absence of complement regulatory proteins such as factor H (FH). FH is a multifunctional 155 kDa protein, present in plasma at 200–600 μg/ml. The lectin pathway is initiated by binding of mannan-binding lectin (MBL) or ficolins to carbohydrate structures present on a wide range of micro-organisms including bacteria, viruses, fungi and parasites.

In our previous study we have reported that unmodified SWNTs and double-walled carbon nanotubes (DWNTs) activate the human serum complement system...
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via both the classical and the alternative pathways. The activation of complement induced by unmodified carbon nanotubes might induce inflammation and granuloma formation. Therefore, it was important to determine whether chemical modification of MWNTs could alter the level of complement activation. This involved the study of the direct binding of two key complement proteins C1q and factor H to chemically modified MWNTs. In addition, the study of the adsorption of plasma proteins on surfaces is also of great relevance for biocompatibility issues since bound proteins may influence subsequent interactions of many different types of cells with the surfaces, or may influence activation of enzyme cascades, such as coagulation and complement.

2. RESULTS
2.1. Characterisation of Chemically Modified MWNTs
Chemically modified samples MWNT18C and MWNT18A (Fig. 1(A)) were prepared and characterised by five different techniques as reported in Ref. [22(a)]. Fourier-transform infrared spectroscopy (FTIR) was a technique that allowed for the detection of NH and NH₂ groups in MWNT18C and MWNT18A samples as evidence of the functionalisation of MWNT18 with ε-caprolactam and L-alanine respectively. High resolution transmission electron microscopy (HRTEM), scanning electron microscopy (SEM), atomic force microscopy (AFM) and thermogravimetric analysis (TGA) were techniques used to further characterise the samples. HRTEM, SEM and AFM showed that poly-ε-caprolactam (or Nylon 6) was attached to MWNTs along their sidewalls and that the coverage was relatively uniform. The extent of the functionalisation of MWNT18C was estimated employing TGA technique: the content of organics in this sample was about 13% (w/w);[22(a)] this high value is evidently due to the formation of Nylon 6 polymer. MWNT18A was characterised by FTIR and TGA,[22(a)] The FTIR spectrum exhibited the presence of NH₂ in MWNT18A, and TGA found ca. 9% (w/w) of organic content for this sample.

The characterisation of MWNT16DT (Fig. 1(B)) was performed by HRTEM and energy dispersive X-ray spectroscopy (EDX). HRTEM showed the binding of 1,6-hexanedithiol to MWNTs (Fig. 2(B)), as compared with bare sidewalls of pristine MWNTs (Fig. 2(A)). EDX confirmed the presence of sulfur atoms in the MWNT16DT sample (Fig. 2(D)), indicating the successful functionalisation of MWNTs with 1,6-hexanedithiol, whereas no sulfur was identified in pristine MWNTs (Fig. 2(C)). TGA analysis suggested that MWNT16DT contains ca. 5% (w/w).
of organics (Fig. 2(E)). MWNTODA was characterised by TGA as well (Fig. 2(E)), which showed the organic content of about 5% (w/w).

2.2. Assessment of Complement Activation in Human Serum via the Classical or Alternative Pathways by Exposure to Chemically Modified MWNTs

Firstly, the nanotubes were tested for their ability to form stable suspensions in human serum after ultrasoundication. The pristine and chemically modified samples were incubated in human serum for 1 and 16 h (Fig. 3). The results showed that a large fraction of the nanotubes remain in suspension for a long period, although it was impossible to afford complete debundling (that is breaking the nanotube aggregates). In particular, this was observed at 1 h after ultrasoundication (Fig. 3(A)). After 16 h, pristine MWNT, MWNT16DT and MWNTODA samples showed further noticeable precipitation (Fig. 3(B)). MWNT18, MWNT18C and MWNT18A remained completely in suspension even after 16 h of incubation. The dispersibility of chemically modified MWNTs was also assessed in phosphate-buffered saline (PBS) (Fig. 4) and water (Fig. 5). The samples dispersed in PBS started precipitating after 30 min, as shown in Figure 4. MWNT18C and MWNT18A suspension in water remained stable for more than 16 h (Fig. 5(B)), whereas MWNTODA and MWNT16DT samples were hardly dispersed from the very beginning and rapidly precipitated after finishing ultrasoundication (Fig. 5(A)).

Then, MWNT samples were tested for complement system activation. The extent of complement consumption (activation) by the chemically modified nanotubes was measured using haemolytic assays as described previously by Salvador-Morales et al. The results (Fig. 6) show that like SWNTs and DWCNTs, pristine MWNTs, treated as a positive control, do activate the classical pathway. At the same time, the four chemically modified MWNT samples tested (MWNT18A, MWNT18C, MWNTODA and MWNT16DT) exhibited greatly reduced levels of complement consumption via the classical pathway in comparison to pristine MWNTs. The nanotubes treated with ε-caprolactam (MWNT18C, Fig. 6) generated the lowest level of complement activation via the classical pathway. MWNT18A, MWNT16DT and MWNTODA also induced low complement activation via the classical pathway, although they activated complement more than MWNT18C. All these nanotube samples exhibited lower levels of complement activation than the positive control zymosan did (Fig. 6). The level of complement activation by these samples via the classical pathway was compared with the amount of 125I-C1q bound to them, as reported in Table I. Figure 7 shows a clear correlation between the amount of C1q bound to functionalised carbon nanotubes and the extent of complement consumption that these samples induce (Fig. 6). MWNT18C is the sample that bound the least C1q amount, and it showed the lowest complement consumption via the classical pathway as well. The pristine MWNTs bound the greatest amount of C1q, and this sample induced the highest complement consumption via the classical pathway (Fig. 6).

For the assessment of activation of the alternative pathway, the incubation of serum with the potential activator and the haemolytic assay were conducted in the presence of Mg2+ cations but in the absence of Ca2+. The absence of Ca2+ cations in this assay blocks classical pathway activation. Very low levels of complement activation via the alternative pathway were observed for all the nanotube samples (Fig. 8) in comparison to the level generated by the positive control zymosan. Pristine MWNTs and MWNT16DT did not detectably activate the alternative pathway (Fig. 8). In contrast to the classical pathway, where all the chemically modified nanotubes activate less than pristine MWNTs, chemical modification on average increased complement consumption compared to pristine MWNTs. The observed low percent of complement consumption via the alternative pathway has an approximate inverse correlation with the amount of 125I-factor H (a down-regulator of alternative pathway activation) bound to the nanotube samples (Table I and Fig. 9). MWNT16DT did not fit this trend. It stimulated much less complement consumption than would be expected from the amount of factor H bound. This may be due to several reasons. One is the low extent of organic functionalisation, of ca. 5% by weight as shown by TGA analysis (Fig. 2(E)).
Table I. Binding of \( ^{125}\text{I}-\text{C1q} \) and \( ^{125}\text{I}-\text{Factor H (FH)} \) to pristine and chemically modified MWNTs in the presence of human serum. The nanotube amounts used in this experiment were 1.25 mg.

<table>
<thead>
<tr>
<th>MWN sample</th>
<th>% C1q bound</th>
<th>% FH bound</th>
<th>% C1q bound</th>
<th>% FH bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pristine MWNTs</td>
<td>57</td>
<td>38</td>
<td>0.43</td>
<td>6.0</td>
</tr>
<tr>
<td>MWNT18C</td>
<td>6.0</td>
<td>27</td>
<td>6.0</td>
<td>6.0</td>
</tr>
<tr>
<td>MWNT18A</td>
<td>15</td>
<td>30</td>
<td>0.49</td>
<td>6.0</td>
</tr>
<tr>
<td>MWNT16DT</td>
<td>41</td>
<td>14</td>
<td>0.45</td>
<td>6.0</td>
</tr>
<tr>
<td>MWNT18ODA</td>
<td>27</td>
<td>7.0</td>
<td>1.2</td>
<td>6.0</td>
</tr>
</tbody>
</table>

The less the derivatisation, the more likely that the sample will behave like pristine MWNTs. Similarly to pristine MWNTs, MWNT16DT is less capable of forming a stable suspension than the other functionalised nanotubes studied. The poor suspension of this sample in buffer solution reduced its interaction surface area for complement activation. It is also possible that factor H binds in a different configuration on MWNT16DT, which is more active in down-regulating complement.

2.3. Binding Properties of Complement and Other Plasma Proteins to Chemically Modified MWNTs

Binding of (unlabelled) proteins from serum or plasma to chemically modified nanotubes was tested, and the results are shown in Figure 10. To explain the mechanism of complement activation via the classical pathway and to examine the interaction of complement proteins with chemically modified nanotubes, the binding of C1q and factor H to MWNTs was studied. In Figure 10, the C1q band pattern is not visible in any of the SDS-PAGE tracks representing Coomassie blue stained proteins bound to carbon nanotubes (tracks 3–6, 8–9). This might be due to the fact that C1q polypeptide chains co-run with the ~25 kDa bands of apolipoprotein AI making them difficult to see.

For this reason it was decided to detect C1q by Western blotting. Western blotting (Fig. 11) confirms the binding of C1q to pristine and chemically modified MWNT samples. Very low binding is seen with MWNT18C (Fig. 11, track 3) consistent with the very low percent complement consumption via the classical pathway (Fig. 6), as well as with the very low amount of \( ^{125}\text{I}-\text{C1q} \) bound to it (Table I). The strongest band in the blot of C1q corresponds to pristine MWNTs (Fig. 11, track 1). This is the sample that bound the highest amount of \( ^{125}\text{I}-\text{C1q} \) (Table I) and has the highest complement consumption (Fig. 6).

Factor H was one of the other human plasma proteins that bound to pristine MWNTs (Fig. 10, track 3), but which was not easily identifiable because of the low amount bound. In Figure 10, track 3, factor H may be the band around 155 kDa which coincides with the protein band of the standard FH visible in Figure 10, track 10. In further support of this, Table I shows the binding of

![Fig. 7. Correlation between the binding of \( ^{125}\text{I}-\text{C1q} \) and complement consumption via the classical pathway. This graph was plotted using the values reported in Table I and Figure 6.](image)
Apart from C1q and factor H, other human plasma proteins were bound to pristine and chemically modified MWNTs (MWNT18, MWNT18C and MWNT18A) and were readily visible by Coomassie blue staining (Fig. 10).

After exhaustive washing in the same buffer, samples of the affinity resin were run through affinity columns containing Sepharose (negative control) or Sepharose-MWNTs, each column equilibrated in the same buffer. These proteins (apolipoprotein AIV, AI, CIII) are the same as those which bind to DWNTs.25 Pristine MWNTs, MWNT18, MWNT18C and MWNT18A (Fig. 10, tracks 4 and 5) bound much less protein in total than the other MWNT samples. Apolipoprotein AIV and CIII seem to be absent, and fibrinogen much diminished in these samples. In addition, faint protein bands appear at 6 kDa in tracks 8 and 9. MWNTODA and MWNT16DT were however more difficult to suspend homogeneously in the Sepharose matrix than the other MWNT samples, therefore these samples were more aggregated and less contact surface may have been exposed to the human plasma. As a consequence less plasma proteins may have bound to them. It is notable that more albumin appeared to be bound to all MWNT samples than to SWNTs or DWNTs.25 The greater inner diameter of MWNTs may result in plasma entering the nanotubes by capillary action. This internalised material may be difficult to wash out. Therefore, albumin (which is 50% of the total plasma protein) may be present not because it is bound to the MWNTs, but simply because traces of plasma remain within the nanotubes after exhaustive washing (but are moved out by electrophoresis).

3. DISCUSSION

3.1. Assessment of Complement Activity via the Classical and Alternative Pathway on Human Serum Samples After Their Exposure to Chemically Modified MWNTs

These proteins (apolipoprotein AIV, AI, C-III) are the same as those which bind to DWNTs.25 Pristine MWNTs, MWNT18, MWNT18C and MWNT18A (Fig. 10, tracks 3–6) all bound a similar spectrum of proteins, except for an unidentified low-molecular-weight species (<3.4 kDa) which was present only in the MWNT18 and MWNT18C tracks (tracks 4 and 5). Fibrinogen (α, β and γ chains), apolipoprotein AIV, AI and CIII, and albumin were the proteins that bound to MWNT samples in greatest amount.

The total amount of protein appears to diminish in the order MWNT18 > MWNT18C = MWNT18A, MWNT16DT and MWNTODA samples (Fig. 10, tracks 8 and 9) bound much less protein in total than the other MWNT samples. Apolipoprotein AIV and CIII seem to be absent, and fibrinogen much diminished in these samples. In addition, faint protein bands appear at ~5 and ~18 kDa in tracks 8 and 9. MWNTODA and MWNT16DT were however more difficult to suspend homogeneously in the Sepharose matrix than the other MWNT samples, therefore these samples were more aggregated and less contact surface may have been exposed to the human plasma. As a consequence less plasma proteins may have bound to them. It is notable that more albumin appeared to be bound to all MWNT samples than to SWNTs or DWNTs.25 The greater inner diameter of MWNTs may result in plasma entering the nanotubes by capillary action. This internalised material may be difficult to wash out. Therefore, albumin (which is 50% of the total plasma protein) may be present not because it is bound to the MWNTs, but simply because traces of plasma remain within the nanotubes after exhaustive washing (but are moved out by electrophoresis).

There were considerable differences in the amount of C1q bound to different chemically modified MWNT samples (Fig. 7), and C1q binding correlated with the extent of classical pathway activation. These differences are likely
to be due to the type of functionalisation. For example, the low amount of C1q attached to MWNT18C and MWNT18A may be due to more hydrophilic nature of these samples. The presence of only monomeric amino acid units in MWNT18A sample makes it less hydrophilic than MWNT18C, which has chemically bound polyamide molecules. On the other hand, MWNTODA and MWNT16DT have more hydrophobic functionalising groups.

The activation of the complement system via the alternative pathway induced by chemically modified MWNTs was very low (Fig. 8). Pristine MWNTs and MWNT16DT did not activate it. However, in contrast to what was observed for classical pathway activation, chemical modification of MWNTs in general slightly increased alternative pathway activation. The mechanism involved in the alternative pathway activation by the chemically modified nanotubes relies on the presence of surface electron donor groups, represented by NH and/or NH2 on MWNT18C, MWNT18A and MWNTODA surfaces, and SH groups on MWNT16DT. Alternative pathway activation depends on the deposition of C3b on the activating surface. C3b could strongly bind through multiple hydrogen bonds to NH/NH2 and SH functional groups present on the nanotube surfaces. The alternative pathway is further regulated by the presence or absence of complement regulatory proteins or regulatory protein binding sites on potential target surfaces (in this case MWNTs). In the present study, it was found that the more factor H was bound to the nanotubes, the less was the activation of the alternative pathway, as shown in Figures 8 and 9. The exception to this trend is MWNT16DT which did not show the same correlation between the amount of factor H bound and the levels of complement activation via alternative pathway.

As a whole, the levels of complement activation induced by the four different chemically modified MWNTs were low. Chemical modifications are therefore likely to improve considerably the biocompatibility properties of MWNTs in human blood plasma. MWNT18A and MWNT18C, for example, are much more readily dispersible in plasma than pristine MWNTs, activate the classical pathway much less, and only marginally activate the alternative pathway. The treatment of MWNT18 with e-caprolactam produces a Nylon 6 polymer on the nanotube surface. The “good” biocompatibility aspect of Nylon 6 wrapped to MWNT18C agrees with the “good biocompatibility” performance of Nylon/carbon nanotube composites, recently reported by Endo et al.20

We can compare complement activation between unmodified DWNTs,25 chemically modified MWNTs (Figs. 6 and 8). For classical pathway activation by DWNTs, the binding of C1 occurs followed by activation of C4 and C2. However, activation may not go beyond that, because of lack of suitable covalent binding sites for C4b or C3b. In the case of chemically modified MWNTs, the binding of C1 is diminished due to the chemical modification (Fig. 7). Since classical pathway complement consumption correlates with C1q binding, the most important effect of chemical modification is its effect on C1q binding. With these modified MWNTs, however, when C1 binds and is activated to cleave C4 and C2, there are suitable “acceptor” groups for a strong binding of C4b and C3b so that complement activation can proceed up to C9. These modified MWNTs also bind less FH than pristine MWNTs, so will have less downregulation of the alternative pathway. However the major effect of the chemical modifications is to diminish classical pathway activation by diminishing C1q binding. The binding of human plasma proteins to chemically modified nanotubes has also been useful to investigate protein adsorption phenomenon. It is known that protein adsorption on blood contact surfaces can determine if those surfaces are biocompatible or not.27 The adsorption phenomenon on those materials very often depends on the extent of their functionalisation. The extent of the chemical modification is important since the greater the coverage of the original surface, the lower will be the complement activation. In the present work the functionalisation of MWNT18C, MWNT18A, MWNTODA and MWNT16DT gave a weight contribution of ca. 13% to 16% (Fig. 2(E) and 5% (w/w) (Fig. 2(E)), respectively. The surface area of carbon nanotubes functionalised by the organic material (e-caprolactam, alanine, 1-octadecylamine and 1,6-hexanediol, respectively) may therefore increase to a similar degree. Although the functionalisation of MWNT18C, MWNT18A, MWNTODA and MWNT16DT is low in terms of surface area, the change in interaction between some of the chemically modified nanotubes and proteins might be high since the organic material attached to the nanotubes can bind and interact with water molecules, which increases their volume. The increased volume may sterically hinder C1q binding. Thus, the binding of C1q to MWNTs may be lower than expected from a simple 13% reduction in surface area. This will result in low levels of activation of the classical pathway.

4. CONCLUSIONS

The four different types of chemical functionalisation analysed in this work reduced considerably the level of activation of the human serum complement system via the classical pathway in comparison to pristine MWNTs. This together with improved dispersion properties indicates that these functionalisations may confer to MWNTs a better biocompatibility performance.

Although haemolytic assays have shown that the chemical functionalisations diminish complement activation, it is important to keep in mind that this type of assay is not the only criterion to determine whether or not a biomaterial is biocompatible. To design blood contacting artificial surfaces with improved antithrombogenic properties, several
other biological assays must be considered such as determination of clotting parameters, interaction with platelets and blood cells.

5. EXPERIMENTAL DETAILS

5.1. Chemicals

1,8-Diaminooctane, 1-octadecylamine, 1,6-hexanedithiol, ε-caprolactam and L-alanine (all 99% purity) were purchased from Sigma-Aldrich (Poole, UK) and were used without additional purification. MWNTs prepared by CVD process (97% + purity, diameter of 10–20 nm and length of 10–50 μm) were purchased from ILHIN Nanotech Co., Inc., Korea.

5.2. Chemical Modification of MWNTs

The chemical modification of pristine MWNTs with 1,8-diaminooctane followed by the covalent attachment of ε-caprolactam or L-alanine was carried out as reported by Basiuk et al.22 The functionalisation of MWNTs through the direct reaction of 1-octadecylamine22b, c and 1,6-hexanedithioi22d, e with the closed caps and sidewall defects of pristine MWNTs was done using the gas-phase solvent-free procedure.23 Briefly, 100 mg of MWNTs and 20 mg of the amine or thiol were placed together into the reactor. The reaction was performed at 150–170 °C for 3 h at ca. 1 Torr. The reaction conditions (high temperature and vacuum) helped to minimize the amount of 1-octadecylamine and 1,6-hexanedithiol physically adsorbed on the nanotube surface. During this procedure, amine/thiol vapors reacted with MWNTs, and the unreacted organic molecules were spontaneously removed from the product under further heating/pumping, and condensed in the upper cold part of the reactor. Before extracting the chemically modified nanotube material, to avoid contamination, the excess amine or thiol was removed from the upper reactor part with a cotton wool wet with ethanol.

5.3. Characterisation of Chemically Modified Carbon Nanotube Samples

The characterisation of MWNT18C and MWNT18A samples was conducted as reported by Basiuk et al.22b,c using FTIR, HRTEM, SEM, AFM and TGA techniques. HRTEM characterisation of MWNT16DT was conducted using a HRTEM JEOL 4000 EX instrument operating at 200 kV. TGA profiles of MWNTODA and MWNT16DT were recorded using a DuPont Thermal Analyzer 951. Experiments were carried out in air flow of 100 ml/min at a heating rate of 10 °C/min up to 1000 °C. EDX studies were performed with a JEOL 119 JSM-5900 scanning electron microscope operating at 20 kV.

5.4. Preparation of Rabbit and Sheep Erythrocytes, Antibody-Sensitised Erythrocytes and Human Serum and Plasma

These materials were prepared as described in Ref. [25].

5.5. Haemolytic Assays (Classical and Alternative Pathways)

Complement activation/consumption via the classical and alternative pathways were assayed as reported in Ref. [25]. Briefly, the carbon nanotubes were incubated for 1 h at 37 °C with human serum, then centrifuged, and the complement activity in the supernatant assayed by its capacity to lyse red blood cells.

5.6. 125I-C1q and 125I-FH Binding to Chemically Modified MWNTs

C1q was isolated from pooled human plasma using affinity chromatography on IgG Sepharose.26 Factor H was isolated by immunoaffinity chromatography using the immobilised monoclonal antibody MRCOX23.29 Iodination of C1q was carried out as described by Salvador-Morales et al.25 while iodination of factor H was done with a standard iodogen-catalysed reaction31 using 200 μg iodogen, 50 μg of factor H, 0.5 mCi Na-125I (Amersham Bioscience UK limited) in the buffer PBS-0.5 mM EDTA. The specific activity of 125I-FH was 9 × 10⁶ cpm/μg. The binding of 125I-C1q and 125I-FH to MWNT samples was examined as follows. Approximately 5 × 10⁵ cpm (40 ng) of 125I-C1q was added to 100 μl of 20 mM HEPES, 50 mM NaCl, 5 mM EDTA, pH 7.0 followed by the addition of 100 μl of human serum. Subsequently this mixture was added to 1.25 mg of MWNT and sonicated for 3 min. The samples were incubated for 1 h at room temperature. The samples were then centrifuged at 13000 rpm for 5 min and the supernatants were discarded. The nanotubes were washed several times with 20 mM HEPES, 50 mM NaCl, 5 mM EDTA, pH 7.0 until the 125I washed into the supernatant fell to background level. MWNTs were placed in a Mini-Assay type 6-20 manual γ counter (Mini Instruments, Burnham-on-Crouch, Essex, UK) to measure the amount of 125I-C1q bound. For the negative control experiments, all the nanotube samples were incubated with 125I-C1q and serum in a buffer containing 4 M NaCl, 10 mM CAPS, pH 11.1.

The amount of 125I-Factor H bound to MWNT samples was measured as above. 3 × 10⁵ cpm (34 ng) of factor H was employed in these reactions.

5.7. Western Blotting to Detect C1q

Western blotting of C1q was carried out as described in Ref. [25].
5.8. Selective Binding of Human Plasma Proteins to MWNTs

The experimental procedure for the selective binding of the human plasma proteins to chemically modified MWNTs and SDS-PAGE procedure is reported in Salvador-Morales et al.25

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References and Notes


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