The Development of Carbon Nanotubes as Cancer Therapeutics: From Pharmacokinetics and Toxicology to Therapy

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Declaration

This thesis describes research conducted in the School of Pharmacy, University of London between October 2006 and December 2009 under the supervision of Prof. Kostas Kostarelos. I certify that the research described is original and that any parts of the work that have been conducted by collaboration are clearly indicated. I also certify that I have written all the text herein and have clearly indicated by suitable citation any part of this dissertation that has already appeared in publication.

Signature: [Signature] Date 19/07/2010
MUM AND DAD

THANK YOU FOR YOUR ENDLESS LOVE AND SUPPORT

A Toi Chère Mère et à Toi Cher Père

Je Dédie Cette Thèse
SUCCESS IS A JOURNEY AND NOT A DESTINATION

ARTHUR ROBERT ASHE, JR
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ABSTRACT

The central hypothesis for this thesis is that functionalised carbon nanotubes (CNT) can be developed for cancer therapy. This was based on current proof-of-principle studies shown that CNT have an extraordinary ability to cross the plasma membrane and deliver therapeutic molecules such as protein and DNA independent of the functional group and the cell type in addition to their unique physicochemical properties. At the same time it is well known that the therapeutic efficacy of many potent and promising anticancer drugs has been limited due to their low water solubility, increased drug resistance and high cytotoxic side effects. Therefore, it was hypothesised that the engineering of novel CNT: anticancer drug hybrids represent a valuable alternative to achieve enhanced cellular uptake and increased efficacy of such therapeutic agents.

The aim of this thesis was to elucidate the important and critical parameters in CNT pharmacokinetics and toxicology and to take this further into establishing effective CNT nanovectors for cancer therapeutics as gaps still exist in the accurate understanding of CNT impact and interaction with the biological milieu. This was achieved by: (1) investigating the effect of surface functionalisation, coating and dispersion properties on CNT biodistribution after intravenous administration; (2) studying the interference between CNT and the widely used cytotoxic assays (MTT, LDH and Annexin V/PI) and subsequently developing a reliable toxicity assay; (3) exploring the effect of chemical functionalisation strategies on the toxicity of CNT in vitro and in vivo; (4) evaluating the cellular uptake and efficacy of CNT: Doxorubicin complexes and CNT-Methotrexate constructs both in in vitro cell monolayers and in vivo tumour models.

It was found that the biodistribution of CNT is highly dependent on their physicochemical characteristics mainly the dispersion properties and individualisation of the CNT. It was also concluded that the systemic administration of CNT is still in its infancy and that focus at this stage should only be on local administration since RES organ uptake is highly observed with CNT. In addition, as hypothesised CNT were found to interfere with the MTT, LDH and Annexin V/PI assays and based on the pitfalls of those assay we have developed a reliable cytotoxic assay. Based on the developed assay, chemically functionalised CNT were found non-cytotoxic compared to polymer coated CNT. Moreover, the in vivo toxicological profile of chemically functionalised MWNT was further investigated using a structure-activity paradigm of toxicity based on the direct exposure of the abdominal cavity of mice to CNT. It was found that chemical functionalisation strategies that debundled and disaggregated CNT led to a sharp reduction of their effective length and completely alleviated the asbestos-like pathogenic behavior observed with their long pristine counterparts.

The engineering of CNT for use as cancer therapeutics was also achieved using CNT: Doxorubicin complexes and CNT-Methotrexate constructs. Both novel nanohybrids showed a better in vitro cytotoxic activity compared to the drug alone especially when a tetrapeptide linker was introduced with the CNT-Methotrexate constructs. However, no therapeutic efficacy or tumour reduction was observed in vivo using tumour models compared to the drug alone after intra-tumoral administration.
Overall, key factors in CNT biodistribution together with the *in vitro* and *in vivo* toxicological profile of chemically functionalised MWNT were reliably determined and thereafter functionalised MWNT were developed as delivery systems for cancer therapeutics.
Published Articles


1 Parts of the work presented in this thesis have been published in some of the cited publications
Submitted Articles


Book Chapters


Abstracts and Proceedings in Conferences


TABLE OF CONTENTS

ACKNOWLEDGEMENTS .................................................................................................................. 4

ABSTRACT .......................................................................................................................................... 5

PUBLICATIONS .................................................................................................................................. 7

TABLE OF CONTENTS .................................................................................................................... 9

LIST OF FIGURES ............................................................................................................................ 15

LIST OF SCHEMES .......................................................................................................................... 18

LIST OF TABLES ................................................................................................................................ 20

LIST OF ABBREVIATIONS ............................................................................................................... 21

CHAPTER I ......................................................................................................................................... 24

INTRODUCTION

1.1. Carbon nanotubes ..................................................................................................................... 25

1.2. CNT functionalisation ............................................................................................................. 27

1.3. CNT biodistribution and pharmacokinetics in vivo ................................................................. 30

1.3.1. Tissue biodistribution of non-covalently coated CNT .......................................................... 30

1.3.2. Tissue biodistribution of covalently functionalised CNT: .................................................... 32

1.4. CNT cytotoxicity in vitro and in vivo ....................................................................................... 37

1.4.1. Cytotoxicity of CNT in vitro .............................................................................................. 37

1.4.1.1. CNT functionalisation and dispersibility ........................................................................ 38

1.4.1.2. Composition of functional group or coating ................................................................... 39

1.4.1.3. Nature of CNT surface (charge, hydrophobicity/ hydrophilicity) .................................... 39

1.4.1.4. CNT size (diameter and length) and aspect ratio ............................................................. 40

1.4.1.5. CNT metal impurities ........................................................................................................ 41

1.4.2. Cytotoxicity of CNT in vivo ............................................................................................... 47

1.4.2.1. Effects following pulmonary exposure ............................................................................ 47

1.4.2.2. Effects following skin exposure ....................................................................................... 50
1.4.2.3. Effects following systemic exposure ..........................................................50
1.5. CNT in the delivery of cancer therapeutics ..........................................................57

1.5.1. Delivery of anticancer agents through non-covalent functionalisation with the CNT ..........................................................57

1.5.1.1. CNT: Anticancer drugs .............................................................................57
1.5.1.2. CNT: Small interference RNA (siRNA) .............................................60
1.5.2. Delivery of anticancer agents through covalent conjugation to CNT .......61
1.5.3. NIR and radiofrequency ablation (hyperthermia) ........................................63

CHAPTER II ..............................................................................................................65

PROJECT AIM AND OBJECTIVES

CHAPTER III ..............................................................................................................68

MATERIALS AND METHODS

3.1. Evaluation of the biodistribution profile of functionalised MWNT ..........68

3.1.1. Chemical functionalisation of MWNT (Aryl f-MWNT) .........................68

3.1.1.1. Oxidation of pristine MWNT (MWNT-COOH) .................................68
3.1.1.2. Aryl Functionalised MWNT-NHBOC ..............................................69
3.1.1.3. Aryl Functionalised MWNT-NH$_3^+$ (BOC deprotection procedure) ..69
3.1.1.4. Quantitative Kaiser test .........................................................................70
3.1.1.5. Preparation of DTPA-aryl f-MWNT .................................................71
3.1.1.6. Preparation of $^{111}$In radiolabelled DTPA-aryl f-MWNT ...............71
3.1.1.7. Transmission Electron Microscopy of aryl f-MWNT .......................72

3.1.2. Tissue biodistribution studies of aryl f-MWNT .........................................72

3.1.2.1. Whole body imaging of $^{[111]}$In]-DTPA-aryl f-MWNT using SPECT/CT ..................................................................................73
3.1.2.2. Quantitative radioactivity analysis of $^{[111]}$In] DTPA-aryl f-MWNT. 73
3.1.2.3. Tissue histology following administration of aryl f-MWNT ..........74

3.2. Evaluation of the biodistribution profile of carbohydrate functionalised and iodine filled SWNT .........................................................................................74

3.2.1. Filling and functionalisation of SWNT (Carbohydrate functionalised and iodine filled SWNT) .................................................................................74
3.2.1.1. Filling of SWNT with Na^{125}I (Na^{125}I@SWNT) .................................75
3.2.1.3. Glycosylation of f-Na^{125}I@SWNT (GlcNAcD-Na^{125}I@SWNT) 676
3.2.1.4. Preparation of GlcNAcD-Na^{125}I@SWNT aqueous dispersions .........76
3.2.1.5. Transmission Electron Microscopy .....................................................76
3.2.2. Cytotoxicity assessment of carbohydrate functionalised and iodine filled SWNT (GlcNAcD-NaI@SWNT) \textit{in vitro} .................................................................77
3.2.2.1. The ‘modified LDH’ mLDH assay of GlcNAcD-NaI@SWNT ........77
3.2.3. Tissue biodistribution studies of GlcNAcD-Na^{125}I@SWNT ..............78
3.2.3.1. Whole body imaging of GlcNAcD-Na^{125}I@SWNT and Na^{125}I using SPECT/CT .................................................................78
3.2.3.2. Quantitative radioactivity analysis of GlcNAcD-Na^{125}I@SWNT or Na^{125}I ........................................................................79
3.2.3.3. Tissue histology following administration GlcNAcD-NaI@SWNT 79
3.3. The Development of accurate and reliable cytotoxicity assay to determine CNT toxicity .................................................................................................................80
3.3.1. Preparation of chemically functionalised and Pluronic coated MWNT...80
3.3.2. The cellular uptake and internalisation both types of MWNT ..........82
3.3.2.1. Flow cytometry (Side scatter) and light microscopy .................82
3.3.2.2. Transmission electron microscopy of cell sections .......................82
3.3.3. Cytotoxicity assessment of both types of MWNT .........................83
3.3.3.1. MTT assay .........................................................................................83
3.3.3.2. Annexin V-FITC/ PI assay .................................................................85
3.3.3.3. The original LDH assay .................................................................87
3.4. Can chemical functionalisation alleviate the asbestos-like carcinogenicity risk associated with long pristine MWNT? .................................................................93
3.4.1. Functionalisation of long pristine MWNT (NT\textsubscript{2} Pristine) ........93
3.4.2. Toxicological effects of intraperitoneally injected NT\textsubscript{2} samples ....95
3.4.2.1. Inflammatory reaction after injections of NT\textsubscript{2} samples .........95
3.4.2.2. The assessment of granuloma formation after 7 days post-injection 96
3.5. CNT: Doxorubicin non-covalent supramolecular complexes for cancer therapeutics .................................................................................................................97
3.5.1. The formation and characterisation of CNT: Doxorubicin complexes ....97
3.5.2. Cytotoxicity assessment of MWNT: Doxorubicin complexes *in vitro* Cell culture ............................................................................................................................99

3.5.3. Therapeutic efficacy of MWNT: Doxorubicin complexes *in vivo* ..........100

  3.5.3.1. Syngeneic tumour model........................................................................100
  3.5.3.2. Intratumoral administration of MWNT: Doxorubicin complexes ... 100
  3.5.3.3. Tissue histology following administration of MWNT: Dox complexes .................................................................101

3.6. Chemically conjugated CNT-Methotrexate conjugates for cancer therapeutics ................................................................................................................................101

  3.6.1. The synthesis and characterisation of CNT-Methotrexate conjugates ...101
  3.6.2. Cellular uptake and internalisation of the MWNT-MTX conjugates .... 103
  3.6.3. Cytotoxicity assessment of the MWNT-MTX conjugates *in vitro* ......104
  3.6.4. Therapeutic efficacy of the MWNT-MTX conjugates *in vivo* ..........104

  3.6.3.1. Intratumoral administration of MWNT-MTX constructs ................105

3.7. Statistical analysis .................................................................................................106

CHAPTER IV ...................................................................................................................107

*THE PHARMACOKINETIC PROFILE OF DIFFERENT CARBON NANOTUBES IN VIVO*

4.1. Evaluation of the biodistribution profile of functionalised MWNT.........108

  4.1.1. Aryl functionalised MWNT: Functionalisation and characterisation....109
  4.1.2. Tissue biodistribution of aryl functionalised MWNT ...............................111
  4.1.3. Tissue histology following administration of aryl-f-MWNT ..........115
  4.1.4. DISCUSSION...............................................................................................118

4.2. Evaluation of the biodistribution profile of carbohydrate functionalised and iodine filled SWNT ..................................................................................................................123

  4.2.1. Carbohydrate functionalised and iodine filled SWNT: Functionalisation and characterisation ..............................................................124
  4.2.2. Cytotoxicity assessment of carbohydrate functionalised and iodine filled SWNT (GlcNAcD-NaI@SWNT) *in vitro* ............................126
4.2.3. The biodistribution and imagining of carbohydrate functionalised and iodine filled SWNT (GlcNAcD-NaI25I@SWNT) in vivo........................................127

4.2.4. Tissue histology following administration of carbohydrate functionalised and iodine filled SWNT (GlcNAcD-NaI@SWNT)........................................130

4.2.5. DISCUSSION..................................................................................................132

4.3. CONCLUSION ....................................................................................................136

CHAPTER V ....................................................................................................................138

THE TOXICOLOGICAL ASSESSMENT OF FUNCTIONALISED CARBON NANOTUBES IN VITRO AND IN VIVO

5.1. Development of accurate and reliable cytotoxicity assay to determine carbon nanotubes toxicity..................................................................................................................139

5.1.1. CNT characteristics and functionalisation.......................................................140

5.1.2. The cellular uptake and internalisation functionalised MWNT ................142

5.1.3. Toxicological assessment of both types of MWNT using established assays .................................................................................................................................146

5.1.3.1. MTT assay: ..............................................................................................146

5.1.3.2. Annexin V-FITC/ PI assay: ....................................................................147

5.1.3.3. Original LDH assay: ...............................................................................150

5.1.3.4. The modified LDH assay: ......................................................................151

5.1.4. DISCUSSION..................................................................................................155

5.2. Can chemical functionalisation alleviate the asbestos-like carcinogenicity risk associated with long pristine MWNT? .................................................................161

5.2.1. CNT characteristics and functionalisation.......................................................162

5.2.2. Toxicological effects of intraperitoneally injected NT 2 samples ..........165

5.2.3. DISCUSSION..................................................................................................170

5.3. CONCLUSION ......................................................................................................174

CHAPTER VI ...................................................................................................................175

CANCER THERAPY USING CARBON NANOTUBES AS DELIVERY VECTORS

6.1. CNT: Doxorubicin supramolecular complexes for cancer therapeutics........176
6.1.1. The formation and characterisation of CNT: Doxorubicin complexes...177
6.1.2. Cytotoxicity assessment of MWNT: Dox complexes in vitro ............181
6.1.3 Therapeutic efficacy of MWNT: Dox complexes in vivo.....................182
6.1.4. Tumour histology following administration of MWNT: Dox complexes ..........................................................186
6.1.5. DISCUSSION..........................................................................................188
6.2. CNT-Methotrexate conjugates for cancer therapeutics.........................193
6.2.1. The preparation and characterisation of CNT-Methotrexate conjugates 194
6.2.2. Cellular uptake and internalisation of the MWNT-MTX conjugates ....197
6.2.3. Cytotoxicity assessment of the MWNT-MTX conjugates in vitro........198
6.2.4. Therapeutic efficacy of the MWNT-MTX conjugates in vivo .............201
6.2.5. DISCUSSION..........................................................................................203
6.3. CONCLUSION ..............................................................................................207

CHAPTER VII ........................................................................................................210

SUMMARY AND FUTURE WORK

7.1. MAIN LESSONS AND MESSAGES FROM THIS THESIS....................210
7.2. FUTURE WORK............................................................................................215

APPENDICES........................................................................................................219

BIBLIOGRAPHY.................................................................................................231
LIST OF FIGURES

CHAPTER I

Figure 1.1.1: Schematic representation of the two types of CNT (A) Single walled carbon nanotubes (SWNT) and (B) Multi-walled carbon nanotubes (MWNT). ........................................ 26

Figure 1.1.2: Different types of carbon nanotubes studied in biomedical applications. ........................................................................................................................... 28

CHAPTER IV

Figure 4.1.1: Characterisation of the aryl functionalised MWNT-COOH. .................. 110

Figure 4.1.2: Images of TLC strips after the labelling reaction and dilution of $^{[111]}$ In] DTPA-MWNT in PBS. .................................................................................................................. 111

Figure 4.1.3: Biodistribution profile of aryl functionalised MWNT ..................... 112

Figure 4.1.4: Biodistribution of $^{[111]}$ In] DTPA-MWNT in BALB/C mice after i.v. administration for 30 min, 4 hrs and 24 hrs .............................................................. 114

Figure 4.1.5: Tissue histology of mice injected with aryl f-MWNT dispersed in 5 % dextrose............................................................................................................................ 116

Figure 4.1.6: Tissue histology of mice injected with pristine MWNT (pMWNT) dispersed in Pluronic F127 and bovine serum albumin (BSA)................................. 117

Figure 4.2.1: Characterisation of GlcNAc-D-Na@SWNT .................................. 125

Figure 4.2.2: In vitro cytotoxicity assessment of GlcNAc-D-Na@SWNT by modified LDH assay .................................................................................................................. 126

Figure 4.2.3: Whole body SPECT/CT imaging was performed after 30 min, 4 hrs and 24 hrs post-intravenous injections with (A) 50μg and 250 μg (0.2MBq, 0.8MBq) of GlcNAc-D-Na$I@SWNT$; and (B) Na$^{125}$I (1.8MBq) with a scanning time of 40-60min. ...................................................................................... 128

Figure 4.2.4: Whole body SPECT/CT imaging was performed after 7 days post intravenous injection with (A) 250μg (0.8MBq) of GlcNAc-D-Na$I@SWNT$s and (B) Na$^{125}$I (1.8MBq) with a scanning time of 40-60min. .................................................. 128

Figure 4.2.5: Tissue biodistribution (A, B) and blood clearance profile (C, D) of GlcNAc-D-Na$I@SWNT$ (50μg, 0.2 MBq; A, C) or Na$^{125}$I (0.36 MBq; B, D) in mice after tail vain injection.................................................. 130
Figure 4.2.6: Tissue histology of mice injected with 'cold' GlcNAc^D-NaI@SWNT dispersed in BSA/saline.

CHAPTER V

Figure 5.1.1: A) Schematic of the structures of the ammonium functionalised MWNT (MWNT-NH_3^+) and Pluronic F127 coated MWNT respectively. B) Dispersion of MWNT-NH_3^+ and MWNT: F127 in aqueous media showing a good dispersibility of MWNT and C) Transmission Electron Microscopy (TEM) images of MWNT-NH_3^+ and MWNT: F127 respectively.

Figure 5.1.2: Analysis of cellular uptake of f-MWNT by light microscopy.

Figure 5.1.3: Analysis of cellular uptake of f-MWNT by light scattering using flow cytometry.

Figure 5.1.4: Cellular internalisation of MWNT inside human lung carcinoma A549 cell section observed by transmission electron microscopy.

Figure 5.1.5: Cell viability of human lung carcinoma A549 cells after 24 hrs incubation with f-MWNT.

Figure 5.1.6: Formazan –MWNT interaction by spiking the formazan with f-MWNT dispersions at 0-250μg/ml final concentrations.

Figure 5.1.7: Cell viability of human lung carcinoma A549 cells after 24hrs incubation with MWNT assessed by Annexin V-FITC/PI staining and quantified by flow cytometry after 24 hrs incubation with (A) MWNT-NH_3^+, (B) MWNT: F127 at a concentration of 0-125 μg/ml and (C) Pluronic F127 alone.

Figure 5.1.8: Cytotoxicity of human lung carcinoma A549 cells after 24hrs incubation with f-MWNT as assessed by the original LDH assay.

Figure 5.1.9: Validation of the modified LDH assay by comparing it to the MTT and Annexin V/PI (FACS) assays using cationic liposomes and DMSO as positive controls.

Figure 5.1.10: Cell survival of human lung carcinoma A549 cells after 24hrs incubation with MWNT-NH_3^+, MWNT: F127 and Pluronic F127 alone as assessed by the modified LDH assay (A). TEM of ultra-thin cell sections shown mitochondrial damage with cells treated with MWNT: F127 but not with Pluronic F127 alone (B).

Figure 5.2.1: Fibre characterisation using Transmission electron microscopy (TEM) and atomic force microscopy (AFM).

Figure 5.2.2: Inflammatory reaction in the peritoneal cavity 24 hrs post injection with fibres, exudation) after 24 hrs.
Figure 5.2.3: Inflammatory reaction in the peritoneal cavity 7 days post injection with fibres. ............................................................................................................................ 167

Figure 5.2.4: The effect of fibre on the diaphragms after 7 days. (A) SEM images of the diaphragm surface at low magnification and at high magnification (B) and (C) Histology using H & E staining. .......................................................................................... 169

CHAPTER VI

Figure 6.1.1: Normalised fluorescence intensity of MWNT: Dox complexes....... 178

Figure 6.1.2: TEM images of MWNT: Dox complexes. .............................. 180

Figure 6.1.3: The percentage cell viability of MCF-7 cells after 24 hr incubation at a final concentration of Dox (600 nM); MWNT:F127 (651.5 ng/ml); Pluronic (6.5 ng/ml) and Pluronic: Dox and MWNT: Dox at a mass ratio of 2:1............... 182

Figure 6.1.4: Growth curves after intratumoral administration of MWNT: Dox complexes in B16F10 melanoma tumours................................................................. 184

Figure 6.1.5: Survival curves after intratumoral administration of MWNT: Dox complexes in B16F10 melanoma tumours................................................................. 185

Figure 6.1.6: Hematoxylin and eosin-stained sections of tumour lesions........ 187

Figure 6.2.1: Dispersion photographs and TEM images of the different MWNT-MTX conjugates .................................................................................................................................. 196

Figure 6.2.2: Confocal microscopy images of MCF7 cells incubated in the absence (naive) and the presence of 10 μg/ml of rhodiumine B labelled MWNT –MTX construct ................................................................................................................... 198

Figure 6.2.3: Cell survival of human breast MCF7 cells after 3, 6 and 24 hrs incubation with the different MWNT-MTX conjugates......................................................... 199

Figure 6.2.4: Light microscopy images of human breast MCF7 cells after 3, 6 and 24 hrs incubation with the different MWNT-MTX conjugates......................................... 200

Figure 6.2.5: Growth curves after intratumoral administration of MWNT-MTX conjugates in DU145 prostate xenograft tumours............................................................ 202
LIST OF SCHEMES

**Scheme 3.1.1:** Functionalisation scheme of *in situ* generated aryl functionalised MWNT and formation of ammonium functionalised MWNT-NH$_3^+$ .................................................. 70

**Scheme 3.1.2:** Illustration of the functionalisation reaction of $^{111}$In radiolabelled DTPA-aryl $f$-MWNT .......................................................................................................................... 71

**Scheme 3.1.3:** Schematic representation of the different steps involved in the chemical functionalisation of SWNT ........................................................................................................... 75

**Scheme 3.1.4:** Schematic representation of the preparation of the two types of (A) the non-covalently functionalised MWNT and (B) chemically functionalised MWNT-NH$_3^+$ .......................................................................................................................... 81

**Scheme 3.1.5:** Representation of the protocol from the MTT assay with its chemical reaction. .................................................................................................................................................. 85

**Scheme 3.1.6:** The basis of the Annexin V-FITC /PI assay ........................................... 87

**Scheme 3.1.7:** Lactate dehydrogenase (LDH) enzyme mediated chemical reaction. .................................................................................................................................................. 88

**Scheme 3.1.8:** Representation of the protocol from the original LDH assay .......... 90

**Scheme 3.1.9:** Representation of the protocol for the modified LDH assay .......... 92

**Scheme 3.1.10:** Chemical functionalisation of the long pristine MWNT (NT$_2$ Pristine). ............................................................................................................................................... 93

**Scheme 3.1.11:** Chemical synthesis of the different MWNT-MTX conjugates. ... 102

**Scheme 4.2.1:** Schematic representation of the carbohydrate functionalised and iodine filled GlcNAc$^D$-NaI@SWNT construct that will be biologically tested........... 125

**Scheme 5.2.1:** Chemical functionalisation of long pristine MWNT (NT$_2$ Pristine). Reaction 1 depicts the introduction of an Alkyl group onto the surface of the NT$_2$. Reaction 2 represents the functionalisation of NT$_2$ through the 1, 3 dipolar cycloaddition and the introduction of TEG moieties onto the NT$_2$ surface......... 162

**Scheme 5.2.2:** The effect of CNT length on their clearance by phagocytosis........... 172

**Scheme 6.2.1:** Chemical synthesis of the different MWNT-MTX conjugates....... 195
Scheme 7.1: A representation of the factors affecting CNT dispersibility and individualisation and subsequently the biodistribution profile of CNT. 211

Scheme 7.2: A representation of doxorubicin stacking onto the surface of MWNT leading to star like complexes as shown by the TEM image. 213

Scheme 7.3: A representation of CNT-MTX construct with the presence of a cleavable linker and the fluorescent probe (Rhodamine B). 214
LIST OF TABLES

Table 1.1.1: Selection of the most important *in vitro* cytotoxicity studies with CNT classified according to type of material used (pristine, coated and chemically-functionalised CNT) .................................................................43

Table 1.1.2: Selection of the most important *in vivo* toxicity studies using CNT reported today classified according to the exposure route (pulmonary, dermal and systemic). ..................................................................................................................53

Table 5.2.1: Length distribution of NT$_2$ samples and LFA ........................................ 163

Table 6.2.1: Kaiser test and loading of MTX onto the MWNT. .................................. 197

Table 6.2.2: Comparison between the non-covalent and covalent functionalisation methodologies used for the preparation of effective anticancer drug delivery systems. ..............................................................................................................208
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Absorbance</td>
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<tr>
<td>AFM</td>
<td>Atomic Force Microscope</td>
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<tr>
<td>BCA</td>
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<td>BOC</td>
<td>tert-Butyloxycarbonyl protecting group</td>
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<td>F127</td>
<td>Block copolymer Pluronic</td>
</tr>
<tr>
<td>FA</td>
<td>Folic Acid</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>f-MWNT</td>
<td>Functionalised Multi-Walled Carbon Nanotubes</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
</tbody>
</table>
GlcNAcD  N-acetyl-D-glycoseamine Dendrons
HCPT   Hydroxy-Camptothecin
H&E   Hematoxylin and Eosin
hrs   Hours
HRTEM High Resolution Transmission Electron Microscopy
I   Iodine
ID   Injected Dose
In   Indium
InCl   Indium Chloride
i.t   Intratracheal instillation/ intratumoural injection
i.p   Intraperitoneal injection
LDH   Lactate Dehydrogenase
LFA   Long Fibres Amosites
KCN   Potassium Cyanide
M   Molar
min   Minutes
mg   Milligram
ml   Millilitre
µl   Microlitre
µm   Micrometer
µmol   Micromoles
MBq   Mega Bequerel
MeOH   Methanol
MTT   3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MTX   Methotrexate
MWNT   Multi-Walled Carbon Nanotubes
NaI   Sodium Iodide
NH₃⁺   Ammonium group
NIR   Near Infrared
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Solution</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene Glycol</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethyleneimine</td>
</tr>
<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>PMN</td>
<td>PolyMorphoNuclear Leucocytes</td>
</tr>
<tr>
<td>PTX</td>
<td>Paclitaxel</td>
</tr>
<tr>
<td>pMWNT</td>
<td>Pristine MWNT</td>
</tr>
<tr>
<td>PL-PEG</td>
<td>Phospholipid-PEG</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphotidylserine</td>
</tr>
<tr>
<td>RES</td>
<td>Reticularendothelial system</td>
</tr>
<tr>
<td>RGD</td>
<td>Arginine-Glycine-Aspartic acid peptide</td>
</tr>
<tr>
<td>Rhd</td>
<td>Rhoadamine B</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodEcyl sulfate</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small Interference RNA</td>
</tr>
<tr>
<td>SPECT</td>
<td>Single Photon Emission Computed Tomography</td>
</tr>
<tr>
<td>STEM</td>
<td>Scanning Transmission Electron Microscopy</td>
</tr>
<tr>
<td>SWNT</td>
<td>Single-Walled Carbon Nanotubes</td>
</tr>
<tr>
<td>Te</td>
<td>Technetium</td>
</tr>
<tr>
<td>TEG</td>
<td>Triethyleneglycol</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TGA</td>
<td>Thermal Gravimetric Analysis</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin Layer Chromatography</td>
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</tbody>
</table>
CHAPTER I

INTRODUCTION
Nanotechnology is the engineering and the manufacturing of materials at the atomic and molecular scale. The National Nanotechnology Initiative defines nanotechnology by referring to structures ranging between 1-100 nm in size [1]. Although this strict size definition, nanotechnology can also refer to entities that are up to several hundred nanometers at least on one dimension. With the witnessing of the increased development of novel nanomaterials for use in drug delivery, it is expected that the landscape of pharmaceutical and biotechnology industries are to be changed in the near future. The novel cylindrical nanostructures known as carbon nanotubes have exceeded expectations in terms of usage both in the engineering and the biomedical fields due to their extraordinary properties. Examples include their high electrical and thermal conductivity in addition to their amazing ability to cross the plasma membrane [2, 3]. Proof-of-principle studies have shown that the biomedical applications of carbon nanotubes (CNT) range from their use as biosensors into drug delivery vectors for diagnostics and therapeutics. However, before either of these applications find their way into the clinical setting, the engineering of these nanocarriers is needed alongside a systematic toxicological and pharmacokinetic assessments.

1.1. Carbon nanotubes

Rediscovered in 1991 by Sumio Iijima, CNT consist of sheets of carbon atoms that are rolled up into a hollow tubular structure [2, 3]. CNT belong to the family of fullerenes, the third allotropic form of carbon along with graphite and diamond. Carbon nanotubes can either be single-walled (SWNT) consisting of one layer of graphene sheet or multi-walled (MWNT) which are characterised by multiple concentric graphene layers (Figure 1.1.1) [4, 5]. While the former have diameters
from 0.4 to 2.0 nm and lengths in the range of 20-1000 nm, the latter are bigger entities with diameters in the range 1.4-100 nm and lengths from 1 to several μm.

Key features that make CNT unique nanomaterials is a combination of distinctive properties namely high aspect ratio, mechanical strength, electrical conductivity and large surface area [3, 6, 7]. CNT hold the possibility of offering greater advantages over existing delivery systems in terms of the large surface area available for conjugation with different functionalities. The inner nanotube volume can also exhibit the capacity to be encapsulated with different moieties. Both of these strategies can be exploited for therapeutic, imaging and targeting purposes. The use of CNT as components for DNA and protein biosensors [8, 9], ion channel blockers [10] and scaffolds for tissue engineering [11] has already been explored. Further properties which make CNT attractive for biomedical applications for use as delivery vectors include their ability to cross biological barriers and their high stability in vivo [12].

![Figure 1.1.1: Schematic representation of the two types of CNT (A) Single walled carbon nanotubes (SWNT) and (B) Multi-walled carbon nanotubes (MWNT).](image-url)
1.2. CNT functionalisation

Although carbon nanotubes and in particular pristine (non-modified) CNT suffer from the drawback of extreme hydrophobicity and hence their non-biocompatibility with the aqueous biological milieu, several strategies have been adopted to render these fascinating nanomaterials more hydrophilic through surface functionalisation which can be achieved via two methodologies either with:

- The non-covalent surface modification by the adsorption of different amphiphilic macromolecules such as lipids, surfactants, copolymers or single stranded DNA with the hydrophobic surface of CNT as shown in Figure 1.1.2 b-d [12-16].

- or through chemical functionalisation by covalently attaching different chemical moieties. This is achieved by surface modification of the pristine material using cycloaddition reactions or strong acid treatment yielding ammonium and carboxylated CNT respectively [6, 17-21] (Figure 1.1.2 e-f). This chemistry rendered CNT dispersed in aqueous and was tested in different biological experiments [22-24]. Scheme 1.1.1 shows the diverse chemical functionalisation strategies implemented to render CNT dispersible in aqueous or organic solvents [21]. Chemical functionalisation offers the possibility for further derivatization of CNT with imaging, diagnostic or therapeutic molecules.
Figure 1.1.2: Different types of carbon nanotubes studied in biomedical applications. The pristine as produced CNT (a) can either be functionalised non-covalently by (b) PEGylated lipid or other modified lipid molecules or (c) copolymer or surfactant molecules. PEO is polyethylene oxide; PPO is polypropylene oxide. (d) Single-stranded DNA (ssDNA) or chemically via (e) 1, 3 dipolar cycloaddition and (f) acid oxidation. Adapted from reference [12].
Scheme 1.1.1: Different chemistries used for the covalent functionalisation of carbon nanotubes. (Adapted from reference [21]).
1.3. CNT biodistribution and pharmacokinetics in vivo

The need to understand the pharmacokinetic profile of CNT-based technologies is perquisite at this stage of their development. Although various groups have now moved forwards and studied the in vivo biodistribution of CNT, comparison between these studies is difficult to make because of: A) the differences in their surface functionalisation properties and the use of different tracking modalities; B) the dosing regimens and the administration routes used.

CNT biodistribution studies performed so far can be categorised in two general classes depending on the type of carbon nanotubes used. Some groups have studied the tissue distribution profiles of non-covalently functionalised CNT and others have investigated the in vivo fate of covalently functionalised CNT.

1.3.1. Tissue biodistribution of non-covalently coated CNT

The first study using non-covalently coated CNT was that of Cherukuri et al. who used intrinsic Near Infrared Fluorescence as a tracking method for the intracellular accumulation of the Pluronic F108 coated SWNT in rabbits [25]. It was found that SWNT were mainly accumulated in the liver and exhibited a very rapid blood clearance (t ½ < 1 hr) which was attributed to the formation of SWNT-protein complexes or SWNT aggregates after protein competition with the Pluronic coating. In another study, the biodistribution of 13C-enriched backbone SWNT coated with Tween 80 was investigated taking advantage of isotopic abundance 13C/12C to quantify CNT in organs via isotope mass spectroscopy [26]. It was found that SWNT exhibited high affinity for the liver, lungs and spleen and remained in these organs for 28 days [26]. The elimination route was thought not to be through the bladder but instead there was a decrease in the amount accumulated in the lung (from
15 to 9.4 %) towards the spleen. Neither of these studies, however, performed a systematic in vivo toxicity or physiology assessment.

Liu et al. have investigated the biodistribution of SWNT coated with pegylated phospholipid by Positron Emission Tomography (PET) and Raman Spectroscopy [13]. The effect of the polyethylene glycol (PEG) chain length (2000 or 5400) in targeting cancer using the RGD peptide was investigated. Significant accumulation of all types of lipid coated SWNT was observed in the liver and spleen. While these lipid-coated SWNT showed no tumour uptake, the more integrin targeted constructs (SWNT-PEG\textsubscript{5400}-RGD) demonstrated a higher tumour accumulation of 10-15 % of ID per gram tissue. In a more recent study, Liu et al. further investigated the effect of length and branching of PEG on the biodistribution of PEG-Lipid coated SWNT [27]. Although, the liver and spleen were still the main organs of accumulation, there was an increase in blood circulation (up to 15 hr) and a reduction in the liver uptake with the branched (7 kDa) PEG coated-SWNT. In addition to the biliary excretion being the main elimination route for these non-covalently pegylated SWNT over the 2 months period, the authors also suggested the possibility that SWNT of smaller dimensions (< 50 nm in length, 1-2 nm in diameter) being excreted via the urine at early time points.

It has to be stressed that all the mentioned studies used the same type of nanotubes (SWNT) and the same strategy of non-covalent functionalisation using different types of coatings. It is also important to note that most of the techniques employed to study the biodistribution profiles of these non-covalently functionalised CNT require the preservation of the pristine carbon backbone which is defected on chemical functionalisation since any damage to its electronic properties would not
allow the use of near infrared or Raman spectroscopy. Overall, it has been found that pristine, non-covalently functionalised SWNT accumulate in RES organs (mainly the liver).

1.3.2. Tissue biodistribution of covalently functionalised CNT:

Several studies have been published so far describing the biodistribution of covalently functionalised CNT in vivo, most of which used SWNT [28-33]. The first study was that of Wang et al. using Iodine-125 labelled hydroxylated SWNT (\(^{125}\)I-SWNT-OH) injected intraperitoneally (i.p) [30]. A rapid distribution of \(^{125}\)I-SWNT-OH was reported in most organs with the highest affinity to the stomach, kidneys and bones. More significantly, \(^{125}\)I-SWNT-OH were found to be excreted mainly via the urine (94%). No significant differences in tissue distribution were reported for the different administration routes (subcutaneous, stomach gavage and intravenous) assessed. In a recent study, Wang et al. looked at the in vivo fate of \(^{131}\)I-SWNT-OH shortly after i.v and i.p administrations and reported their fast distribution into organs as observed from 2-60 min post-injections [33]. Interestingly, the kidneys showed the highest uptake with 24 % ID after 2 min post-injection with a slight reduction in the signal within the 60 minutes time frame of the experiment.

Guo et al. have reported a very similar biodistribution study to that of Wang et al. in which MWNT were functionalised with glucosamine, subsequently labelled with Technetium -99 (\(^{99m}\)Tc-MWNT-glu) and injected i.p. into mice [29]. \(^{99m}\)Tc-MWNT-glu exhibited a rapid organ biodistribution and a blood circulation half life (\(t_{1/2}\)) of 5.5 hr. In addition, significant amounts of MWNT were retained in the kidneys over 24 hr with more than 75 % of radioactivity found in the urine and faeces. However, in a later study using taurine functionalised MWNT (\(^{14}\)C-taurine-
MWNT) the same group observed high affinity to liver (80% of I.D) after i.v administration and residual retention for 28 days with gradual elimination within 3 months [32]. Deng et al. also investigated the effect of alternative administration routes other than i.p (stomach gavage and intratracheal instillation (i.t) to compare the in vivo fate of f-MWNT [32]. They reported great accumulation in the small and large intestine for the stomach gavage and higher affinity to lungs after i.t administration. Moreover, similar findings of major liver accumulation were reported recently by the same group [34] in which a novel radiotracing technique was adopted yielding 125I-taurine- MWNT. These findings contradicted the previous reports by the same group using 125I-SWNT-OH discussed above. Moreover, Yang et al. have produced covalently pegylated SWNT and 30% of the injected dose remained in the blood one day post-injection as quantified by isotope-MS measurement [35].

Other studies looking at the tissue biodistribution of functionalised f-CNT have been performed using very similar CNT surface functionalisation chemistry via the 1, 3 dipolar cycloaddition yielding ammonium functionalised CNT (f-CNT). In the study carried out by Singh et al. SWNT-NH₃⁺ were further functionalised with the chelating molecule diethylentriaminepentaacetic (DTPA) and subsequently radiolabelled with 111 Indium ([¹¹¹In] DTPA-SWNT) which was then intravenously injected into mice [28]. The study demonstrated distribution of f-SWNT in the kidney, muscle, skin, bone and blood after 30 min post injection. Subsequently, f-SWNT were cleared from the body with a maximum blood circulation half-life of 3.5 hrs.
In a subsequent study, $[^{111}\text{In}]$ DTPA-MWWNT were administered i.v. in rats and found to be mainly localised in the kidneys within 30 min post-injection and subsequently excreted via the urine as studied by dynamic whole body single photon emission computed tomography [36]. In two recently published papers [37, 38], our laboratory has suggested the importance of the degree functionalisation as a determinant of the extent of nanotube individualisation which appeared to be a prerequisite criterion in urinary excretion. These observations were in agreement with the hydroxylated ($^{125}\text{I}$-SWNT-OH) and glucosamine functionalised (MWNT $^{99m}\text{Tc}$-MWNT-glu) despite the very different chemical processing and treatment of the material.

Following these initial studies, McDevitt et al. used the same functionalisation chemistry as Singh et al. [28] and Lacerda et al. [36, 37] to produce ammonium functionalised SWNT labelled with $^{111}\text{In}$, using 2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclodecane (DOTA) as the chelating molecule [31]. Furthermore, in this study monoclonal antibodies (Rituximab) were conjugated on the SWNT to offer tissue targeting. The differences in tissue biodistribution of $[^{111}\text{In}]$ DOTA-SWNT-NH$_3^+$ and the targeted construct ($[^{111}\text{In}]$ DOTA-SWNT-Rituximab) were assessed. The $[^{111}\text{In}]$ DOTA-SWNT accumulated mainly in the kidney, spleen, liver and bone showing rapid blood clearance. Antibody conjugation affected the tissue biodistribution as evidenced by a decrease in kidney residence and a 2-fold increase in liver affinity with no change in spleen accumulation. This study and a subsequent similar investigation by the same group [39] are generally in good agreement with the original observations of Singh et al. that rapid clearance and urinary excretion of CNT occurs.
What can be generally concluded so far from these studies is that pristine CNT exhibit a different \textit{in vivo} biodistribution profile compared to covalently functionalised CNT which can only be explained by the difference in their dispersion properties. While the former acted as other nanomaterials and showed higher affinity to RES organs mainly liver, the more water soluble and chemically modified CNT behaved similar to small molecules and were excreted via the urine (Scheme 1.1.2). This clearly shows the importance of covalent functionalisation in rendering the CNT biologically compatible by enhancing their urinary excretion and reducing their accumulation in RES organs. Surface characteristics; in particular the type of functional groups and surface charge density are thought to determine the degree of non-specific protein binding and opsonisations prior to capturing by RES organs mainly liver [40]. In addition, the observed different CNT affinities to organs could also be explained by the different degrees of covalent functionalisation of CNT which seems to dictate the individualisation/aggregation statues of CNT. Our laboratory recent data for instance indicates the importance of the high degree of functionalisation to ensure adequate CNT individualisation and subsequent urinary excretion with minimal unwanted tissue accumulation [37, 41]. Therefore, it should be emphasised that the biological outcomes that would be observed with pristine CNT should always be distinguished from that of their functionalised counterparts.
Scheme 1.1.2: A summary selection of the earliest CNT biodistribution studies. The different CNT surface functionalisation and the organs of accumulation or excretion route(s) are highlighted.
1.4. CNT cytotoxicity *in vitro and in vivo*

With the increased use of CNT in different fields and their rapid advance in biomedical applications [6, 14, 20, 22, 42, 43] in addition to their structural similarity with known carcinogens such as asbestos, the potential impact of carbon nanotubes on human health and environment is viewed with apprehension [44]. Hence, there is an emergent necessity for a fundamental understanding of their toxicological aspects which is a critical step in their development, especially for therapeutic and diagnostic purposes.

Several important physicochemical properties of CNT such as size, shape, aggregation, chemical composition and dispersion play an important role in determining their *in vitro* and *in vivo* cytotoxic profile. It should be mentioned however that most toxicological studies to date have focused mainly on their effect following exposure through inhalation, dermal contact or ingestion for which researchers have used non-functionalised, unpurified pristine CNT in an attempt to mimic those CNT used in workplaces and production units. Little has been studied following intravenous administration as it not considered as relevant to public health. However, this route is of tremendous importance for the development of CNT in biomedical applications and more systematic studies should therefore be performed following systemic administration of CNT.

1.4.1. Cytotoxicity of CNT *in vitro*

Most toxicological studies performed *in vitro* with CNT have investigated their effects on lung-derived cells, macrophages and keratinocytes since the lung and skin are the main portals of entry of nanoparticles into the body. Table 1.1.1 offers a summary of the most relevant *in vitro* toxicity studies to date. Differences in
dispersibility, surface charge, aspect ratio and impurities are all factors that can contribute to the potential cytotoxicity of carbon nanotubes, but also to the difficulty in drawing credible comparisons between the currently published data.

1.4.1.1. CNT functionalisation and dispersibility

One of the main factors that plays a pivotal role in determining the cytotoxicity profile of CNT is their surface functionalisation and dispersibility. Sayes et al.[45] reported that increased functionalisation and hence improved water dispersibility render the SWNT-Phenyl-SO$_2$H and SWNT-Phenyl-(COOH)$_2$ less toxic. Moreover, Zhang et al. [46] found that human epidermal keratinocyte (HEK) viability was enhanced upon the improved dispersibility of the chemically functionalised 6 aminohexanoic acid SWNT (AHA-SWNT) in pluronic F127. In addition, Dumortier et al. [47] showed that the well dispersed ammonium functionalised SWNT (via the 1, 3 dipolar cycloaddition reaction) were not cytotoxic compared to PEGylated SWNT that also exhibited reduced dispersibility. The latter affected primary immune cells and macrophages not only by activating them, but also by affecting their ability to respond to physiological stimuli. Wick et al.[48] have also reported that the aggregation of CNT is responsible for their toxicity indicating that purified SWNT agglomerates induced more toxicity on human mesothelium-derived cells than its non-purified counterpart which was dispersed in Tween 80. This was attributed to their improved dispersibility, suggesting that cytotoxicity is dependent not only on increased functionalisation but also on the type of functionalisation.
1.4.1.2. Composition of functional group or coating

The type of functionalisation or coating used to improve the dispersibility of the nanotubes should be carefully chosen as cytotoxicity can occur due to the possible toxic or lytic activity of the surfactant molecules coating the CNT. For example, Dong et al. [49] found that the cytotoxicity observed with SWNT coated with sodium dodecyl sulphate (SDS) and sodium dodecylbenzene sulfonate (SDBS) was due to the intrinsic toxicity of the surfactants used. However, the cell viability of human astrocytoma cells (1321N1) was not affected when SWNT were coated by sodium cholate or single stranded DNA [49]. Monteiro-Riviere and co-workers [50] attempted to identify the best surfactant to confer adequate aqueous dispersibility to the CNT with minimal cytotoxicity. They found that Pluronic F127 was the best copolymer having surfactant-like properties to disperse CNT with negligible cytotoxicity. Their study showed that MWNT dispersed in F127 were less irritant to human keratinocytes than the pristine non-coated MWNT. Other groups have described cytotoxicity with their dispersed nanotubes coated with dipalmitoylphosphatidylcholine (DPPC) [51] or phosphatidylserine [52]. Interestingly, Becker et al. [53] also mentioned the importance of dispersibility and that of individualisation achieved by dispersing their nanotubes with DNA.

1.4.1.3. Nature of CNT surface (charge, hydrophobicity/ hydrophilicity)

As with other nanoparticles, surface charge, aspect ratio and length also contribute to the toxicity of CNT. A systematic study published by Shen et al. [54] found that the positively charged polyethyleneimine (PEI) functionalised MWNT are toxic at low concentrations (up to 10 µg/ml) compared to their neutral and anionic converted counterparts (MWNT-PEI-Acetylated & MWNT-PEI-carboxylated)].
addition, Nimmagadda et al.[55] described how the more hydrophilic and purified, glucosamine-functionalised SWNT were less toxic than the hydrophobic pristine and purified SWNT.

14.1.4. CNT size (diameter and length) and aspect ratio

The importance of CNT size in determining their cytotoxicity profile has also been emphasised. Becker et al.[53] reported a dose-dependent cytotoxicity above 20μg/ml, but also a length-dependent uptake of SWNT with the shortest nanotubes (187+/− 17 nm) causing the highest cell death in human lung fibroblasts. In addition, Magrez et al.[56] examined the effect of diameter and aspect ratio of carbon based materials on their cytotoxicity profile and reported that purified MWNT are less toxic than carbon black and carbon nanofibers even at longer exposure times with lung carcinoma cells. However, Tian et al.[57] compared SWNT and MWNT to carbon black and activated carbon and found that cytotoxicity was highest with SWNT followed by activated carbon (SWNT ≥ activated carbon ≥ carbon black ≥ MWNT) in human dermis fibroblast cells. However, when total surface area was compared, purified SWNT proved to be more toxic than the non-purified counterpart, explained by the presence of bundles of pristine material with large surface area than the well-individualised and dispersed SWNT. When zinc oxide (ZnO), silicon oxide (SiO2) nanoparticles and MWNT were compared in a different study, ZnO nanoparticles showed more detrimental effects to primary mouse embryo fibroblasts although ZnO and SiO2 have similar structural characteristics and particle size [58]. Interestingly, SWNT showed 50 % reduction in oxidative stress compared to 91 % with ZnO and only 36 % for SiO2 and greater genotoxicity was observed with SWNT.
1.4.1.5. CNT metal impurities

One of the biggest concerns regarding CNT toxicology is the presence of metal catalysts that may contribute to the overall toxicity of the material. One of the first cytotoxicity studies using CNT was carried out by Shvedova et al.[59] in which they investigated the effect of unrefined SWNT on human epidermal keratinocytes (HaCaT). SWNT caused accelerated oxidative stress, loss of viability and morphological changes which were attributed to the high level of iron catalysts (approx 30%) present inside the SWNT. Pulskamp and co-workers [60] observed that even though no signs of acute toxicity were obtained with commercially available, non-purified CNT an increase in intracellular reactive oxygen species and a decrease in the mitochondrial potential, which was not the case with its purified counterpart without metal catalysts. Vittorio et al.[61] studied the effect of purity and oxidation of MWNT on the cytotoxicity to human neuroblastoma cells. At low concentrations (up to 10µg/ml) cell viability was not affected regardless of the material impurities, however there was a dose-dependent reduction in cell viability as the concentration was increased above 10µg/ml.

Choi et al.[62] found that purified, SWNT affected cell proliferation compared to other inorganic nanoparticles (such as iron oxide and silica) after 24hrs incubation with human lung carcinoma cells (A549). SWNT induced oxidative stress followed by apoptosis at doses up to 250-500µg/ml, suggesting that the different inorganic nanoparticles can cause cytotoxicity through different mechanisms. Furthermore, Deckers et al.[63] found that regardless of size or purity, MWNT caused dose-dependent increase in LDH release as an indication for membrane damage. Different effects have also been described using non-purified CNT. For
instance Cui et al. [64] found that SWNT can inhibit HEK293 cell proliferation by inducing cell apoptosis and decreasing cell adhesive proteins. Monteiro-Riviere et al. [65] also showed that non-functionalised MWNT were found to internalise into cells and cause an irritating response in HEK cells as indicated by the release of pro-inflammatory cytokines (such as IL-8). DNA damage has also been described as the result of MWNT interaction with mouse embryonic stem cells leading to toxicity together with activation of the tumour suppressor protein p53 [66].

In summary, the low dispersibility and agglomeration of as-produced pristine CNT seem to be the most important contributing factor determining the cytotoxicity of CNT. In comparison, whenever chemically-functionalised material were studied, cytotoxicity has been dramatically reduced due to improvements in CNT dispersibility. In addition, the presence of residual metal catalyst within the nanotube walls is another factor that complicates the overall reported cytotoxicity of CNT. It should also be stressed that most of the currently published data are based on colorimetry- and fluorescence-based assays which have been found to interfere with CNT and give rise to unreliable data [67-72]. The need for more systematic studies in which detailed physicochemical characterisation of CNT and their aqueous colloidal dispersions that ultimately interact with cells is required, along with improvements in the protocols used for colorimetric and fluorescence assays to determine levels of cytotoxicity.
<table>
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<tr>
<th>CNT Type</th>
<th>Surface Modification</th>
<th>Concentration used</th>
<th>Cell Line</th>
<th>Assays</th>
<th>Incubation Time</th>
<th>Conclusions</th>
<th>Ref</th>
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<tbody>
<tr>
<td>SWNT</td>
<td>NA</td>
<td>0.78±0.25 - 200µg/ml</td>
<td>Human Epidermal Keratinocytes (HEK)</td>
<td>MTT assay, Trypan Blue</td>
<td>1-5 days</td>
<td>SWNT can inhibit the proliferation of HEK cells, induce cell apoptosis and decrease cell adhesive ability</td>
<td>[64]</td>
</tr>
<tr>
<td>MWNT</td>
<td>NA</td>
<td>100, 200, 400µg/ml</td>
<td>Human Epidermal Keratinocytes (HEK)</td>
<td>Neutral Red, Inflammatory mediators</td>
<td>1.2, 4, 8, 12, 24 hrs</td>
<td>70% cell viability with 200-400µg/ml Induction of the release of pro-inflammatory mediators (IL-8) in time dependent manner</td>
<td>[65]</td>
</tr>
<tr>
<td>SWNT, MWNT</td>
<td>NA</td>
<td>0.8, 1.61...up to 100µg/ml</td>
<td>Human Dermin Fibroblast Cells</td>
<td>MTT assay, Cell adhesion assay, Cell death assay</td>
<td>1-5 days</td>
<td>SWNT induced the strongest effects, apoptosis and necrosis. Refined SWNT are more toxic than unrefined SWNT</td>
<td>[57]</td>
</tr>
<tr>
<td>Activated Carbon</td>
<td>NA</td>
<td></td>
<td></td>
<td>Cell death assay</td>
<td></td>
<td>Surface area and surface chemistry important</td>
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<td>Cell death assay</td>
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<tr>
<td>SWNT, Carbon Graphite</td>
<td>Pristine (Raw)</td>
<td>15.30, 75µg/ml</td>
<td>Mesothelial derived cells (MSTO-211H)</td>
<td>Hoescht proliferation assay, MTT assay</td>
<td>3 days</td>
<td>Agglomerates most toxic CNT pellets toxic due to carboneous portion CNT bundles most dispersed and safe</td>
<td>[48]</td>
</tr>
<tr>
<td>Activated Carbon</td>
<td>Oxidised (Agglomerates)</td>
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<td></td>
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<tr>
<td>SWNT</td>
<td>Pristine in serum and no serum containing media</td>
<td>1.56 - 800µg/ml</td>
<td>Human Lung Carcinoma (A549)</td>
<td>Alamar blue (AB), Neutral Red, MTT Coomassie Blue</td>
<td>24 hrs</td>
<td>SWNT interfere with assays AB most reproducible Greater toxicity in the absence of serum</td>
<td>[72]</td>
</tr>
<tr>
<td>Carbon Black</td>
<td>Pristine in serum and no serum containing media</td>
<td></td>
<td></td>
<td>Cell death assay</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MWNT</td>
<td>NA</td>
<td>0.04, 0.4, 1.5, 5 µg/ml</td>
<td>Human Aortic Endothelial Cells (HAEC)</td>
<td>WST-1, LDH assays, Tubule formation, Actin Stress labelling</td>
<td>3, 24 hrs</td>
<td>Dose and time dependent reduction in cell viability Reduced tubule formation and cytoskeleton Actin disruption.</td>
<td>[73]</td>
</tr>
<tr>
<td>SWNT</td>
<td>Dispersed in PBS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SWNT, Carbon Black, Zinc Oxide (ZnO), Silicon Oxide (SiO2)</td>
<td>NA</td>
<td>$5, 10, 50 &amp; 100µg/ml</td>
<td>Primary Mouse Embryo Fibroblasts (PMEF)</td>
<td>MTT assay, WST assay, LDH assay, Comet assay Intracellular reactive oxygen species, Oxidative damage</td>
<td>24 hrs</td>
<td>Unsuitability of using MTT assay Dose dependent toxicity @ 100µg/ml Reduction in oxidative stress by 50% @100µg/ml</td>
<td>[58]</td>
</tr>
<tr>
<td>SWNT, Carbon Black, Zinc Oxide (ZnO), Silicon Oxide (SiO2)</td>
<td>Dispersed in Fetal Bovine Serum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SWNT, MWNT</td>
<td>Coating</td>
<td>400 µg/ml</td>
<td>HEK</td>
<td>Neutral Red</td>
<td>24 hrs</td>
<td>MWNT-F127 caused 70% cell viability similar to pristine MWNT.</td>
<td></td>
</tr>
<tr>
<td>-----------</td>
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<td>-----------</td>
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<td>--------</td>
<td>-------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>MWNT</td>
<td>Coating</td>
<td>0.002-0.2 µg/ml</td>
<td>Human Lung Carcinoma</td>
<td>MTT assay</td>
<td>4 days</td>
<td>CNT less toxic than carbon fibers and carbon black.</td>
<td></td>
</tr>
<tr>
<td>Carbon nanofibers</td>
<td>Gelatin dispersed</td>
<td></td>
<td>H596, Calo I</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbon Black</td>
<td>Oxidised</td>
<td></td>
<td>H446</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SWNT</td>
<td>Coating</td>
<td>1.97 x 10^6-3.6 x 10^7 mg/ml</td>
<td>Human Lung Fibroblast (IMR 90)</td>
<td>WST-1</td>
<td>16 hrs</td>
<td>Below 20 µg/ml: No toxicity.</td>
<td></td>
</tr>
<tr>
<td>MWNT, short &amp; Long</td>
<td>Coating</td>
<td>0.25-100 µg/ml</td>
<td>Human Lung Carcinoma</td>
<td>MTT assay</td>
<td>1 hr - 72 hrs</td>
<td>Dose dependent increase in LDH release.</td>
<td></td>
</tr>
<tr>
<td>Purified and Non-purified</td>
<td>Arabic gum (0.25%)</td>
<td></td>
<td>(A549)</td>
<td></td>
<td></td>
<td>No dependence on length.</td>
<td></td>
</tr>
</tbody>
</table>

**Tissue Culture Assay**

**MTT**

Pristine SWNT: Increase in reactive oxygen species and decrease in mitochondrial potential.

No acute toxicity.

Impurities have an effect on the observed toxicity.

No effect with acid treated SWNT

Impurities have an effect on the observed toxicity.
<table>
<thead>
<tr>
<th>Material</th>
<th>Coating</th>
<th>Concentration</th>
<th>Cell Type</th>
<th>Assay</th>
<th>Time</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>MWNT</td>
<td>Coating</td>
<td>1% F68 in media</td>
<td>Mouse Macrophage Cells</td>
<td>Wst-8</td>
<td>16-32 hrs</td>
<td>Concentration dependent reduction in cell viability of macrophages. MWNT injure macrophage plasma membrane</td>
</tr>
<tr>
<td>SWNT</td>
<td>Coating</td>
<td>50, 100, 200pg/ml</td>
<td>Human Astrocytoma (1321 N1)</td>
<td>MTS assay (Cell Titer solution)</td>
<td>72 hrs</td>
<td>SWNT Toxicity due to surfactants (SDS, SDBS) SWNT-SC /DNA non-toxic</td>
</tr>
<tr>
<td>MWNT 99%</td>
<td>Coating</td>
<td>5-10pg/ml</td>
<td>Human Neuroblastoma SH-SY5Y</td>
<td>MTT . Wst I assay</td>
<td>Short (72hrs)</td>
<td>Dose dependent decrease in cell viability with increased concentration above 10pg/ml</td>
</tr>
<tr>
<td>MWNT 97%</td>
<td>Coating</td>
<td>5-500pg/ml</td>
<td>Human Neuroblastoma SH-SY5Y</td>
<td>MTT . Wst I assay</td>
<td>Long (2 weeks)</td>
<td>Dose dependent decrease in cell viability with increased concentration above 10pg/ml</td>
</tr>
<tr>
<td>MWNT (97%)-oxidised</td>
<td>Pluronic F127</td>
<td>5-100pg/ml</td>
<td>Human Neuroblastoma SH-SY5Y</td>
<td>MTT . Wst I assay</td>
<td>Long (2 weeks)</td>
<td>Dose dependent decrease in cell viability with increased concentration above 10pg/ml</td>
</tr>
</tbody>
</table>

**Chemically-functionalised SWNT**

<table>
<thead>
<tr>
<th>SWNT</th>
<th>Covalent</th>
<th>3 pg/ml-30 mg/ml</th>
<th>Human Dermal Fibroblasts</th>
<th>Calcein AM</th>
<th>MTT assay</th>
<th>48 hrs</th>
<th>As the degree of functionalisation is increased, SWNT became less cytotoxic. Covalent functionalisation is less toxic than surfactant stabilised/coating.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SWNT</td>
<td>-Phenyl SO3H</td>
<td>3 pg/ml-30 mg/ml</td>
<td>Human Dermal Fibroblasts</td>
<td>Calcein AM</td>
<td>MTT assay</td>
<td>48 hrs</td>
<td>As the degree of functionalisation is increased, SWNT became less cytotoxic. Covalent functionalisation is less toxic than surfactant stabilised/coating.</td>
</tr>
<tr>
<td>SWNT</td>
<td>-PhenylySO3Na</td>
<td>3 pg/ml-30 mg/ml</td>
<td>Human Dermal Fibroblasts</td>
<td>Calcein AM</td>
<td>MTT assay</td>
<td>48 hrs</td>
<td>As the degree of functionalisation is increased, SWNT became less cytotoxic. Covalent functionalisation is less toxic than surfactant stabilised/coating.</td>
</tr>
<tr>
<td>SWNT</td>
<td>Pristine-F108</td>
<td>3 pg/ml-30 mg/ml</td>
<td>Human Dermal Fibroblasts</td>
<td>Calcein AM</td>
<td>MTT assay</td>
<td>48 hrs</td>
<td>As the degree of functionalisation is increased, SWNT became less cytotoxic. Covalent functionalisation is less toxic than surfactant stabilised/coating.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SWNT</th>
<th>Covalent</th>
<th>3 pg/ml-30 mg/ml</th>
<th>Human Dermal Fibroblasts</th>
<th>Calcein AM</th>
<th>MTT assay</th>
<th>48 hrs</th>
<th>As the degree of functionalisation is increased, SWNT became less cytotoxic. Covalent functionalisation is less toxic than surfactant stabilised/coating.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SWNT</td>
<td>-Phenyl SO3H</td>
<td>3 pg/ml-30 mg/ml</td>
<td>Human Dermal Fibroblasts</td>
<td>Calcein AM</td>
<td>MTT assay</td>
<td>48 hrs</td>
<td>As the degree of functionalisation is increased, SWNT became less cytotoxic. Covalent functionalisation is less toxic than surfactant stabilised/coating.</td>
</tr>
<tr>
<td>SWNT</td>
<td>-PhenylySO3Na</td>
<td>3 pg/ml-30 mg/ml</td>
<td>Human Dermal Fibroblasts</td>
<td>Calcein AM</td>
<td>MTT assay</td>
<td>48 hrs</td>
<td>As the degree of functionalisation is increased, SWNT became less cytotoxic. Covalent functionalisation is less toxic than surfactant stabilised/coating.</td>
</tr>
<tr>
<td>SWNT</td>
<td>Pristine-F108</td>
<td>3 pg/ml-30 mg/ml</td>
<td>Human Dermal Fibroblasts</td>
<td>Calcein AM</td>
<td>MTT assay</td>
<td>48 hrs</td>
<td>As the degree of functionalisation is increased, SWNT became less cytotoxic. Covalent functionalisation is less toxic than surfactant stabilised/coating.</td>
</tr>
</tbody>
</table>

**Notes:**

- MWNT injure macrophage plasma membrane
- SWNT Toxicity due to surfactants (SDS, SDBS)
- SWNT-SC /DNA non-toxic
- Dose dependent decrease in cell viability with increased concentration above 10pg/ml
- No ROS @ 5 pg/ml (72 hrs) and 2 weeks.

**Materials:**

- MWNT: Multi-Wall Carbon Nanotubes
- SWNT: Single-Wall Carbon Nanotubes
- SDS, SDBS, SC, ssDNA, aDNA:
- MTT: 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt
- Wst-8:
- MTS assay (Cell Titer solution)
- Annexin V PI
- Calcein AM
- Ammonium Functionalised
- PEGylated
- Oxidation/amidation
- 1-10µg/ml
<table>
<thead>
<tr>
<th>Nanostructures</th>
<th>Modification</th>
<th>Concentration</th>
<th>Cell Line</th>
<th>Assay</th>
<th>Time</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SWNT</strong></td>
<td>Covalent</td>
<td>0.000000005 - 0.05 mg/ml</td>
<td>HEK</td>
<td>MTT assay</td>
<td>24-48 hrs</td>
<td>Dose dependent decrease in cell viability.</td>
</tr>
<tr>
<td><strong>Fluorinated (C/F ratio 2:4:1)</strong></td>
<td>6 aminohexanoic derived (AH-SWNT)</td>
<td>Improved viability as AH-SWNT is dispersed in F127 due to improved dispersion.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>AH-SWNT-F127</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>MWNT</strong></td>
<td>Covalent</td>
<td>0-100μg/ml</td>
<td>Human Thyroid Cancer cells (FRO)</td>
<td>MTT assay</td>
<td>24 hrs</td>
<td>MWNT-PEI (+ve charged) toxic @ 10μg/ml</td>
</tr>
<tr>
<td></td>
<td>PEI (+)</td>
<td></td>
<td>Human Epithelial Carcinoma (KB)</td>
<td></td>
<td></td>
<td>Neutral and anionic PEI derivatives, not toxic up to 100μg/ml</td>
</tr>
<tr>
<td></td>
<td>PEI-SAHE (-)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PEI-Ac (-/-)</td>
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</tr>
</tbody>
</table>
1.4.2. Cytotoxicity of CNT in vivo

Several laboratories have studied the in vivo toxicity of CNT using animal models in an attempt to investigate whether they cause any adverse effects on human health. Concerns have mainly focused on the pulmonary and dermal tissue as was also the case with the in vitro studies. Only a few studies have investigated the effects of nanotubes administered systemically for the development of therapeutics and diagnostics. The in vivo cytotoxicity studies using CNT can be classified according to the route of exposure (pulmonary, dermal or systemic). Table 1.1.2 summarises the most important studies that have investigated the in vivo toxicological profile of CNT to date based on this classification.

1.4.2.1. Effects following pulmonary exposure

Most in vivo toxicological studies have focused on the pulmonary route of exposure to CNT because of its importance to public and occupational health. Maynard and co-workers [75] have investigated the release of particles from unrefined SWNT into the air and its effect on workers at a small scale production facility. They reported very low airborne particle concentrations were generated as a consequence of handling unrefined materials with no effects. That is the only published work to date studying direct human exposure with CNT. Li et al. [76] reported that MWNT caused proliferation and thickening of lung alveolar walls as a result of the size and aggregation of MWNT in mice. Mitchell and co-workers [77] observed no lung damage after 14-day exposure of mice to MWNT, but instead detected suppression of systemic immunity. More recently, the same group have also offered a mechanistic explanation of this observation suggesting that protein
signalling from the lung (where the CNT reside) activate pathways in the spleen leading to suppression of immunity [78].

Intra-tracheal instillation (i.t) is one of the main administration routes used to study the pulmonary toxicity of CNT. Carrero-Sanchez et al. [79] stated that N-doped MWNT were safer than non-doped pristine nanotubes after i.t injection which might be due to their improved dispersibility. In addition, Li et al. [76] observed that aggregated MWNT can reach the lung directly by bypassing the mucociliary system, leading to persistent pathological lesions in bronchi and alveoli. Other groups have also reported that the agglomeration of SWNT in the airways is the primary cause of morbidity [80] and granuloma formation [81]. Using guinea pigs, Huczko et al. [82, 83] examined the effect of CNT characteristics and the duration of exposure on the degree of respiratory distress observed together with any induced lung pathology. Elgrabli et al. [84] used bovine serum albumin (BSA) to improve the dispersibility of MWNT and observed no inflammatory, physiological or histological pathologies, however BSA-coated MWNT were found inside alveolar macrophages which became apoptotic in an attempt to eliminate the CNT. Lastly, metal catalyst impurities seem to also play a role on the in vivo toxicity as studied by Lam et al. [81] using SWNT that contained residual catalyst.

Although intraperitoneal (i.p) administration is not directly relevant to human pulmonary exposure, it is considered toxicologically relevant in determining the carcinogenic potential of inhaled particles at the mesothelium. Poland et al. [85] found that rigid and long MWNT (>20µm in length) can cause granuloma lesion formation 7 days after i.p injection of 50µg MWNT dispersed in BSA, comparable to that of asbestos fibers. This is thought to be due to the inability of macrophages to
engulf such long fibers. Whether the observed granuloma will develop into a tumour was not investigated, but the similarity of the induced effect seen between long, pristine MWNT and long fibre amosite (asbestos) indicated that tumour formation could be a possibility. In a similar study, Takagi and co-workers [86] injected i.p. 3mg of MWNT (dispersed in Tween80/methylcellulose) into mice heterozygous to a mutation in p53, therefore more susceptible to mesothelioma (tumor of the mesothelium). The carcinogenic potential of MWNT was observed 25 weeks post-injection and was comparable to asbestos, even though it was difficult to determine the most important underlying factor(s) responsible for tumor development between CNT structure, metal impurities and poor dispersibility of injected material. Both above studies used long, pristine MWNT however, these results should be considered with caution and must not be used as conclusive facts about the toxicity associated with all CNT material. For example, Muller et al. [87] found that MWNT (dispersed in PBS) and i.p injected into rats, after 2 years did not cause carcinogenicity because the MWNT were less than 1 micrometer in length. Such studies, as well as with the majority of efforts to develop CNT for biomedical applications, indicate the toxicologically safer profile of shorter CNT.

As mentioned above, other studies have also reported that CNT can bypass the pulmonary defence mechanisms and reach the systemic circulation [77]; [88]. It may therefore be the case that pulmonary toxicity as well as systemic side effects can occur as a result of low level chronic inhalation exposure to CNT [88]. In the same context, Erdely et al. [89] have recently emphasised the possible inter-connections between pulmonary exposure and systemic circulation by reporting that exposure to SWNT and MWNT induced acute lung and systemic effects as was characterised by
an increase in lung and blood gene and protein expression markers [89]. More studies to further explore possible complex mechanisms triggered are needed and will surely appear.

1.4.2.2. Effects following skin exposure

Due to the difficulty in assessing dermal effects on rodents and humans, fewer studies, compared to pulmonary exposure, have been performed today. Maynard et al. [75] investigated the effect of handling unrefined CNT in a laboratory production facility and found about 0.2-6 milligram per hand of CNT deposits on gloves, indicating that dermal exposure can occur on unprotected body regions. Authors therefore stressed the use of protective clothing in order to minimise dermal exposure to CNT. Interestingly, Huczko et al.[90] found no association between working with soot containing CNT and the risk from possible skin allergies as assessed by some dermatological tests on human volunteers and rabbits.

1.4.2.3. Effects following systemic exposure

Most studies exploring the toxicological impact of CNT after systemic administration have been conducted in the context of developing CNT for biomedical applications. Following subcutaneous injection of oxidised MWNT, Sato et al. [91] found that longer nanotubes (825 nm) mediated a stronger immune response compared to their shorter MWNT (220 nm in length). The results were explained also in this case on the basis of macrophages being unable to easily engulf the longer MWNT. Recently, Koyama et al. [92] studied the effect of metal impurities on the toxicological profile of subcutaneously injected non-purified and purified MWNT and observed the induction of immunological toxicity and localised alopecia with as-produced, non-purified MWNT. In another study, Carrero-Sanchez
et al. [79] described no signs of toxicity or biochemical and histopathological alterations after oral and intraperitoneal administration of pristine and N-doped MWNT. However, when SWNT and MWNT dispersed in Tyrode solution were intravenously injected by Radomski and co-workers [93], platelets were easily stimulated by CNT causing an enhancement in the rate of carotid artery thrombosis formation.

Focusing more on studies that used chemically-functionalised CNT for biomedical applications, Yang et al. [26] found that neither the pristine, nor the PEGylated SWNT showed signs of acute toxicity even at higher doses of 80mg pristine and 24mg PEGylated SWNT per kg body mouse weight. Interestingly, Lacerda et al. [37] found that intravenously injected ammonium functionalised MWNT did not cause any physiological or pathological abnormalities after 24 hrs post-injection even with high injected MWNT doses (20 mg/kg) in Balb/C mice compared to serum-suspended pristine MWNT. This work emphasised the importance of adequate chemical functionalisation leading to individually dispersed CNT as a critical factor for the prevention of toxicity and organ accumulation. In addition, Schipper et al. [94] showed no haematological or histopathological alterations when SWNT coated with PEGylated lipids were i.v injected and assessed up to 4 months post-injection. However, when Yang et al. [95] injected purified SWNT (coated with 1% Tween 80) i.v. in (male CD-ICR) mice, serum biochemical changes and pulmonary inflammation were observed in the absence of induction of apoptosis or changes in immunological markers 3 months post-injection. In order to study whether there is any splenic toxicity (spleen being one of the major organs of CNT accumulation), Deng et al. [96] found that glucosamine-functionalised MWNT
(i.v injected) did not cause any change in the phagocytic activity nor a reduction in glutathione, superoxide dismutase of the spleen over 2 months.

All types of nanoparticles, including CNT, are novel and complex structures with unknown biological activities, hence their toxicity cannot merely be determined based on a single parameter. A collection of different parameters which may result in an additive or synergistic toxicological impact have to be assessed. Although comparisons between the currently published data on CNT toxicology are difficult because of differences in several important material parameters such as size, dispersibility, functionalisation, metal catalyst contaminants, one can clearly see that agglomeration and low dispersibility of as-produced, pristine CNT is a major contributing factor for toxicity. Chemical functionalisation that improves CNT dispersibility leads to dramatically reduced cytotoxicity both \textit{in vitro} and \textit{in vivo}. Another major contributing factor is the presence of metal catalysts which complicates the cytotoxicity profile as the intrinsic nanotube contribution to toxicity is difficult to identify. Hence, detailed chemical and structural characteristics should always be provided in toxicological assessments of CNT to allow a clearer determination of contributing factors to toxicity. More systematic studies should also be done with chemically functionalised CNT developed for biomedical applications, to determine the effect of material characteristics (such as shortening) that make CNT biocompatible with the biological milieu. Lastly, a concern for \textit{in vitro} toxicological assessment of CNT is the need for accurate assays that do not interfere with the CNT and will allow high-throughput and accurate \textit{in vitro} assessments of the cytotoxic risks associated with different types of CNT materials.
Table 1.1.2: Selection of the most important *in vivo* toxicity studies using CNT reported today classified according to the exposure route (pulmonary, dermal and systemic).

<table>
<thead>
<tr>
<th>CNT Type</th>
<th>Functionalisation/Exposure condition</th>
<th>Dose</th>
<th>Model</th>
<th>Exposure duration</th>
<th>Conclusions</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pristine MWNT</td>
<td>Inhalation, particles in the air (aerosol)</td>
<td></td>
<td>Healthy volunteers</td>
<td>30 min</td>
<td>Nanotube concentrations from 0.7 to 53 µg/m³</td>
<td>[75]</td>
</tr>
<tr>
<td>Aerosolised MWNT</td>
<td>Inhalation</td>
<td>32.61 mg/m³</td>
<td>Female Kunming mice</td>
<td>5, 10, and 15 days</td>
<td>Aggregations of MWNT induced proliferation and thickening of the alveolar walls</td>
<td>[76]</td>
</tr>
<tr>
<td>Pristine MWNT (respirable aggregates)</td>
<td>Inhalation</td>
<td>0.3, 1 and 5 mg/cm³</td>
<td>Male C57BL/6 mice</td>
<td>7 and 14 days</td>
<td>No significant lung inflammation or tissue damage</td>
<td>[77]</td>
</tr>
<tr>
<td></td>
<td>Intratracheal instillation, suspension in saline with Tween</td>
<td>25 mg</td>
<td>Male Dunkin Hartley guinea pigs</td>
<td>4 weeks</td>
<td>Do not induce any abnormalities of pulmonary function or measurable inflammation</td>
<td>[82]</td>
</tr>
<tr>
<td>Pristine Arc-CNT (respirable aggregates)</td>
<td>Intratracheal instillation, suspension in saline with Tween</td>
<td>1 and 5 mg/kg</td>
<td>Male Cr:CD(SD)IGS BR rats</td>
<td>24 h, 1 week, 1 and 3 months</td>
<td>High dose exposure causes mortality within 24 post-instillation</td>
<td>[80]</td>
</tr>
<tr>
<td>Pristine-laser SWNT</td>
<td>Intratracheal instillation, suspension in PBS with 1% Tween 80</td>
<td>0.1 and 0.5 mg/mouse</td>
<td>Male B6C3F1 mice</td>
<td>7 and 90 days</td>
<td>Dose dependent epithelioid granulomas</td>
<td>[81]</td>
</tr>
<tr>
<td>Raw &amp; purified SWNT</td>
<td>Intratracheal instillation, dispersion in mouse serum</td>
<td>0.5, 2 and 5 mg/rat</td>
<td>Female Sprague-Dawley rats</td>
<td>1 and 2 months (single bolus of 500 µl/Rat)</td>
<td>Ground MWNT are clearly rapidly, while un-ground accumulate in the airways</td>
<td>[97]</td>
</tr>
<tr>
<td>Pulmonary Lesions after 2 months</td>
<td></td>
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<td>----------------------------------</td>
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<tr>
<td><strong>CVD- and Arc-MWNT</strong> (suspension in saline with SDS)</td>
<td></td>
<td></td>
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<tr>
<td>Purified and N-doped MWNT (suspension in PBS)</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Raw MWNT (suspension in saline with 1% Tween 80)</td>
<td></td>
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<td></td>
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<tr>
<td>Long MWNT (Dispersed in Tween 80)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Short &amp; Long MWNT (dispersed in BSA)</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>MWNT with defects (Dispersed in PBS)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>MWNT without defects</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>CNT Patch test (filter paper saturated with water suspension of soot)</td>
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</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Material</th>
<th>Route of Administration</th>
<th>Dose</th>
<th>Species</th>
<th>Duration</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MWNT</td>
<td>Intratracheal instillation</td>
<td>15 mg</td>
<td>Guinea pigs</td>
<td>90 days</td>
<td>Pulmonary lesions, pneumonitis</td>
</tr>
<tr>
<td>Purified and N-doped MWNT</td>
<td>Nasal and intratracheal</td>
<td>1, 2.5, and 5 mg/Kg</td>
<td>Male CD1 mice</td>
<td>24, 48, and 72 hrs</td>
<td>Pristine MWNT cause dose dependent death after i.t administration.</td>
</tr>
<tr>
<td>Raw MWNT</td>
<td>Intratracheal instillation</td>
<td>0.05 mg/mouse</td>
<td>Female Kunming mice</td>
<td>8, 16 and 24 days</td>
<td>MWNT clumps distribute in bronchi and alveoli</td>
</tr>
<tr>
<td>Long MWNT</td>
<td>Intraperitoneal injection</td>
<td>3 mg</td>
<td>P53 Heterozygous mouse</td>
<td>25 weeks</td>
<td>Carcinogenic effects after 25 weeks.</td>
</tr>
<tr>
<td>Short &amp; Long MWNT</td>
<td>Intraperitoneal injection</td>
<td>50 µg</td>
<td>Female C57BL6 mice</td>
<td>24 hrs, 7 days (50 µg per mouse)</td>
<td>Increase Polymorphonuclear leukocytes and protein levels</td>
</tr>
<tr>
<td>MWNT with defects</td>
<td>Intraperitoneal injection</td>
<td>2-20 mg</td>
<td>Wistar rats</td>
<td>2 years</td>
<td>No carcinogenicity detected after 2 years post-injections</td>
</tr>
<tr>
<td>MWNT without defects</td>
<td>Intraperitoneal injection</td>
<td>20 mg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNT</td>
<td>Patch test</td>
<td>Soot with high content of CNT</td>
<td>Healthy volunteers</td>
<td>96 hrs</td>
<td>CNT don’t cause skin irritation or allergies</td>
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</table>

References: [79, 83, 86, 85, 87, 90]
<table>
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<tr>
<th>Dermal</th>
<th>Ocular instillation</th>
<th>Soot with high content of CNT</th>
<th>Albino rabbits</th>
<th>24, 48 and 72 h (0.2 ml of water suspension of soot)</th>
<th>CNT don’t cause skin irritation or allergies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pristine SWNT</td>
<td>Dermal exposure</td>
<td>Particles in the air (aerosol)</td>
<td>Healthy volunteers</td>
<td>11-16 h</td>
<td>Deposition on individual gloves from 0.2 to 6 mg (visible contamination)</td>
</tr>
</tbody>
</table>

| Systemic        |                         |                                |                |                                                                 |                                             |

| MWNTox          | Clusters were implanted in the tissue (thoracic region) | Clusters of 0.1 mg | Male Wistar rats | 1 and 4 weeks | Granulomatous inflammation, Inflammatory response around 220 nm was slighter than 825 nm |

| Purified open SWNT and MWNT | Intravenous administration | 50 µg/ml | Wistar-Kyoto rats | 15 min | Accelerate the time and rate of development of carotid artery thrombosis |

| Purified and N-doped MWNT | Oral and Intraperitoneal administration | 1, 2.5, and 5 mg/Kg | Male CD1 mice | 7 and 30 days | Both types of MWNT do not induce signs of distress or tissues changes; No changes observed in the biochemistry parameters |

| PEG-coated SWNT | Intravenous administration | 151 mg/mouse | Female and male nude mice | 4 months | Both types of SWNT persisted in the liver and spleen without acute or chronic toxicity |

| SwNTox-PEG       | Intravenous administration | 47 mg/mouse | (single dose of 100 µl on days 0 and 7) |                      |                                            |

| Pristine MWNT    | Intravenous administration | Suspensions in Serum | Female BalB/C | 24 hrs | No physiological or pathological abnormalities with functionalised MWNT compared to pristine MWNT. The higher degree of functionalisation, the lower accumulation in organs (Lungs, liver, spleen) |

| MWNT-NH₂        | 5 % dextrose |                      |                |                        |                                             |
1.5. CNT in the delivery of cancer therapeutics

The discovery that carbon nanotubes are capable of traversing the plasma membrane [24] and promoting the cellular uptake of small molecules and macromolecules (e.g. nucleic acids and peptides [98, 99] has opened up opportunities for various biological applications including cancer therapy [23]. Although most of the existing anticancer drugs are very potent small molecules, their efficacy is constrained not only by their systemic toxicity and narrow therapeutic window but also due to drug resistance and limited cellular entry. For this reason the development of efficient delivery systems with the ability to reach target cells and enhance cellular uptake of existing potent drugs is needed. Functionalised CNT have shown even greater promise as novel delivery systems based on their ability to cross biological barriers independent of the cell type they interact with and the functional group at their surface [24]. In addition to their high surface area which provides multiple attachment sites for molecules, their intrinsic physical properties have proven at least in proof-of-principle studies, to be another advantage of using CNT in cancer therapeutics [100].

1.5.1. Delivery of anticancer agents through non-covalent functionalisation with the CNT

1.5.1.1. CNT: Anticancer drugs

One of the latest strategies used with CNT in cancer therapy is the capacity of polycyclic aromatic molecules to form supramolecular complexes with the backbone of CNT through π-π stacking. The most investigated anticancer drug in this context is the anthracycline; doxorubicin. The earliest investigations in this regard were carried out in this thesis and initially published by us [14] and Liu et al. [101] at the
same time. Both groups proved the ability of doxorubicin to form supramolecular complexes when mixed with CNT by clear quenching of doxorubicin fluorescence and the formation of star like complexes as observed by TEM [14, 101]. While Liu et al. [101] used SWNT coated with phospholipid–polyethylene glycol (PL-PEG) molecules; we worked with MWNT coated with Pluronic F127 which renders the MWNT dispersed in aqueous media. Liu and coworkers described a pH and diameter dependent loading and release of doxorubicin from SWNT, however no improvement in the cell killing capacity of the complexes compared to drug alone was observed [101].

In a subsequent therapeutic in vivo study, Liu and coworkers showed that the SWNT: PL-PEG: Doxorubicin complexes led to a significant enhancement in the therapeutic efficacy and reduced side effects in a Raji lymphoma xenograft model in vivo after the systemic administration of the complexes; however, no statistically significant difference from doxorubicin alone or Doxil was observed [102]. Other chemically functionalised CNT were also able to strongly bind doxorubicin similar to what was described by Heister et al. [103] who used carboxylated and bovine serum albumin (BSA) coated SWNT. Cellular uptake studies of the constructs were shown by confocal microscopy using WiDr cancer cells which over-express carcinoembryonic (CEA) antigens. In addition, monoclonal antibodies targeting the CEA antigens were used but no comparison of their cellular uptake with the non-targeted constructs was given.

Moreover, oxidised SWNT and polysaccharide coated SWNT [104] were complexed with doxorubicin at pH 7.4 in contrast to what was suggested by Liu and colleagues [101] with a pH dependent binding and release of doxorubicin from
SWNT. Folic acid (FA) was used as a targeting moiety and FA-SWNT: Doxorubicin complexes were more efficiently uptaken by human epithelial cervical HeLa cells compared with the non-targeted complexes. Moreover, there was a dose and time dependent toxicity with the FA targeted complexes. The latest studies of this type were conducted by Chaudhuri et al.[105] who described the use of pegylated SWNT for the non-covalent attachment of doxorubicin but this time doxorubicin was linked to SWNT through a carbamate linker in an attempt for the controlled release of doxorubicin. Interestingly, there was a time dependent cell death in B16-F10 melanoma cells with a maximum efficacy after 72 hrs which might be due to effective enzymatic cleavage of the linker. In addition, there was a reduced B16-F10 tumour growth after the systemic administration of the SWNT: Doxorubicin complexes but no improvement compared with the drug alone was observed.

Using the CNT coating strategy with PL-PEG, Feazell et al.[106] covalently linked Platinium Pt (IV) to the distal group of the PEG molecule along with a tracking moiety (fluorescein). The release of the drug and its reduction to its active form (cisplatin) occurs only at lower pH environments such as endocytic vesicles which meant the release only occurred inside cells [106]. The authors observed a 100 fold enhancement in the cytotoxicity against testicular carcinoma cells once the platinum compound was tethered to PL-PEG coated SWNT. The strategy of platinum prodrug delivery was again assessed by the same group but with the introduction of folic acid (FA) as targeting ligands [107]. The authors described the selective destruction of FA positive cell lines using the targeted SWNT-Pt (IV) construct but not to FA negative cell lines, however this contradicts their previous study in which the non-targeted SWNT-Pt (IV) construct was uptaken by FA negative cell lines and showed an enhanced cytotoxicity.
Paclitaxel has also been attached to SWNT through its covalent linkage to the distal regions of the PL-PEG coating of the SWNT [108]. In a paclitaxel-resistant breast carcinoma in vivo model, the SWNT: paclitaxel construct showed higher efficacy and tumour growth suppression compared with the clinically used Taxol after systemic administration which is thought to be a result of the long blood circulation given by the CNT. It should also be mentioned that the construct was also uptaken by RES organs, but no toxicity was observed [108].

In a completely different approach, Hampel et al. [109] showed that carboplatin-filled CNT inhibited the growth of EJ28 bladder cells compared to carboplatin and CNT alone in a dose dependent manner [109].

1.5.1.2. CNT: Small interference RNA (siRNA)

An approach that has been studied extensively in cancer therapy is the use of small interference RNA. Complexation between siRNA and carbon nanotubes have been described in proof-of-principle studies as an alternative approach for the effective delivery of siRNA that suffers from low cellular uptake and nuclease degradation. The use of CNT as delivery vectors to mediate the uptake of Telomerase Reverse Transcriptase (TERT) siRNA into murine and human tumour cells was attempted by Zhang et al.[110]. They observed that treatment with the ammonium functionalised SWNT (H$_3$N-SWNT): TERTsiRNA complexes led to suppression of cell growth due to silencing of the TERT gene which is important in the development and growth of tumours, however no therapeutic data were shown.

In addition, SWNT with the PL-PEG coating were chemically linked through disulfide linker to siRNA encoding the lamin A/C protein found inside the nuclear lamina of the cells and is essential in nuclear stability, chromatin structure and gene
expression. A highly efficient uptake of the siRNA by SWNT was observed compared to conventional transfection agents (lipofactamine) and gene silencing was observed by the reduction of lamin A/C protein expression.

In a different study, ammonium functionalised SWNT were complexed with siRNA targeting the cyclin A2 protein [111]. The cyclin A2 play a critical role in DNA replication, transcription and cell regulation and is over-expressed in different tumours mainly leukemia. *In vitro* studies showed that SWNT-NH₃⁺: siRNA complexes are efficiently uptaken into human erythroleukemic cells resulting in the down-regulation of the over-expressed cyclin A2 oncogene that subsequently affected cellular proliferation [111].

The most comprehensive and promising study to date was that run by Podesta *et al.* [43] using a cytotoxic inducing proprietary siRNA sequence (siTox®). In this comparative study, siTox was complexed to both ammonium functionalised MWNT and cationic liposomes. It was found that only MWNT-NH₃⁺: siTox complexes can elicit delayed tumour growth and increased survival of human lung xenograft bearing mice after intratumoral administration [43]. This direct comparison with liposomes demonstrates the potential use of carbon nanotubes for the delivery of siRNA for cancer therapy.

1.5.2. Delivery of anticancer agents through covalent conjugation to CNT

Because of the CNT high aspect ratio, attachment of multiple copies of different moieties along the CNT backbone can be achieved through chemical conjugation. For instance, Pastorin *et al.* first developed double functionalised CNT-methotrexate conjugates with imaging probes via covalent linkages for the use of CNT as multimodal drug delivery systems [22]. However, the cell-kill efficacy of the
MWNT- methotrexate conjugate was not improved as compared to methotrexate alone which is thought to be due to the high stability of the linking amide bond between the drug and the CNT backbone leading to slow intracellular release of the drug from the tubes. The same group has recently improved on the MWNT-methotrexate construct not only in terms of dispersibility in aqueous media but also by introducing cleavable linkers namely ester and peptide sensitive linkers [112]. The latter, which is sensitive to the over-expressed cathepsin B enzyme in tumour cells, has shown statistically significant enhancement in the cytotoxicity of the drug compared to drug alone.

Bhirde et al.[113] used cisplatin was chemically conjugated to carboxylated SWNT to which epidermal growth factor (EGF) was also covalently attached as a targeting ligand. Selective uptake and enhanced cell killing with the targeting construct into head and neck cell lines in vitro and in vivo was obtained. In addition, rapid regression in head and neck tumours treated with the EGF targeted SWNT-cisplatin construct was achieved after systemic administration [113].

Taxoid was also chemically linked through disulfide bonds to carboxylated and ammonium functionalised SWNT which was also linked to biotin as the targeting ligand [114]. While the non-targeted SWNT-taxoid construct showed an energy independent uptake, the biotin targeted construct showed an endocytosis based cellular uptake. A clear specificity of the targeted construct was also observed against biotin positive cell lines and the subsequent cytotoxicity observed was merely due to the released taxoid inside the cells [114].

While most studies used SWNT, the third study using MWNT was conducted by Wu et al.[115] who conjugated MWNT to 10-hydroxy-camptothecin (HCPT)
with a cleavable ester bond. MWNT-HCPT showed a superior cell killing activity in vitro and in vivo compared to clinically used HCPT formulations. In addition, the blood half life of the drug was increased from only 30 min to 3.6 hrs after conjugation to MWNT.

CNT have also been used as delivery vectors for boron compounds as shown in a proof of principle study run by Yinghuai et al., illustrating the possibility of using nanotubes as vehicles for effective boron neutron capture therapy for cancer treatment [116]. Their substituted C2B10 carborane cage-modified CNT showed preferential accumulation in tumour cells compared to blood and other organs, however, no reported mechanism of accumulation was proposed or shown in this study.

1.5.3. NIR and radiofrequency ablation (hyperthermia)

One of the most investigated applications of CNT in cancer therapy is the use of their intrinsic optical properties that allow for development of hyperthermia treatment. For instance, the strong optical absorbance exhibited by CNT in the near infrared (NIR) region allowed Kam et al. to achieve tumour cell death by localized hyperthermia after continuous NIR radiation of CNT [117]. Selective targeting was achieved by linking folic acid (FA) moieties to PL-PEG coating the nanotubes. An alternative approach was recently reported using Kentera® (a polymer composed of polyphenelyne ethynelyne) coated SWNT for cancer hyperthermia by radiofrequency irradiation [15]. Exposure of CNT to electromagnetic field led to heat generation causing subsequent cell necrosis and death when CNT were inside tumour cells. This approach was proposed as a non-invasive method compared to radiofrequency ablation usually used in cancer treatment in which electrodes are
introduced directly into the tumour mass. Gannon et al. showed that thermal
destruction occurred not only in vitro in two resistant and aggressive cell lines
(hepatocellular “HepG2 and Hep3B” and pancreatic “Panc 1” cell lines) but also
SWNT treated tumors revealed necrosis after 48 hrs as indicated by histology
analysis [15]. This study did not contain any overall tumour regression but more
experimental studies are expected.

Overall, these initial studies have shown what carbon nanotubes can potentially
offer a novel tool for the development of advanced cancer therapeutics; however
more systematic work is still required in order to identify the full capabilities and
limitations of CNT in the biomedical field.
CHAPTER II

PROJECT AIM AND OBJECTIVES

Carbon nanotubes have received considerable attention in recent years because of their extraordinary properties and their ability to cross biological barriers. Although recent advances have been made in the use of CNT as delivery vectors for therapeutic and diagnostic purposes, gaps still exist in the accurate understanding of their impact on biological systems. This means that there is a need for a fundamental and systemic understanding of their pharmacological and toxicological profiles as this is a critical step in the early stages of development for any pharmaceutical agent. 

The aim of this project was therefore to elucidate the important and critical parameters in CNT pharmacokinetics and toxicology and to take this further into establishing effective CNT nanovectors for cancer therapeutics.

The main objectives and focus of this project were:

- Determination of key parameters that play a pivotal role in the *in vivo* biodistribution of functionalised MWNT injected systemically.

- Evaluation of the *in vitro* and *in vivo* cytotoxicity of functionalised CNT. Particular attention was given to the establishment of a reliable technique for the accurate evaluation of the *in vitro* cytotoxicity of CNT.

- The development of CNT-anticancer drug constructs that can be readily uptaken by cells and improve the efficacy of drug activity.
Current CNT biodistribution data suffers from discrepancies and lack of reproducible conclusions which is mainly due to the variability in several factors that can affect the biodistribution and pharmacokinetics of CNT when systemically injected into animal models. In an attempt to identify those critical factors, different types of CNT chemically and non-covalently functionalised were studied. A close look at the effect of surface functionalisation, coating, filling and dispersion properties on the biodistribution was also attempted. This aspect of the research will be discussed in detail in Chapter IV.

Another broader issue with CNT in the recent years is the accurate determination of their toxicity profiles in vitro. This has proven difficult because of the interference that CNT have posed with the widely used cytotoxicity reagents and assays. In this project (Chapter V, Section 5.1) the development of a reliable cytotoxicity assay was attempted based on the pitfalls and drawbacks seen in the use of CNT with established in vitro cytotoxicity assays such as MTT, LDH and Annexin V/PI. In addition, a comparison between the safety profiles of chemically and non-covalently functionalised CNT was highlighted in this study.

Moreover, the effect of chemical functionalisation on alleviating the carcinogenic risks associated with long rigid MWNT was also studied in this project using a structure-activity paradigm of toxicity based on the direct exposure of the abdominal cavity of mice to MWNT. Two types of chemical functionalisation were investigated and more insights into the determinant factors for granuloma formation were given in Chapter V, Section 5.2.

Advancing this research further, the engineering of MWNT for use as cancer therapeutics was attempted. Although the field of oncology pharmaceuticals does not
lack effective therapeutic molecules, its primary and ongoing problem has been systemic toxicity, drug resistance and limited cellular entry of drugs. The intrinsic ability of CNT to translocate biological barriers was hypothesised to offer an alternative transport mechanism for anticancer drug agents to reach their target sites and release their cargo in a controlled manner. In this regard, two different approaches were explored in this project (Chapter VI). The first attempt was based on the structural characteristics of CNT allowing for the non-covalent attachment of doxorubicin molecules onto their surfaces due to π-π stacking (Section 6.1). The second approach was dependent on the chemical derivatization of the functional groups at the surface of CNT with the anticancer drug, methotrexate along the introduction of cleavable linkers for the development of a prodrug like system (Section 6.2). A thorough investigation onto the efficacy of these novel CNT-anticancer drug constructs was attempted both in *in vitro* cell monolayers and *in vivo* tumour models.
CHAPTER III

MATERIALS AND METHODS

3.1. Evaluation of the biodistribution profile of functionalised MWNT

3.1.1. Chemical functionalisation of MWNT (Aryl f- MWNT)

The preparation of aryl functionalised MWNT was carried out with the assistance of Dr. Nikos Karousis (Theoritical and Physical Chemistry Institute, Nationali Hellenic Research Foundation, Athens, Greece).

3.1.1.1. Oxidation of pristine MWNT (MWNT-COOH)

20 mg of MWNT (MWNT, Nanocyl) were suspended in 5 ml mixture of concentrated sulphuric (H₂SO₄) and nitric acid (HNO₃) in a 3/1 v/v ratio respectively in a 20 ml test tube and sonicated in a water bath (Ultrasonic cleaner, VWR) for 5 hrs maintaining the water temperature between 35 and 40°C. The obtained mixture was further diluted with 15 ml of water and filtered through Millipore filter paper (pore size of 0.22 μm). The black solid collected from the top of the filter paper was mixed with 30 ml of deionised water and sonicated for 5 min and filtered again through a filter paper of the same pore size. The black solid was washed thoroughly with deionised water until the pH reached 6. We are also aware of the possibility of forming polyaromatic hydrocarbon fragments as a consequence of the oxidation procedure as recently described by Shaffer et al. [118] and Whitbey et al. [119]. This means that any subsequent functionalisation of the carboxyl groups can also occur on the polyaromatic hydrocarbons.
3.1.1.2. Aryl Functionalised MWNT-NHBOC

15 mg of the prepared MWNT-COOH was suspended in 40 ml of deionised water and sonicated for 20 min. A solution of the aniline derivative prepared by Dr. Nikos Karousis (1.85 mmol, Scheme 3.1.1) in 10 ml of absolute ethanol was added. After degassing the reaction mixture and bubbling with nitrogen, 2.5 mmol of isoamyl nitrite was quickly added and the suspension was stirred in an oil bath at 85 °C overnight. After cooling to RT, the reaction mixture was diluted with 50 ml of dimethylformamide (DMF), filtered with a Millipore (0.22 μm) membrane filter and washed extensively with DMF and CHCl₃ to remove any unbound organic material. Then, the black solid material on top of the filter was dispersed in 50 ml DMF with 5 min sonication and again filtered through a Nylon membrane filter (0.22 μm), washing it extensively with deionised water and methanol (MeOH). The black aryl functionalised HOOC-MWNT-NHBOC was then removed from the filter and dispersed in dichloromethane (DCM, CH₂Cl₂).

3.1.1.3. Aryl Functionalised MWNT-NH₃⁺ (BOC deprotection procedure)

The BOC protecting group of the functionalised MWNT-NHBOC was cleaved by treatment with trifluoroacetic acid (TFA, C₂HF₃O₂) at room temperature for 6 hrs. The reaction mixture was then diluted with chloroform (CHCl₃) and filtered through Millipore membrane filter (0.22 μm) washing the black solid with CHCl₃, water and dichloromethane: methanol (1:1) mixture.
3.1.1.4. Quantitative Kaiser test

This test was used to establish the amount of free amino groups (-NH$_3^+$) generated around the sidewalls of the nanotubes. 1 mg of MWNT-NH$_3^+$ was weighed and in a test tube the following solutions were carefully added in this specific order: 75 µl of solution 1 (10 g of phenol dissolved in 20 ml of ethanol), 100 µl of solution 2 (2 ml of 1 mM potassium cyanide (KCN) dissolved in 98 ml of pyridine) and 75 µl of solution 3 (1 g of ninhydrine dissolved in 20 ml of ethanol. The test tube was heated to 100 °C for 7 min and once removed it was diluted with 4.8 ml of 70 % ethanol to a final volume of 5 ml, then carefully mixed.

A dispersion of pristine (non-amino functionalised) MWNT was also run as a blank. The loading of the amino groups was calculated using the following equation and expressed as micromole of amino groups per gram material.

\[
\mu \text{mol/gram} = \frac{[\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}}] \times \text{Dilution (ml)} \times 10^6}{\text{Extinction coefficient} \times \text{sample weight (mg)}}
\]

(Where Dilution = 5 ml & Extinction coefficient = 15000 M$^{-1}$cm$^{-1}$)
3.1.1.5. Preparation of DTPA-aryl f-MWNT

MWNT-NH$_3^+$ were mixed with DMF in a round bottom flask and sonicated for 10 min. Diisopropylethylamine (DIEA, C$_8$H$_{19}$N) was then added and the mixture stirred for 10 min before 2-4-Isothiocyanatobenzyl-diethyketriaminepentaacetic acid (p-SCN-Bn-DTPA) solution in water or DMF was added. The reaction mixture was then left stirring for 2 days or longer depending on the reaction efficiency as checked by the Kaiser test. At the end of the reaction, the black solution was filtered through a Nylon membrane filter (0.22 µm) while washed with DMF and DCM. Finally, the efficiency of the reaction was again quantified by the Kaiser test which indicated the number of remaining free amino groups and hence the amount of DTPA conjugated to MWNT.

Scheme 3.1.2: Illustration of the functionalisation reaction of $^{111}$In radiolabelled DTPA-aryl f-MWNT.

3.1.1.6. Preparation of $^{111}$In radiolabelled DTPA-aryl f-MWNT

General protocol involved the dilution of DTPA-MWNT (Scheme 3.1.2, Material 3) and DTPA alone in an equal volume of 0.2 M ammonium acetate buffer (pH 5.5), to
which indium chloride ($^{111}$InCl$_3$) was added. The indium was left to react with the 
DTPA- MWNT and DTPA alone for 10 minutes, after which the reaction was
quenched by the addition of 0.1 M EDTA chelating solution (1/20 the reaction
volume is added). $^{111}$InCl$_3$ alone, used as a control, was also subjected to the same
conditions of the labelling reaction. Aliquots of each final product were diluted five
folds in PBS and then 1 µl spotted on silica gel impregnated glass fiber sheets
(PALL Life Sciences, UK). The strips were developed with a mobile phase of 50
mM EDTA in 0.1 M ammonium acetate and allowed to dry before analysis. This
was then developed and the autoradioactivity quantitatively counted using a Cyclone
phosphor detector (Packard Biosciences, UK). The immobile spot on the TLC strips
indicated the percentage of radiolabelled $[^{111}$In] DTPA-MWNT conjugate (Scheme
3.1.2, material 4), while the free $^{111}$In or $[^{111}$In] DTPA were shown by the mobile
spot.

3.1.1.7. Transmission Electron Microscopy of aryl f-MWNT

A drop of the f-MWNT dispersion was placed on a grid with a support film of
Formvar/carbon, excess material was blotted off with a filter paper and the MWNT
dispersions were examined under a FEI CM120 BioTwin Transmission Electron
Microscope (Philips/FEI, USA) using a 80 KV electron beam current. Images were
captured using an AMT Digital Camera. All TEM images were taken with the
assistance of Mr. David McCarthy (The School of Pharmacy, London).

3.1.2. Tissue biodistribution studies of aryl f-MWNT

Six- to eight-week-old C57BL6 mice were obtained from Harlan (Oxfordshire, UK),
allowed to acclimatise for 1 week and were kept in groups of 3 for the duration of
the experiments and given food and water. All experiments were conducted with prior approval from the UK Home office.

3.1.2.1. Whole body imaging of $[^{111}\text{In}]-\text{DTPA-aryl f-MWNT}$ using SPECT/CT

C57BL6 mice were anaesthesised by isofluorane inhalation. Each animal was injected via the tail vein with 200 $\mu$l of $[^{111}\text{In}]-\text{DTPA-MWNT}$ in 5% dextrose containing 1.12MBq $^{111}\text{In}$ and 50 $\mu$g of DTPA-MWNT. 30 min, 4 hrs and 24 hrs post-injection, mice were imaged by using SPECT/CT scanner (Bioscan, Washington DC, WA, USA). The SPECT image was obtained in 16 projections over 40 min using a 4-head scanner with a 1.4 mm pinhole Collimators, to obtain 45000 counts per second. CT scans were taken at the end of each SPECT acquisition and all images were reconstructed using the MEDISO software (Medical Imaging Systems). Fusion of SPECT and CT images was carried out using the PMOD software. All SPECT/CT experiments were carried out with assistance of Dr. Khuloud Al-Jamal (The School of Pharmacy, London).

3.1.2.2. Quantitative radioactivity analysis of $[^{111}\text{In}]-\text{DTPA-aryl f-MWNT}$

Each animal was injected with 200 $\mu$l of $[^{111}\text{In}]-\text{DTPA-MWNT}$ in 5% dextrose containing 0.2-0.5 MBq $^{111}\text{In}$ and 50 $\mu$g of DTPA-MWNT via the tail vein injection. At 30 min, 4 hrs and 24 hrs after injection, four mice per group were killed, blood collected. Heart, lungs, liver, spleen, kidneys, stomach and small and large intestines were sampled, each sample being weighed and counted on a gamma counter (Perkin Elmer, USA), together with a dilution of the injection sample. The percentage of injected dose per gram of tissue (%ID/g) and per organ (%ID/organ) was calculated for each tissue type. The biodsitribution experiment was carried out with the assistance of Dr. Khuloud Al-Jamal (The School of Pharmacy, London).
3.1.2.3. Tissue histology following administration of aryl f-MWNT

Animals were injected with non-radiolabelled DTPA-MWNT and 200 μl of DTPA-MWNT in 5% dextrose containing 50 μg of MWNT and 200 μl of the vehicle alone were injected. In addition, 50 μg of pristine MWNT dispersed in the copolymer F127 (0.25 % final concentration, See Section 3.3.1 for details about preparation) and bovine serum albumin (BSA; 0.5 % final concentration) were also injected as controls. Animals were sacrificed 24 hrs post-injections and lungs were fixed under inflation with 10 % buffered formalin and the liver, spleen and kidneys were harvested. The tissues were fixed in 10 % buffered formalin and processed for routine histology with Hematoxylin and Eosin stain by the Laboratory Diagnostic Service of the Royal Veterinary College (London, UK). Microscopic observation of tissues was carried out with Nikon Microphot-FXA microscope coupled with Infinity 2 digital camera.

3.2. Evaluation of the biodistribution profile of carbohydrate functionalised and iodine filled SWNT

3.2.1. Filling and functionalisation of SWNT (Carbohydrate functionalised and iodine filled SWNT)

The preparation of carbohydrate functionalised and iodine filled SWNT was carried out by Dr. Sung You Hong (Chemistry Research Laboratory, University of Oxford, UK) and Dr. Gerard Tobias (Inorganic Chemistry Laboratory, University of Oxford, UK) and shown in Scheme 3.1.3. More details about the chemistry are found in reference [121] and are described below.
3.2.1.1. Filling of SWNT with Na$^{125}$I (Na$^{125}$I$@$SWNT)

SWNT were purified and shortened with steam at 900 °C [122]. SWNT (1 mg), Na$^{125}$I (47 μL, 4.7 mCi, 1x10$^{-5}$ M NaOH) and NaI (10 μL, 12 mg/mL) were transferred into a silica ampoule and heated to 70 °C to remove water. The ampoule was sealed under vacuum and annealed at 900 °C for 4 hrs. The mixture was then washed with water to remove the non-filled material, filtered and dried. The cold sample was prepared under the same conditions replacing Na$^{125}$I with NaI.

3.2.1.2. The 1, 3-dipolar cycloaddition on Na$^{125}$I$@$SWNT (f-Na$^{125}$I$@$SWNT)

Na$^{125}$I$@$SWNT (1 mg), NBoc-aminoacid (1.0 mg, 3.3 μmol) and 2,3,5-triiodobenzaldehyde (1.6 mg, 3.3 μmol) were dispersed in DMF (dry, 2 mL) with the aid of sonication (1-2 min). The reaction mixture was refluxed at 120-130 °C for 96 hrs and then cooled to room temperature and concentrated in vacuo. The residue was re-dispersed and washed with MeOH. The sample was filtered on polycarbonate film (pore size: 0.2 μm) and dried in vacuo. The cold sample was prepared under the same conditions. TLC (DCM:MeOH=8:2) indicated the clear removal of reagents.

Scheme 3.1.3: Schematic representation of the different steps involved in the chemical functionalisation of SWNT (Adapted from reference [121]).
3.2.1.3. Glycosylation of $f$-Na$^{125}$I@SWNT (GlcNAcD-Na$^{125}$I@SWNT)

$f$-Na$^{125}$I@SWNT (1 mg) were dispersed in DCM (dry, 2 mL) by sonication for 2 min. To the suspension was added TFA (0.5 mL) and the reaction mixture was stirred at room temperature for 1 hrs to afford amine terminus by the cleavage of NBoc protecting groups. The mixture was concentrated in vacuo and dried under vacuum. Amine terminated SWNTs were treated with N-acetyl-D-glycosamine (GlcNAcD; 2 mg), DIPEA (1 µL) and HATU (1 mg) in DMF (dry, 2.1 mL). The reaction mixture was sonicated for 2 min and stirred under Ar at room temperature. After 24 hrs, the reaction mixture was concentration in vacuo, the residue was re-dispersed, filtered and rinsed with MeOH followed by water. The sample was dried in vacuo. The cold sample was prepared under the same conditions. TLC (water: isopropanol: ethyl acetate, 1:2:2) indicated the removal of reagents.

3.2.1.4. Preparation of GlcNAcD-NA$^{125}$I@SWNT aqueous dispersions

For biological purposes the GlcNAcD-NA$^{125}$I@SWNT and cold derivative were dispersed at a final concentration of 1 mg/ml in 2.5% Bovine serum albumin (BSA; Sigma-Aldrich, UK) by bath sonication for 45 min. Further dilutions of this stock were carried out using 5% dextrose for in vitro or PBS for in vivo experiments.

3.2.1.5. Transmission Electron Microscopy of GlcNAcD-NA$^{125}$I@SWNT

A drop of the $f$-MWNT dispersion was placed on a grid with a support film of Formvar/carbon, excess material was blotted off with a filter paper and the MWNT dispersions were examined under a FEI CM120 BioTwin Transmission Electron Microscope (Philips/FEI, USA) using a 80 KV electron beam current. Images were
captured using an AMT Digital Camera. All TEM images were taken with the assistance of Mr. David McCarthy (The School of Pharmacy, London).

3.2.2. Cytotoxicity assessment of carbohydrate functionalised and iodine filled SWNT (GlcNAcD-NaI@SWNT) in vitro

Cell culture: Lung carcinoma cells (A549; ATCC®, CCL-185™, UK) were grown in F12 Ham media (Invitrogen, UK) supplemented with 10% fetal bovine serum (FBS, Invitrogen, UK) and 50 U/ml penicillin (Invitrogen, UK) and 50 μg/ml streptomycin (Invitrogen, UK) at 37 °C in 5% CO2. Cells were passaged when reaching 80% confluency.

3.2.2.1. The ‘modified LDH’ mLDH assay of GlcNAcD-NaI@SWNT

The lactate dehydrogenase (LDH) assay was used to assess the cytotoxicity of ‘cold’ GlcNAcD-NaI@SWNT in A549 cells after 24 hrs incubation with different concentrations of GlcNAcD-NaI@SWNT (7.8, 31.25 and 125μg/ml) containing 0.312, 0.078 and 0.019% BSA respectively. In brief, the Promega Cytotox 96® Non-radioactive cytotoxicity assay (Promega UK Ltd) was used according to the manufacturer instructions. The assay was modified to avoid interference with SWNT in which the LDH of healthy cells that survived treatment was assessed by artificially lysing the cells instead of looking at the LDH released due to cytotoxicity (See Section 3.3.3.4 for full description). Cells were seeded in 96 well plates at a seeding density of 12500 cells per well. Cells were treated with the different concentrations of GlcNAcD-NaI@SWNT for 24 hrs before running the assay. Cells were lysed with 10μl of lysis buffer (9% Triton X100) and 100μl of serum free media and left for 45-60min at 37°C. 50μL of cell lysate after centrifugation (13000 rpm, 5min) were mixed with 50μl of the substrate mix in a microtitter plate and
incubated for 15min at room temperature. Absorbance was read at 490 nm using Victor® Multilabel plate reader (PerkinElmer, USA). The amount of LDH released was an indication of the number of cells which survived treatment. Hence the percentage cell survival (n=4 ± SD) is expressed using this formula:

\[
\text{% Cell Survival} = \left( \frac{A_{490\ nm\ of\ treated\ cells}}{A_{490\ nm\ of\ untreated\ cells}} \right) \times 100
\]

3.2.3. Tissue biodistribution studies of GlcNAcD-Na\textsuperscript{125}I@SWNT

Six- to eight-week-old C57BL6 mice were obtained from Harlan (Oxfordshire, UK), allowed to acclimatise for 1 week and were kept in groups of 5 for the duration of the experiments and given food and water. All experiments were conducted with prior approval from the UK Home Office.

3.2.3.1. Whole body imaging of GlcNAcD-Na\textsuperscript{125}I@SWNT and Na\textsuperscript{125}I using SPECT/CT

C57BL6 mice were anaesthetised by isofluorane inhalation. Each animal was injected via the tail vein injection with 250 μl containing 50μg or 250μg of GlcNAcD-Na\textsuperscript{125}I@SWNTs containing approximately 0.8MBq or 0.2MBq, respectively. Free Na\textsuperscript{125}I (1.8MBq) was injected for comparison. 30 min, 4 hrs and 24 hrs post-injection, mice were imaged using the Nano-SPECT/CT scanner (Bioscan, USA). SPECT images were obtained in 16 projections over 40-60 min using a 4-head scanner with 1.4 mm pinhole collimators. CT scans were taken at the end of each SPECT acquisition and all images were reconstructed with MEDISO software (Medical Imaging Systems). Fusion of SPECT and CT images was carried
out using the PMOD software. All SPECT/CT experiments were carried out with the assistance of Dr. Khuloud Al-Jamal (The School of Pharmacy, London).

3.2.3.2. Quantitative radioactivity analysis of GlcNAcD-Na\textsuperscript{125}I@SWNT or Na\textsuperscript{125}I

Animals (n=4-6) were injected with 250µl containing 50 µg (0.2 MBq) of GlcNAcD-Na\textsuperscript{125}I@SWNT in 0.5% BSA or Na\textsuperscript{125}I (0.36 MBq) in 5% dextrose containing 0.2-0.5 MBq via tail vein injection. Blood was collected by bleeding 50µl from the superficial tail vein at 2 min, 10 min, 30 min, 4 hrs and 24 hrs. Each animal was bled only twice during the whole experiment. At 30 min, 4 hrs and 24 hrs after injection, mice were killed, and blood was collected. Thyroid, heart, lungs, liver, spleen, kidneys, stomach and intestines were sampled, each sample being weighed and counted on a Gamma Counter (Perkin Elmer, USA), together with a dilution of the injected dose with deadtime limit below 60%. The percentage injected dose per gram tissue or the percentage injected dose per organ was calculated for each tissue. The biodistribution experiment was carried out with the assistance of Dr. Khuloud Al-Jamal (The School of Pharmacy, London).

3.2.3.3. Tissue histology following administration GlcNAcD-NaI@SWNT

Animals were injected with 200 µl containing 50µg of the ‘cold’ GlcNAcD-NaI@SWNT in 0.5% BSA or 0.5% BSA as a vehicle. Animals were sacrificed at 24 hrs or 30 days post-injection, lungs were fixed under inflation with 10% buffered formalin. Liver, spleen and kidneys were harvested and post-fixed. Organs were processed for routine histology with hematoxylin and eosin stain by the Laboratory Diagnostic Service of the Royal Veterinary College (London, UK). Microscopic
observation of tissues was carried out with Nikon Microphot-FXA microscope coupled with Infinity 2 digital camera.

3.3. The Development of accurate and reliable cytotoxicity assay to determine CNT toxicity

3.3.1. Preparation of chemically functionalised and Pluronic coated MWNT

Two types of functionalised MWNT were used in this study; the non-covalently coated MWNT with Pluronic F127 and the chemically functionalised MWNT (MWNT-NH$_3^+$). The Pluronic coated MWNT (MWNT: F127) were prepared as previously described in [14]. Briefly, pristine MWNT (Nanocyl® 3150) were dispersed using 1% of tri-block copolymer Pluronic F127 (Sigma, UK, Structure shown in page 98) by bath sonication (Ultrasonic cleaner, VWR) for 30 minutes into a final MWNT concentration of 1 mg/ml (Scheme 3.1.4A).

The chemically functionalised type (MWNT: NH$_3^+$, Scheme 3.1.4B) used in this part of the thesis were prepared by Dr. Alberto Bianco (CNRS, Strasbourg, France).

Briefly, oxidised MWNT were prepared as reported in reference [123]. Then, 200 mg were heated in 10 ml of neat oxalyl chloride at 62 °C for 24 hrs. After evaporation in vacuo the resulting nanotubes were dispersed in a solution of Boc-monoprotected diamino-triethyleneglycol (TEG) (670 mg) in distilled THF (10 mL) and heated at reflux for 48 hrs. The nanotubes were re-precipitated several times from methanol/diethyl ether by successive sonication and centrifugation. The Boc protecting groups were removed overnight using 4 M HCl in dioxane (10 ml) to afford ammonium functionalised MWNT (MWNT-TEG-NH$_3^+$) (110 mg) following
evaporation of the acid solution and re-precipitation in diethyl ether. Kaiser test (described in Section 3.1.1.3) showed a loading of 0.320 mmol/g. The MWNT-NH$_3^+$ were nicely dispersed as a 0.5mg/ml dispersion in 5% dextrose after 30 min bath sonication (Ultrasonic cleaner; VWR, UK). Scheme 3.1.4 shows a brief description of both functionalisation methodologies.

Scheme 3.1.4: Schematic representation of the preparation of the two types of (A) the non-covalently functionalised MWNT and (B) chemically functionalised MWNT-NH$_3^+$.  

**Transmission Electron Microscopy:** A drop of the $f$-MWNT dispersion was placed on a grid with a support film of Formvar/carbon, excess material was blotted off with a filter paper and the MWNT dispersions were examined under a FEI CM120 BioTwin Transmission Electron Microscope (Philips/FEI, USA) using a 80 KV electron beam current. Images were captured using an AMT Digital Camera. All
TEM images were taken with the assistance of Mr. David McCarthy (The School of Pharmacy, London).

3.3.2. The cellular uptake and internalisation both types of MWNT

Cell culture: Lung carcinoma cells (A549; ATCC® CCL-185™, UK) were grown in F12 Ham media (Invitrogen, UK) supplemented with 10% fetal bovine serum (FBS, Invitrogen, UK) and 50 U/ml penicillin (Invitrogen, UK) and 50 µg/ml streptomycin (Invitrogen, UK) at 37 °C in 5% CO₂. Cells were passaged to reach 80% confluency.

3.3.2.1. Flow cytometry (Side scatter) and light microscopy

A549 cells were seeded into 24-well plates (Corning Costar Corporation®, USA) at 50000 cells per well and left to attach overnight before incubation with both types of MWNT. Cells were then incubated with MWNT-NH₄⁺, MWNT: F₁₂₇ (0-125 µg/ml) and the equivalent Pluronic F₁₂₇ concentration (0-1250 µg/ml) in complete media. Cells were incubated with the MWNT for 24 hrs at 37 °C in a humidified atmosphere (5% CO₂) incubator. Monolayers were photographed before trypsinization to compare the results obtained from light microscopy with sideward-scattering light analysis. For light scatter analysis by flow cytometry, cells were washed with PBS to remove unbound MWNT, trypsinized, centrifuged at 1000 rpm for 5min at 4°C and re-suspended in PBS then transferred to 1.5 ml microcentrifuge tubes and kept on ice for immediate analysis by flow cytometry.

3.3.2.2. Transmission electron microscopy of cell sections

These studies were carried out with the assistance of Dr. Karin H Muller (The Nanoscience Centre, University of Cambridge, UK) and TEM images were taken by
Dr. Karin H Muller and Dr. Alexandra A Porter (Department of Materials, Imperial College London, UK).

A549 cells were incubated with 50 μg/ml of MWNT: F127 and MWNT-NH₃⁺ for 24 hrs. Following exposure, cell monolayers were washed with PBS (Gibco, UK) and then were fixed with 4% gluteraldehyde in PIPES buffer (0.1 M, pH 7.4) for 1 h at 4 °C. Cells were then scraped and pelleted and processed as pellets for TEM embedding. Cells were treated with graded solutions of ethanol (70, 95, and 100%) for 5 min in each solution. Samples were then prepared in and in the absence of osmication and infiltrated under vacuum in quetol resin (Agar Scientific, UK) for 3 days. The fixed and embedded cells were sectioned with a diamond knife (Diatome 45°, Leica, UK) on an ultramicrotome at 70 nm thickness for TEM imaging. Samples were post-stained with uranyl acetate and lead citrate for 5 min in each to enhance contrast from cell membranes. These heavy metal stains obscure contrast from MWNT inside the cell, making it difficult to image individual MWNT. In order to image individual MWNT: F127, unstained cell sections were also imaged [124, 125].

3.3.3. Cytotoxicity assessment of both types of MWNT

3.3.3.1. MTT assay

The colorimetric MTT assay is used to measure cell viability [126]. The yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, tetrazole, MTT) is reduced by mitochondrial reductase in living and metabolically active cells to purple, water insoluble formazan crystals, which can then be dispersed using DMSO or other detergents (Scheme 3.1.5). A decrease in absorbance at 570
nm compared to untreated control cells is then a measure of the cell viability or the amount of apoptosis or necrosis that has been caused by the test material.

The MTT solution (Sigma, UK) was prepared as 5mg/mL in sterile PBS and subsequently sterilised via 0.2µm filter (Millipore, USA) and was stored in 2 ml aliquots at −20 °C. Dimethyl sulphoxide (DMSO, Sigma, UK) was used as the solubilization solution. A549 cells were seeded at a density of 10,000 cells/well in a flat bottomed 96-well plates (Corning Costar Corporation®, USA) to a volume of 100 µL. Cells were left to adhere and grow by incubating them at 37 °C in 5% CO2 for 24 hrs. Cells were then treated with the MWNT-NH₃⁺ and MWNT: F127 dispersions diluted in complete media at a concentration range from 7.9 to 125µg/ml. Control wells were treated with complete media and 10 % DMSO (Hybri-Max™, sterile filtered, hybridoma tested, Sigma, UK) as a positive control for toxicity.

After 24 hrs, the medium was removed and replaced by 120 µL of MTT/media (20 µl MTT+100 µl complete media) and incubated for 3:30 hrs to allow MTT reduction. A 100 µL of DMSO was then added to each well and left for 10-15 min at 37 °C to allow complete solubilization of the formazan product (Scheme 3.1.5). The plate was then measured for the optical densities at 570 nm using Victor® Multilabel plate reader (PerkinElmer, USA) to determine the cell viability. The results were expressed as the percentage of cell viability (n=8 ±SD) compared to untreated wells. The percentage cell viability is calculated using this formula:

\[
\text{% Cell Viability} = \frac{A_{570\ nm\ of\ treated\ cells}}{A_{570\ nm\ of\ untreated\ cells}} \times 100
\]
*Formazan-MTT interaction (Spiking experiment)*: The MTT solution was added to another non-treated well plate for 3:30 hrs, after which MWNT different concentrations were added. After solubilisation, the absorbance was read at 570 nm using Victor® Multilabel plate reader (PerkinElmer, USA).

![Scheme 3.1.5: Representation of the protocol from the MTT assay with its chemical reaction.](image)

**Scheme 3.1.5**: Representation of the protocol from the MTT assay with its chemical reaction.

### 3.3.3.2. Annexin V-FITC/ PI assay

The Annexin-V-FITC/PI staining kit was performed according to the instructions of the manufacturer (Roche Applied Science, Germany). Briefly, this assay identifies early changes in apoptosis which is linked to exposing phosphotidylserine (PS) from the interior of the plasma membrane to its exterior (*Scheme 3.1.6*). The protein
Annexin V (fluorescently labelled with FITC, green) binds with high affinity to PS which can be identified through the FITC fluorescent and quantified by flow cytometry. In addition, at the late stages of apoptosis and early necrosis, the membrane integrity is also compromised which allows the inclusion of exclusion dyes such as propidium iodide (PI, red) and their intercalation with DNA. Therefore, the dually labelled cells with FITC and PI indicate late apoptotic and necrotic cells (Scheme 3.1.6).

A549 cells were seeded into 24-well plates (Corning Costar Corporation®, USA) at 50000 cells per well and left to attach overnight before incubation with both types of MWNT. Cells were then incubated with MWNT-NH₂⁺, MWNT: F127 (0-125 µg/ml) and the equivalent Pluronic F127 concentration (0-1250 µg/ml) in complete media. Cationic liposomes (DOTAP: Cholesterol 2:1 molar ratio) were used as positive control for toxicity. Cells were incubated with the f-MWNT and cationic liposomes for 24 hrs at 37 °C in a humidified atmosphere (5% CO₂) incubator. Cells were media aspirated and washed once with PBS and then trypsinised by adding 0.2 ml Trypsin per well. Complete media was then added to each well and these transferred to eppendorf tubes. The cell suspension was then centrifuged for 5 min at 1500 rpm and the supernatant was removed. To the pellets, 1 ml of ice cold PBS was added and this was again centrifuged as described above. Once the supernatant was removed, 100 µl of staining solution (100 µl incubation buffer + 1 µl Annexin V-FITC + 15 µl Propidium iodide (PI) (Roche Applied Science, Germany) was added to each Eppendorf tube and incubated at 15-25 °C for 10 to 15 min. Before analysis, 0.5 ml of incubation buffer is added to each Eppendorf tube. Samples were analysed on a flow cytometer using 488 nm excitation and a 515 nm bandpass filter for fluorescein detection and a filter 615 nm for PI detection. Electronic compensation
of the instrument was performed to exclude overlapping of the two emission spectra. Cell death was expressed as percentage cell population stained with Annexin V or with PI staining. A CyAn ADP flow cytometer (DakoCytomation) was used to analyse 20000 cells per sample.

Scheme 3.1.6: The basis of the Annexin V-FITC/PI assay.

3.3.3.3 The original LDH assay

The Lactate dehydrogenase (LDH) is a stable cytosolic enzyme that is released from the cell upon cell lysis. The lactate dehydrogenase assay is based on quantitatively measuring released LDH using a coupled enzymatic assay, in which LDH plays a
role in the conversion of a tetrazolium salt (INT) into a red soluble formazan product which then can be measured colorimetrically (Scheme 3.1.7). The amount of LDH released is proportional to the number of lysed cells [128, 129].

The cytotoxicity assessment of the MWNT-NH$_3^+$ and MWNT: F127 was further determined using the LDH assay. This assay was performed using the Promega Cytotox 96 ® Non-radioactive cytotoxicity assay (Promega, UK Ltd) according to the manufacturer instructions (Scheme 3.1.8).

![Scheme 3.1.7: Lactate dehydrogenase (LDH) enzyme mediated chemical reaction.](image)

A549 cells were seeded at 10000 cells in a 96-well plate and left to attach overnight. Cells were then treated with the MWNT: NH$_3^+$ and MWNT: F127 at range of concentrations (0, 1.9, 7.8, 31.25, and 125µg/ml). In addition, 10 % DMSO was used as a positive control for cytotoxicity. After 24 hrs incubation, 50 µL cell media is
mixed with 50 μl substrate mix in a new microtiter plate and incubated for 30 minutes at room temperature. Absorbance at 490 nm was read using Victor® Multilabel plate reader (PerkinElmer, USA). The amount of LDH released was an indication of the number of lysed cells due to cytotoxicity. The results were expressed as the percentage LDH released (n= 4±SD) compared to maximum LDH released from the untreated control cell. The percentage LDH released (% cytotoxicity) is calculated using this formula:

\[
\% \text{ LDH released} = \frac{A_{490 \text{ nm}} \text{ of treated & untreated cells} - A_{490 \text{ nm}} \text{ of media alone}}{A_{490 \text{ nm}} \text{ of Maximum of untreated cells} - A_{490 \text{ nm}} \text{ of media alone}} \times 100
\]

The absorbance data at 490 nm also can be shown without converting it into percentage LDH release to highlight the interference of CNT with the results of the assay. The absorbance of the MWNT dispersions at the same concentrations was therefore read at 490 nm for comparison with the LDH release data.
3.3.3.4. The ‘modified LDH’ mLDH assay

The original colorimetric LDH assay was modified to avoid interference of the components used in the assay with the CNT. The survived cells after treatment are artificially lysed with Triton X-100, and the cell lysate is centrifuged in order to precipitate the CNT. The released LDH is therefore an indication of the number of the viable cells that survived treatment with CNT (Scheme 3.1.9).

A549 cells were seeded at 10000 cells in a 96-well plate and left to attach overnight. Cells were then treated with the MWNT: NH₃⁺ and MWNT: F127 at a range of...
concentrations (0, 1.9, 7.8, 31.25, 125 µg/ml). In addition, 10 % DMSO was used as a positive control for cytotoxicity. After 24hrs treatment, cells were lysed with 10 µl of lysis buffer per 100 µl serum free media and left for 45-60 min at 37 °C. 50 µL of cell lysate after centrifuging at 13000 rpm for 5 min was mixed with 50 µl substrate mix in a new microtiter plate and incubated for 15 minutes at room temperature (Scheme 3.1.9). Absorbance at 490 nm was read using Victor® Multilabel plate reader. The amount of LDH released was an indication of the number of cells which survived treatment. Hence the percentage cell survival (n=4 ± SD) is expressed using this formula:

\[
\% \text{ Cell Survival} = \left( \frac{A_{490 \text{ nm of treated cells}}}{A_{490 \text{ nm of untreated cells}}} \right) \times 100
\]

Validation of the mLDH assay: This assay was assessed with a range of concentrations (0.0075-0.48mM) of cationic liposomes (DOTAP: Cholesterol 2:1 molar ratio) and different concentrations of DMSO (5 and 20 %), both materials is expected not to interfere with the assay. In addition, the results were compared with the MTT and Annexin V-FITC/PI assays.
Aspirate the media
Lyse the cells (lysis solution)
Transfer to eppendorfs
Centrifuge to pellet the CNT
50 µl cell lysate is added into a new 96 w/p
50 µl substrate mix added
15 min @ 37 °C
50 µl stop solution added
Absorbance 490 nm

Scheme 3.1.9: Representation of the protocol for the modified LDH assay.
3.4. Can chemical functionalisation alleviate the asbestos-like pathogenicity risk associated with long pristine MWNT?

3.4.1. Functionalisation of long pristine MWNT (NT₂ Pristine)

This part of the project was done in collaboration with Prof. Kent Donaldson (ELEGi Laboratory, Queen’s Medical Research Institute, Edinburgh).

Long pristine MWNT which were used in the Poland study [85] and denoted as NT₂ Pristine were functionalised using two different chemical reactions. As shown in Scheme 3.1.10, the long NT₂ Pristine were functionalised using octyl-iodide (octyl functionalisation) [130, 131] generating NT₂-Alkyl as shown by reaction 1 and through the 1, 3 dipolar cyclo-addition [20, 132] generating NT₂-TEG (reaction 2).

Scheme 3.1.10: Chemical functionalisation of the long pristine MWNT (NT₂ Pristine). Reaction 1 depicts the introduction of alkyl groups onto the surface of the pristine NT₂. Reaction 2 represents the functionalisation of NT₂ through the 1, 3 dipolar cycloaddition and the introduction of TEG moieties onto the NT₂ surface.
**Preparation of NT\textsubscript{2} aqueous dispersions:** For biological purposes all NT\textsubscript{2} samples (NT\textsubscript{2} pristine, NT\textsubscript{2}-Alkyl and NT\textsubscript{2}-TEG) and long asbestos amosite (LFA) used as positive control were dispersed at a final concentration of 100\(\mu\)g/ml in 0.5% bovine serum albumin (BSA; Sigma-Aldrich, UK)/ saline solution by bath sonication (Ultrasonic cleaner, VWR) for 2 hrs.

**Transmission Electron Microscopy:** A drop of the NT\textsubscript{2} and LFA dispersions was placed on a grid with a support film of Formvar/carbon, excess material was blotted off with a filter paper and the MWNT dispersions were examined under a FEI CM120 BioTwin Transmission Electron Microscope (Philips/FEI, USA) using a 80 KV electron beam current. Images were captured using an AMT Digital Camera. All TEM images were taken with the assistance of Mr. David McCarthy (The School of Pharmacy, London).

**Atomic Force Microscopy (AFM):** A drop of the NT\textsubscript{2} and LFA dispersions was deposited on the surface of freshly cleaved mica (Agar Scientific, Essex, UK) and allowed to adsorb for 5 min. Unbound structures were removed by washing with 0.22 \(\mu\)m filtered deionized H\textsubscript{2}O, then dried under a nitrogen stream. Imaging was carried out in TappingMode using a Multimode AFM, E-type scanner, Nanoscope IV controller, Nanoscope 5.12b control software (Veeco, Cambridge, UK) and a silicon tapping tip, made of crystallized silicon (NSG01, NTI-Europe, Apeldoorn, The Netherlands) of curvature radius of 10 nm. The tip was mounted on tapping-mode silicon cantilever with a typical resonant frequency of 150 kHz and a force constant of 5.5 N/m, to image 5 \(\mu\)m \(\times\) 5 \(\mu\)m square areas of the mica surface, with a resolution of 512 \(\times\) 512 pixels and a scan rate of 1 Hz. All AFM images were performed in air and were taken with the assistance of Mr. Tian Bowen (The School of Pharmacy, London).
3.4.2. Toxicological effects of intraperitoneally injected NT\textsubscript{2} samples

Six- to eight-week-old C57BL6 mice were obtained from Harlan (Oxfordshire, UK), allowed to acclimatise for 1 week and were kept in groups of 5 for the duration of the experiments and given food and water. All experiments were conducted with prior approval from the UK Home Office. Two groups of animals (n=4-6) were intraperitoneally injected with 0.5 ml containing 50 \mu g of NT\textsubscript{2} pristine, NT\textsubscript{2}-Alkyl, NT\textsubscript{2}-TEG and LFA in 0.5 % BSA/Saline (Vehicle control; 0.5 ml). One group of animals was scarified after 24 hrs and the other 7 days post-injections.

3.4.2.1. Inflammatory reaction after injections of NT\textsubscript{2} samples

24 hrs and 7 days post-injection, mice were sacrificed by cervical dislocation and the peritoneum lavaged three times using 2 ml washes of sterile ice-cold PBS. The lavages were pooled together and placed on ice for the entire duration of the processing. The lavage fluid was then centrifuged at 1000 rpm for 5 minutes at 4\degree C in a Mistral 3000i centrifuge (Thermo Fisher Scientific, Inc., MA, USA) and an aliquot of the supernatant was retained for total protein measurement. The remaining supernatant was discarded and the cell pellet re-suspended in 0.5 ml of 0.1 % BSA/sterile saline solution and mixed with fluorescently labelled Ly-6C/G (Gr-1) antibody (Invitrogen, UK) that is used to differentiate polymorphonuclear leucocytes (PMN) from other cells. The GR-1 positive PMN leucocytes were quantified using flow cytometry. The assay with the Gr-1 antibody was performed according to the manufacturer’s instructions. It is recommended that between 0.1 and 0.25\mu g of antibody to be used per 1x10\textsuperscript{6} cells in a 100 \mu l staining volume.

Total protein in the lavageate: Total protein concentration of the peritoneal lavage fluid was measured using the bicinchoninic acid (BCA) protein assay (Thermo
Scientific Pierce, UK). The colorimetric assay was performed according to the manufacturer’s instructions.

Sample protein concentrations were established by comparison to a bovine serum albumin BSA standard curve (0 – 1000 µg/ml). The standard solutions and samples (10 µl) were loaded into 96-well plate. The reagent mixture was prepared by adding 1 part of reagent B to 50 parts of reagent A. After the colour change of the mixture (colourless to greenish), 200 µl of the mixture was added to each well. This assay is time dependent, so the samples were 37°C for 30 min before reading their absorbance at 562 nm using Victor® Multilabel plate reader. The protein concentration of each sample was determined via extrapolation from the BSA standard curve.

3.4.2.2. The assessment of granuloma formation after 7 days post-injection

After 7 days, the abdominal wall was dissected free, exposing the peritoneal cavity via a midventral incision with lateral incisions extending to the vertebra column, which was then severed below the diaphragm. The diaphragm was then carefully dissected by cutting through the ribs and chest wall with care taken not to puncture the diaphragm. The diaphragm was gently rinsed three times by emersion in ice-cold sterile PBS and placed overnight into:

- Methacarn fixative (60 % methanol, 30 % chloroform and 10 % glacial acetic acid) for histological staining or
- 3 % glutaraldehyde/ 0.1 M sodium cacodylate (pH 7.2) buffer for scanning electron microscopy (SEM) of the diaphragm surface.
After overnight incubation in fixative, the diaphragm was carefully excised from the surrounding ribs prior to further processing for either histological or SEM analysis [85].

**Histological examination of diaphragms:** The diaphragm was then removed from the ribs and a similar full width section of the upper quadrant of the diaphragm was removed from each animal sampled so as to encompass both muscular and central tendinous regions of the diaphragm. This excised tissue was dehydrated through graded alcohol (ethanol) and imbedded on-edge in paraffin[85]. Four μm sections of the diaphragm were stained with hematoxylin and eosin stain by the Laboratory Diagnostic Service of the Royal Veterinary College (London, UK). Microscopic observation of tissues was carried out with Nikon Microphot-FXA microscope coupled with Infinity 2 digital camera.

**Surface examination of diaphragms using SEM:** The excised diaphragm was stained with osmium tetroxide prior to critical point drying, mounted and gold sputter coated and then examined by scanning electron microscopy (SEM; FEI XL30 TMP, Eindhoven, The Netherlands). All SEM images were taken with the assistance of Mr. David McCarthy (The School of Pharmacy, London).

3.5. CNT: Doxorubicin non-covalent supramolecular complexes for cancer therapeutics

3.5.1. The formation and characterisation of CNT: Doxorubicin complexes
Pristine MWNT were dispersed using 1 % of tri-block copolymer 1 (Pluronic F127, Sigma LTD) by bath sonication (Ultrasonic cleaner, VWR) for 30 minutes into a final
MWNT concentration of 1 mg/ml as described in [14] (See Scheme 3.1.4 above). The MWNT: Doxorubicin complexes were prepared by mixing equal volumes of doxorubicin hydrochloride 2 (20 μg/ml) with increasing MWNT concentration (10, 20, 40 μg/ml). The formed complexes had from 0.5x10^{18} to 2x10^{18} molecules of doxorubicin per mg of MWNT.

Fluorescence Spectrophotometry: Doxorubicin (Dox) is a fluorescent molecule with a chromophore composing of three planar and aromatic anthraquinonic rings. Hence, the supramolecular interaction between doxorubicin and MWNT was studied by monitoring doxorubicin fluorescence using fluorescence spectrophotometry (Perkin Elmer Luminescence Spectrometer LS 50B, UK).

Transmission Electron Microscopy: The structural characteristics of the MWNT: Dox complexes were studied using transmission electron microscopy (TEM). The complexes were prepared as described above but by keeping the final MWNT concentration constant at 0.5 mg/ml in order to visualize the complexes while keeping the same number of Dox molecules per mg of MWNT as used for the fluorescence analysis. A drop of the MWNT: Dox complexes was placed on a grid with a support film of Formvar/carbon, excess material was blotted off with a filter paper and the MWNT dispersions were examined under a FEI CM 120 BioTwin Transmission Electron Microscope (Philips/FEI, USA) using a 80 KV electron beam current. Images were captured using an AMT Digital Camera. All TEM images were
taken with the assistance of Mr. David McCarthy (The School of Pharmacy, London).

3.5.2. Cytotoxicity assessment of MWNT: Doxorubicin complexes in vitro Cell culture

Human breast cancer cells (MCF-7; ATCC® HTB-22™) were cultured in Minimum Essential Medium Eagle (MEM) media supplemented with 10% fetal bovine serum (FBS, Invitrogen, UK) and 50 U/ml penicillin (Invitrogen, UK) and 50 µg/ml streptomycin (Invitrogen, UK) at 37 °C in 5% CO2. Cells were passaged to reach 80% confluency.

**MTT assay:** MCF-7 cells were seeded in flat bottomed 96-well plates and were allowed to attach and grow by incubating them at 37° C in 5% CO2 for 24 hours. Cells were treated with the MWNT: Dox complexes at MWNT: Dox 2:1 mass ratio, equivalent doxorubicin alone (600 nM), (Pluronic 6.5 ng/ml) and CNT (615.5 ng/ml) and the equivalent Pluronic: Dox. Control wells were treated with complete media. After 24 hrs, the medium was removed and replaced by 120 µl of MTT/media (20 µl MTT + 100 µl complete media, see below for preparation) and incubated for 3½ hours to allow MTT reduction. A 150 µl of DMSO was then added to each well and left for 10 min at 37 °C to allow complete solubilisation of the formazan product. The plate well was then measured for the optical densities at 570 nm using Victor® Multilabel plate reader (PerkinElmer, USA) to determine the cell viability. The results were expressed as the percentage of cell viability (n=8 ±SD) compared to untreated wells. The percentage cell viability is calculated using this formula:
3.5.3. Therapeutic efficacy of MWNT: Doxorubicin complexes in vivo

Six- to eight-week-old C57BL6 mice were obtained from Harlan (Oxfordshire, UK), allowed to acclimatise for 1 week and were kept in groups of 5 for the duration of the experiments and given food and water. All experiments were conducted with prior approval from the UK Home Office.

Cell culture: B16F10, a melanoma murine cancer cell line (ATCC®, UK) was cultured in Advanced RPMI-1640 (Gibco, UK) supplemented with 10% FBS, 1% L-Glutamine and 1% penicillin/streptomycin.

3.5.3.1. Syngeneic tumour model

1x10^6 B16F10 cells were subcutaneously inoculated into the shaved right flank of C57BL6 mice using 26G needle in a volume of 100 μl PBS. The tumor volume was estimated by bilateral Vernier calliper measurement three to four times per week and calculated using the formula (width x width) x (length) x (π/6), where length was taken to be the longest diameter across the tumour, as previously described. Intratumoural injections were performed when tumours reached 200 mm^3.

3.5.3.2. Intratumoral administration of MWNT: Doxorubicin complexes

Tumour bearing mice were anaesthetised using inhalation isofluorane and injected with 50 μl of MWNT: Dox complexes at the 2:1 mass ratio. The needle was inserted into the longitudinal direction from the tumour edge and 50 μl of MWNT: Dox (equivalent to 25μg MWNT and 12.5 μg Dox), Dox alone, MWNT: F127 alone, F127: Dox and 5 % dextrose (naive untreated) was administered slowly over 1 min.
In addition, ammonium functionalised MWNT (MWNT-NH$_3^+$) were also complexed with Dox and the intratumoral injections of 50 µl of [MWNT-NH$_3^+$]: Dox complexes were also performed together with MWNT-NH$_3^+$ alone. The needle was left in the tumour for another 5 min to prevent sample leakage. Injections were carried out on days 7, 9, 11, 13, and 15 following tumour inoculation. Mice were sacrificed by cervical dislocation when tumour volume reached 800–1000 mm$^3$.

### 3.5.3.3. Tissue histology following administration of MWNT: Dox complexes

Tumours, lungs, liver, spleen, and kidneys were fixed in 10% buffered formalin and processed for routine histology with hematoxylin and eosin (H&E) stain by the Laboratory Diagnostic Service of the Royal Veterinary College (London, UK). Microscopic observation of tissues was carried out with Nikon Microphot-FXA microscope coupled with Infinity 2 digital camera.

### 3.6. Chemically conjugated CNT-Methotrexate conjugates for cancer therapeutics

#### 3.6.1. The synthesis and characterisation of CNT-Methotrexate conjugates

MWNT–Methotrexate (MTX) conjugates were prepared by Dr. Cristian Samori (CNRS, Strasbourg, France) and detailed chemistry can be found in reference [112]. Briefly, pristine MWNT were initially oxidized as previously described and amine functionalities introduced onto the sidewalls using 1, 3 dipolar reaction [18, 132, 133]. Then the conjugation of MTX was introduced in 3 different ways (Scheme 3.1.11):
1. MTX was linked to the amino functionalities without an enzymatic sensitive linker similar to what was previously described [22].

2. MTX was linked to the amino functionalised through a 6-hydroxyhexanoic ester and hence the MWNT-MTX (esterase sensitive linker) [108, 134]. This construct was further modified with rhodamine B for uptake studies using confocal microscopy.

3. MTX was linked to the amino functionalised using tetrapeptide (Gly-Leu-Phe-Gly) that is sensitive to cathepsin B overexpressed in cancer cells and hence the name of the construct (MWNT-MTX with peptidase sensitive linker) [135-137].

![Scheme 3.1.11: Chemical synthesis of the different MWNT-MTX conjugates.](image)

Transmission Electron Microscopy: A drop of the f-MWNT dispersion was placed on a grid with a support film of Formvar/carbon, excess material was blotted off with...
a filter paper and the MWNT dispersions were examined under a FEI CM120 BioTwin Transmission Electron Microscope (Philips/FEI, USA) using a 80 KV electron beam current. Images were captured using an AMT Digital Camera. All TEM images were taken with the assistance of Mr. David McCarthy (The School of Pharmacy, London).

3.6.2. Cellular uptake and internalisation of the MWNT- MTX conjugates

Cell Culture: Human breast cancer cells (MCF-7; ATCC®.HTB-22™) were cultured in Folate -Free RPMI 1640 medium (Invitrogen, UK) and supplemented with 10% fetal bovine serum (FBS, Invitrogen, UK) and 50 U/ml penicillin (Invitrogen, UK) and 50 µg/ml streptomycin (Invitrogen, UK) at 37 °C in 5% CO2. Cells were passaged to reach 80 % confluency.

Confocal microscopy studies: MCF7 cells were plated onto microscope coverslips at a density of 30000 cells and left to attach overnight. The cells were then incubated with the different MWNT conjugates (HOOC-MWNT-NH3+ and MWNT-MTX-rhodamine B) in folate-free medium at a final MWNT concentration of 10µg/ml for 24 hrs. The cells were then washed with phosphate buffer saline (1X PBS, Invitrogen, UK) and fixed using 4 % paraformaldehyde (Sigma,UK) for 20min, followed by 3 cycles of washes with PBS after which the coverslips were mounted onto slides. The cellular uptake of MWNT-MTX-rhodamine B was imaged using a Zeiss Axiovert LSM510 confocal with a 63x oil immersion objective (Carl Zeiss Inc., Thornwood, NY). Rhodamine B was excited at 543 nm laser power with pinhole of 500 nm. All images were collected using identical acquisition parameters. Confocal images were taken with the assistance of Dr. Chang Guo (The School of Pharmacy, London).
3.6.3. Cytotoxicity assessment of the MWNT-MTX conjugates *in vitro*

The cytotoxicity of the MWNT-MTX constructs was assessed using the modified lactate dehydrogenase (LDH) assay (*Section 3.3.3.4*). MCF 7 cells were seeded at 7000 cells in a 96-well plate and left to attach overnight. Cells were then treated with MTX alone and the different MWNT-MTX conjugates at which the concentration of MTX was kept constant at 10μM with and without the MWNT. MWNT without MTX were used as control to exclude any intrinsic toxicity from the MWNT alone. In addition, 10 % DMSO was used as a positive control for cytotoxicity. After 3, 6 and 24 hrs treatment, cells were lysed with 10 μl of lysis buffer per 100 μl serum free media and left for 45-60 min at 37 °C. 50 μL of cell lysate after centrifuging (13000 rpm, 5 min) was mixed with 50 μl substrate mix in a new microtiter plate and incubated for 15 minutes at room temperature. Absorbance at 490 nm was read using Victor® Multilabel plate reader. The amount of LDH released was an indication of the number of cells which survived treatment. Hence the percentage cell survival (n=4 ± SD) is expressed using this formula:

\[
\text{% Cell Survival} = \frac{A_{490 \text{ nm of treated cells}}}{A_{490 \text{ nm of untreated cells}}} \times 100
\]

3.6.4. Therapeutic efficacy of the MWNT-MTX conjugates *in vivo*

Six- to eight-week-old female CD1 nude mice (20-25 g) (Charles River Laboratories, UK) were allowed to acclimatise for 1 week and were kept in groups of 5 for the duration of the experiments and given food and water. All experiments were conducted with prior approval from the UK Home Office.
**Cell culture:** DU145, human prostate carcinoma cell line (ATCC®, UK) was cultured in Advanced RPMI-1640 (Gibco, UK) supplemented with 10% FBS, 1% L-Glutamine and 1% penicillin/streptomycin

**Xenograft tumour model:** 1x10^6 DU145 cells were subcutaneously inoculated into the right flank of CD-1 nude mice using 26G needle in a volume of 100 µl PBS. The tumour volume was estimated by bilateral Vernier calliper measurement three to four times per week and calculated using the formula (width x width) x (length) x (π/6), where length was taken to be the longest diameter across the tumour, as previously described. Intratumoural injections were performed when tumours reached 200 mm³.

**3.6.3.1. Intratumoral administration of MWNT-MTX constructs**

Tumour bearing mice were anaesthetised using inhalation isofluorane and injected with 50 µl of the MWNT-MTX constructs with and without the peptidase sensitive linker. The needle was inserted into the longitudinal direction from the tumour edge and 50 µl of MWNT-MTX with no linker (equivalent to 10.54µg MWNT and 1µg MTX), MWNT-MTX with peptidase sensitive linker (equivalent to 25µg MWNT and 1µg MTX), MTX alone (1µg), MWNT-NH₃⁺ alone (25µg) and 5 % dextrose (naive untreated) was administered slowly over 1 min. The needle was left in the tumour for another 5 min to prevent sample leakage. Injections were carried out on days 34, 41, and 48 following tumour inoculation. Mice were sacrificed by cervical dislocation when tumours reached day 55 post-inoculation due to the very slow growth of the tumour.
3.7. Statistical analysis

Data were analysed using two samples, double sided, student t-test. Differences were considered significant at (*p< 0.05, ** p< 0.01, *** p< 0.001).

* Sources of the different CNT materials used in this thesis are shown in APPENDIX XII.
CHAPTER IV

THE PHARMACOKINETIC PROFILE OF DIFFERENT CARBON NANOTUBES IN VIVO
4.1. Evaluation of the biodistribution profile of functionalised MWNT

The biodistribution profile of chemically functionalised and non-covalently coated MWNT after intravenous administration was determined. The fate of radiolabelled $[^{111}\text{In}]$ DTPA-MWNT was investigated using gamma scintigraphy and dynamic imaging by small animal imaging (Single Photon Emission Computed Tomography, SPECT). The histological examination of tissues was also investigated in an attempt to determine tissue damage caused by MWNT accumulation but also as a non-quantitative method to visualise the MWNT uptake into tissues and organs.
4.1.1. Aryl functionalised MWNT: Functionalisation and characterisation

Pristine MWNT were initially oxidised by acid treatment and further functionalised with amino groups in their side walls (Scheme 3.1.1-2, Materials and Methods). The ammonium functionalised MWNT (f-MWNT-NH$_3^+$) were prepared via in-situ aryl diazonium salt functionalisation. The chemistry followed has been established by Tour and co-workers previously [138-140]. It involves the initial formation of a diazonium salt, via the reaction of the aniline derivative with isoamyl nitrite, which subsequently reacts with the surface of the nanotubes [120]. The presence of the aryl functional groups grafted to surface of the nanotubes was confirmed by thermal decomposition of the f-MWNT-NH$_3^+$ in the temperature range 300-450°C compared to the thermally stable pristine MWNT as shown by thermogravimetric analysis (TGA) (lines 1 and 3, Figure 4.1.1A). The f-MWNT-NH$_3^+$ were then used to covalently link the diethyltriaminepentaacetic (DTPA) chelating agent which is used to cage the $\gamma$ emitting radionuclide (Indium 111) in order to track the in vivo biodistribution of MWNT. As indicated by the Kaiser test, about 57% of amino functionalities were reacted with DTPA, leaving 43% of free un-reacted amino functions at the CNT surface. Functionalised MWNT were characterised by transmission electron microscopy (TEM). Figure 4.1.1B shows the coexistence of fully individualised MWNT as indicated by the white arrows as well as CNT clusters of different dimensions as pointed out by the black arrows. This may be due to $\pi-\pi$ interactions between the aryl groups of the functional moieties bringing the nanotubes together in a cluster formation.
Figure 4.1.1: Characterisation of the aryl functionalised MWNT-COOH. (A) Thermal gravimetric analysis of pristine MWNT (line 1), MWNT-COOH (line 2) and aryl functionalised MWNT-COOH (line 3) under inert atmosphere (nitrogen gas). The weight loss observed in the temperature range 200-250 °C is due to the decomposition of the COOH group while the further weight loss observed in the temperature range 300-450 °C (line 3) is due to the decomposition of the aryl functional groups (TGA carried out by Dr. Nikos Karouis). (B) TEM images of aryl functionalised MWNT-COOH. White arrows indicate individualised MWNT and black arrows point to MWNT aggregates. Scale bar is 100 nm.

Before moving into animal experiments the radiolabelling of the DTPA-MWNT was achieved by reacting the DTPA-MWNT with $^{111}$InCl$_3$ to form radioconjugate as described in Section 3.1.6, Materials and Methods. The radiolabelling efficiency was determined by thin layer chromatography (TLC). Aliquots of $^{111}$In DTPA-MWNT were diluted in PBS and spotted in silica gel impregnated glass fiber strips. The TLC strips were then developed using EDTA/Ammonium acetate as the
mobile phase after which they are dried and the radioactivity was measured using a cyclone phosphor detector.

Free $^{111}$In moved with the solvent front, while the $[^{111}\text{In}]-\text{DTPA-MWNT}$ moved into the middle which is a characteristic of this type of chelating DTPA. As shown in Figure 4.1.2, high $[^{111}\text{In}]$ radiolabelling of DTPA-MWNT (73.5\%) was achieved.

![Figure 4.1.2](image_url)

Figure 4.1.2: Images of TLC strips after the labelling reaction and dilution of $[^{111}\text{In}]$ DTPA-MWNT in PBS. Radioactive efficiency is indicated by the percentage radioactivity in the application point as an indication of the indium caged in the DTPA molecule.

### 4.1.2. Tissue biodistribution of aryl functionalised MWNT

Following intravenously administered $^{111}$In radiolabelled DTPA-MWNT, C57BL6 mice were placed in single photon emission computed tomography (NanoSPECT/CT) scanner and the dynamic distribution of the radioactive MWNT
conjugate was recorded. The SPECT/CT images shown in Figure 4.1.3 indicate the rapid accumulation of the MWNT in the liver, lungs and the bladder within the first few minutes after i.v. injection. At 4 hrs post-injection it was obvious that the CNT that were observed in the bladder at 30 min, were excreted in the urine. A reduction in the radioactivity signals of the lungs could also be observed in Figure 4.1.3B. Similar observations to those for 4 hrs can also be seen at 24 hrs post-injection with further reduction in the radioactivity signals in the lungs (Figure 4.1.3B). This can probably be explained by the fact that the different CNT fragments have different tissue affinities due to their sizes and dimensions. So, the individualised CNT were excreted from the kidneys within a very short period, while relatively small aggregates translocated from the lungs which is the first portal of entry of cationic moieties into reticuloendothelial organs (liver and spleen) over time.

![Figure 4.1.3: Biodistribution profile of aryl functionalised MWNT showing by (A) SPECT/CT fused whole body images, 30 min, 4hrs and 24hrs after tail vein injection of $[^{111\text{In}}]$DTPA-MWNT (1.12 MBq, 50μg) with a scanning time of 40min. (B) Cross sections in the lung, liver, spleen, kidney and bladder at equivalent time points.](image)
Quantitative radioactivity analysis using gamma counting of the harvested organs was then performed in order to confirm the SPECT observation and examine quantitatively the translocation of the CNT between the different organs over time. Quantitative radioactivity analysis after 30 min showed that the highest radioactivity indicated as percentage injected dose (% ID) per tissue was observed in liver followed by the lungs and spleen (Figure 4.1.4A). Furthermore, looking at tissue affinity data as represented by % ID per gram tissue, the lungs and spleen showed the highest radioactivity (80 %) followed by liver at 30 min post-injection. Interestingly, the uptake in spleen peaked at both 4 hrs and 24 hrs time points while the % ID per gram liver tissue remained the same suggesting that the redistribution of $[^{111}\text{In}]$DTPA-MWNT took place from lungs to spleen rather than liver tissues (Figure 4.1.4B). This confirms what was previously described in the SPECT/CT imaging where the radioactivity intensity in the lungs was decreasing over 24 hrs time point (Figure 4.1.4A).

Moreover, the accumulation of MWNT into the lungs and the reticuloendothelial system (RES) seems to be very rapid with only 10 % and 2 % of injected dose found in the blood after 1 and 5 minutes post-injection, respectively, as can be seen in Figure 4.1.4C. Once the MWNT distribute into lungs, liver and spleen they localise inside these organs with the redistribution of probably smaller CNT aggregates from the lungs to the spleen as shown by the SPECT/CT and gamma counting analysis. Interestingly, limited CNT excretion seems to occur via two pathways urinary (3 %) as well as biliary excretion in the faeces (2.5 %) in the first 24 hrs after injection (Figure 4.1.4D).
Figure 4.1.4: Biodistribution of $^{111}$In DTPA-MWNT in BALB/C mice after i.v. administration for 30 min, 4 hrs and 24 hrs (A) represented as percentage injected dose (% ID) per organ, (B) % ID per gram tissue. (C) Blood profile of $^{111}$In DTPA-MWNT, (D) Excretion profile of $^{111}$In DTPA-MWNT ($n = 4$ and error bars for standard deviation).
4.1.3. Tissue histology following administration of aryl-\(f\)-MWNT

Following the dynamic tracking of \([^{111}\text{In}]\) DTPA-MWNT, the histological impact of the non-radiolabelled DTPA-MWNT on the lungs, liver, spleen and the kidneys after 24 hrs post-injection was explored. Mice were injected with the same dosing regimen of 50 \(\mu\)g of DTPA-MWNT in 5 % dextrose per mouse. The histological sections indicate the accumulation of large MWNT aggregates in the lungs (Figure 4.1.5B) and smaller aggregates in the spleen and liver (Figure 4.1.5F and Figure 4.1.5D). This indicates that larger CNT aggregates accumulated in the lung and resided in that tissue for the time span of the experiment (24 hrs), while smaller CNT fractions had a higher affinity for the spleen. Moreover, no necrosis or fibrosis was observed in any of the tissues that MWNT accumulated.

The biodistribution of the precursor pristine MWNT (pMWNT) was also studied using tissue histology since radiolabelling on such material cannot be achieved. pMWNT were not dispersible in aqueous media (such as the 5 % dextrose used to disperse the functionalised MWNT), they were therefore dispersed with the copolymer Pluronic F127 and in bovine serum albumin (BSA) for comparison of the effect of the dispersion on the distribution of the MWNT. Figure 4.1.6 depicts that pMWNT: F127 resided mainly in the spleen, followed by the liver and some in the lungs. However, much bigger pMWNT aggregates were shown in the lung, spleen and the liver once dispersed in BSA (Figure 4.1.6B, D, F). Neither MWNT: F127 or MWNT: BSA caused any damage to organs of uptake as shown by the absence of necrosis in the H & E stained sections.
Figure 4.1.5: Tissue histology of mice injected with aryl f-MWNT dispersed in 5% dextrose. Hematoxylin and eosin stained sections of lungs (A & B), liver (C & D), spleen (E & F) and kidney (G & H) of C57BL/6 mouse tissues at 24 hrs post-administration of 200 μl 5% dextrose (Control A, C, E & G) and 50 μg of aryl f-MWNT (B, D, F & H). Black arrows indicate MWNT aggregates (B,D). 40x magnification.
Figure 4.1.6: Tissue histology of mice injected with pristine MWNT (pMWNT) dispersed in Pluronic F127 and bovine serum albumin (BSA). Hematoxylin and eosin stained sections of lungs (A & B), liver (C & D), spleen (E & F) and kidney (G & H) of C57BL6 mouse tissues at 24 hrs post-administration of 50 μg of pMWNT-F127 (A, C, E & G) and 50 μg of pMWNT-BSA (B, D, F & H). 20 x magnification.
4.1.4. DISCUSSION

In an attempt to determine the factors contributing to the in vivo biodistribution of CNT, several studies have been proposed. CNT have shown either to be excreted in the urine [28-30, 36, 37] or were found mainly in reticuloendothelial organs (RES) which includes the liver and spleen [13, 25-27, 34]. However, striking differences exist between these studies as CNT of different chemical functionalisation or non-covalent coatings (lipids or polymers) are used. In addition, different dosing regimens and animal models were studied that make comparison between those studies challenging. Taking all these into account no clear conclusions on what determines CNT biodistribution profile can easily be made. Our laboratory has already suggested the degree of functionalisation is an important contributor in the biodistribution of CNT as it leads to fully individualised CNT that can be subsequently excreted in the urine [37]. In addition, Lacerda et al.[36] proposed a possible mechanism of urinary excretion that was highly dependent on the individualisation characteristics of CNT. Herein, the effect of individualisation and aggregation of the same CNT dispersion on determining the in vivo biodistribution and tissue affinities of the CNT was further investigated using a different type of chemically functionalised MWNT. In addition, the biodistribution of the pristine MWNT precursor dispersed in Pluronic F127 and BSA was assessed based on the non-quantitative approach of tissue histology.

The most established and reliable methodology for determining the biodistribution of CNT in vivo has been the radio-labelling of CNT using metal chelating molecules such as DTPA and DOTA. The choice of radio-metal depends on its half life which determines the length of the biodistribution study. Herein, ammonium functionalised MWNT prepared following the diazonium salt reaction
were chemically linked to DTPA which allowed its complexation with indium ($^{111}$In) (Scheme 3.1.2, Materials and Methods). This type of chemically functionalised MWNT was previously found non-cytotoxic in vitro to human breast carcinoma (MCF7) cells [120] and its biodistribution profile was therefore assessed in vivo.

It was observed that tissue uptake of carboxylated and aryl functionalised MWNT (HOOC-MWNT-NH$_3^+$) occurs immediately after injection as confirmed by SPECT imaging and gamma counting analysis (Figure 4.1.3 and Figure 4.1.4). The distribution into the organs seems to be dependent on the dimensions of the CNT sample. Fully individualised MWNT that exist in the CNT dispersion as shown in Figure 4.1.1 seem to get excreted via the bladder at early time points confirming the observation of Singh et al.[28] and Lacerda et al.[36]; in which ammonium functionalised SWNT and MWNT were excreted in the urine due to the fact that they were fully individualised. In addition, Lacerda et al.[37] have recently shown that ammonium functionalised MWNT exhibited no tissue accumulation due to their individualisation as a result of their high degree of chemical functionalisation. Furthermore, the effect of the functional group on the in vivo fate was also investigated by comparing the highly functionalised MWNT-NH$_3^+$ with its counterpart MWNT-DTPA (containing four carboxylic groups) and again no organs accumulation was observed with both formulations indicating that the biodistribution of the CNT is not dependent on the functional group as much it is on the individualisation and good dispersion properties of the sample [37]. It should also be mentioned that the observed radioactivity in the bladder at early time points can be due to the coexistence of free $^{111}$In with the individualised fraction of MWNT as shown by the presence of free $^{111}$In in the CNT sample (Figure 4.1.2). In addition, the DTPA-MWNT sample contained aggregates of different dimensions and lung
uptake and RES (spleen and liver) recognition have occurred. The lung uptake is thought to be due to the cationic nature of ammonium functionalised CNT which can bind strongly to plasma proteins and hence become larger in dimensions and get trapped in the pulmonary vascular bed as shown in Figure 4.1.5. Moreover, it is thought that the spleen uptakes nanoparticles of bigger dimensions than 100 nm which could also be the case with CNT [141]. In addition, the liver exhibits 60% uptake of liposomes of less than 50 nm as they are smaller than the pore size of the liver fenestrae [25]. Hence, it is thought that the liver have recognised much smaller CNT aggregates which can explain the difficulty in identifying them in liver histology.

Moreover, as the CNT: plasma proteins complexes uptaken by the lungs dissociate, the CNT aggregates seem to re-distribute to the spleen as shown by the increase in the percentage ID per gram tissue over 4 and 24 hrs post-injection but the CNT aggregates are still not small enough to get into the liver (Figure 4.1.4B). A similar biodistribution profile was observed with another type of chemically functionalised MWNT which was initially oxidised and its carboxyl groups were then converted onto amine functionalities through the amidation reaction (APPENDIX I). Since the pristine precursors of the aryl functionalised and the amidated MWNT are different, the direct comparison between the two types of MWNT remains difficult. The common parameter between the two chemistries is that both were initially oxidised, so one might suggest that the presence of carboxyl groups could have had an effect on the biodistribution of MWNT especially that both types generated ammonium functionalised MWNT. This clearly shows that amine functionalities per se are not the determinant factor for the biodistribution of the CNT but instead the type of chemistry used seems to play a pivotal role.
The effect of the size on the pharmacokinetics and the biodistribution of dextran derivatives was described by Lee et al. [142] in which the *in vivo* fate of dextran derivatives either oxidised or chemically conjugated to drugs was found to be depend on the size of the particles and on the physicochemical characteristics of the attached drugs. In addition, Deng et al. [34] using water dispersed \(^{14}\)C-taurine functionalised MWNT and Liu et al. [27] with PEGylated-Lipid coated SWNT showed over 80% of the injected dose in the liver and the liver and spleen respectively after intravenous administration. It seems that both described nanotube dispersions have probably exhibited poor stability and poor individualisation *in vivo* leading to observed RES biodistribution.

Moreover, once MWNT accumulate in tissues, they remain in the organs for the time frame of the experiment without causing any necrosis or fibrosis side effects as confirmed by the histology analysis (*Figure 4.1.5*). Clearance from the organs seems to be slow as indicated by the presence of CNT in the lungs, liver and spleen after 24 hrs post-injection. Complete organ excretion is thought to occur probably over several months via the biliary route into the faeces as was described for the non-covalently functionalised SWNT [27].

In addition, since the tracking of pristine MWNT precursor is very difficult to achieve because their radiolabelling is impossible, the non-quantitative approach of looking at histology sections of tissues was attempted for pMWNT dispersed in either F127 or BSA. It can be clearly seen that pMWNT: F127 showed a better profile that the pMWNT:BSA as observed by the much smaller pMWNT aggregates in the lungs and liver (*Figure 4.1.6*). Although, the macroscopic dispersion of MWNT: F127 seemed better than that of MWNT:BSA, the possibility of desorption
of the Pluronic or serum coating is thought to happen once inside the animal body as previously suggested by Cherukuri et al.[25] leading to organ accumulation of CNT. However, assuming that the coating stays stable in vivo, the Pluronic coating seem to act as a shealth to protect the rapid recognition of pMWNT by RES organs due to its polymeric nature like PEGylation. On the other hand, opsonisation of the BSA coating can occur more readily which can be the reason behind the bigger aggregates observed with the pMWNT: BSA and their high accumulation in RES organs; however more work is warranted in this area.
4.2. Evaluation of the biodistribution profile of carbohydrate functionalised and iodine filled SWNT

The biodistribution profile of carbohydrate functionalised and iodine filled SWNT after intravenous administration was studied. The tissue distribution and the \textit{in vivo} imaging of Na$^{125}$I glyco-SWNT was investigated using gamma scintigraphy and dynamic imaging by the small animal imaging Single Photon Emission Computed Tomography (SPECT/CT). Tissue histology was also investigated in attempt to visualise the SWNT inside organs and to determine whether tissue damage and necrosis occurred as a result of SWNT accumulation.
4.2.1. Carbohydrate functionalised and iodine filled SWNT: Functionalisation and characterisation

Another advantage of using CNT in biotechnology over other nanomaterials is the possibility of filling their inner volume with different molecules (therapeutics, diagnostic and imaging). Proof-of-principle studies have revealed that molecules can be encapsulated inside CNT [143-145]. Herein, sodium iodide (NaI) salts were encapsulated inside steam purified [146] SWNT by molten phase capillary wetting and the encapsulation was confirmed by high-resolution transmission electron microscopy (HRTEM) and scanning transmission electron microscopy (STEM) as recently described by Hong et al.[121].

To improve the dispersibility of SWNT, the filled SWNT were initially functionalised through the 1,3-dipolar cycloaddition, previously reported by Prato and co-workers [132, 147, 148]. This functionalisation methodology preserves the tubular structure and the closed ends of the nanotubes during treatment which circumvent the release of the filled material. After the successful functionalisation of the filled SWNT without loss of its cargo, the surface of SWNT was further modified with biomolecules in an attempt to improve on the cytotoxicity and dispersibility of SWNT in aqueous media. Glycoconjugates were chosen as molecules of interest due to the essential roles they play in many biological processes like cell signalling and immune response [149-151]. As shown in Scheme 4.2.1 the $^{125}$I filled SWNT were further coupled with N-acetyl-D-glycosamine (GlcNAc) dendrons, to afford GlcNAc$^D$-NaI@SWNT.

124
Scheme 4.2.1: Schematic representation of the carbohydrate functionalised and iodine filled GlcNAc\(^{\beta}\)-NaI@SWNT construct that will be biologically tested. Adapted from reference [121].

Although it is very hard to image SWNT using transmission electron microscopy (TEM) due to their small diameters, Figure 4.2.1A shows GlcNAc\(^{\beta}\)-NaI@SWNT to be aligned closer to each other in bundles. In addition, STEM analysis confirmed the successful filling of SWNT with NaI as shown in Figure 4.2.1B by the white dots due to the high atomic number of the radiometals inside the CNT.

Figure 4.2.1: Characterisation of GlcNAc\(^{\beta}\)-NaI@SWNT (A) TEM image of GlcNAc\(^{\beta}\)-NaI@SWNT dispersed in BSA/saline (B) Z-contrast STEM images of GlcNAc\(^{\beta}\)-NaI@SWNT showing NaI filling as white dots (STEM image taken by Dr. Belen Ballesteros (Inorganic Chemistry Laboratory, University of Oxford [121]).
4.2.2. Cytotoxicity assessment of carbohydrate functionalised and iodine filled SWNT (GlcNAc\textsuperscript{D}-NaI@SWNT) \textit{in vitro}

Before moving into animal studies, the safety profile of GlcNAc\textsuperscript{D}-NaI@SWNT against human lung epithelial cell line (A549) was assessed using an \textit{in vitro} cytotoxicity assay (the modified LDH assay) described in \textbf{Chapter III} and \textbf{Chapter V}. A dose escalation study was performed with the GlcNAc\textsuperscript{D}-NaI@SWNT dispersed in BSA/Saline as the vehicle. As shown in \textbf{Figure 4.2.2}, no cytotoxicity was observed after 24 hrs incubation with GlcNAc\textsuperscript{D}-NaI@SWNT up to the highest dose used of 125 µg/ml.

\begin{figure}[h]
\centering
\includegraphics[width=0.7\textwidth]{cytotoxicity_assessment.png}
\caption{\textit{In vitro} cytotoxicity assessment of GlcNAc\textsuperscript{D}-NaI@SWNT by modified LDH assay. Human lung epithelial cells (A549) were treated for 24 hrs with different concentrations of GlcNAc\textsuperscript{D}-NaI@SWNT (0-125 µg/ml) dispersed in BSA/Saline.}
\end{figure}
4.2.3. The biodistribution and imagining of carbohydrate functionalised and iodine filled SWNT (GlcNAcD-NaI25@SWNT) in vivo

The biodistribution of these constructs was assessed using single positron emission computed tomography (SPECT/CT). Animals were intravenously injected with 50μg and 250μg of GlcNAcD-NaI25@SWNTs containing 0.2MBq and 0.8MBq of radioactivity respectively. After 30 min post-injection, free NaI25 accumulated in the thyroid, stomach and the bladder as shown in Figure 4.2.3B. However, lung accumulation seemed predominant with the GlcNAcD-NaI25@SWNT with no signal of the encapsulated NaI25 elsewhere in the body (Figure 4.2.3A). Moreover, GlcNAcD-NaI25@SWNT retained persistently in the lungs after 4 and 24 hrs post-injection (Figure 4.2.3A). In the other hand, free NaI25 was mainly retained in the thyroid and stomach after 4 hrs and only in the thyroid after 24 hrs (Figure 4.2.3B). After 7 days, GlcNAcD-NaI25@SWNT still showed persistent lung accumulation, while trace signals of NaI25 were observed in the thyroid (Figure 4.2.4A). Interestingly, when a higher dose of GlcNAcD-NaI25@SWNT was injected (250 μg), the same lung accumulation was observed (Figure 4.2.3A, last panel).
Figure 4.2.3: Whole body SPECT/CT imaging was performed after 30 min, 4 hrs and 24 hrs post-intravenous injections with (A) 50μg and 250 μg (0.2MBq, 0.8MBq) of GlcNAc\(^{125}\)I-\text{Na}\(^{125}\)I@SWNT; and (B) \text{Na}\(^{125}\)I (1.8MBq) with a scanning time of 40-60min.

Figure 4.2.4: Whole body SPECT/CT imaging was performed after 7 days post intravenous injection with (A) 250μg (0.8MBq) of GlcNAc\(^{125}\)I-\text{Na}\(^{125}\)I@SWNTs and (B) \text{Na}\(^{125}\)I (1.8MBq) with a scanning time of 40-60min.
The quantitative biodistribution study of the SWNT construct was then performed using gamma counting. A lower radioactivity dose was needed contrary to the SPECT/CT imaging study which requires much higher radioactivity doses. **Figure 4.2.5A** depicts the biodistribution profile of GlcNAc$^D$-Na$^{125}$I@SWNT after 30 min, 4 hrs and 24 hrs. It can be clearly seen that lung accumulation was dominant with GlcNAc$^D$-Na$^{125}$I@SWNT over the time points studied (**Figure 4.2.5A**). However, the main organs of uptake of the free $^{125}$I were thyroid, stomach and intestines after 30 min post-injection as shown by **Figure 4.2.5B**. After 24 hrs, the free $^{125}$I was cleared completely from the body. Blood profile analysis shows that less than 1% of the total injected dose of GlcNAc$^D$-Na$^{125}$I@SWNTs was detected in the blood 3 min post-injection due to the rapid lung accumulation (**Figure 4.2.5C**) and 27% of the total injected dose of Na$^{125}$I was detected in the blood 3 min post-injection (**Figure 4.2.5D**).
4.2.4. Tissue histology following administration of carbohydrate functionalised and iodine filled SWNT (GlcNAc\textsuperscript{D}-Na\textsubscript{I}@SWNT)

Histological examination of the main organs of CNT accumulation was carried out in an attempt to check for any signs of histopathological abnormalities (e.g. tissue necrosis, fibrosis and inflammation). Figure 4.2.6 illustrates the presence of cold GlcNAc\textsuperscript{D}-Na\textsubscript{I}@SWNT in the lung alveoli at 24 hrs post-administration as shown by the black aggregates in the H&E lung-sections but with no signs of tissue damage. Furthermore, no accumulation of SWNT in the liver, spleen or kidney was observed which is in agreement with the SPECT/CT and gamma counting data.
Interestingly, there were no signs of necrosis and fibrosis in the lung tissue 30 days post-injection although SWNT accumulation in the lung was still observed (Figure 4.2.6). Interestingly, there seem to be some degree of redistribution of SWNT construct from the lungs to the liver, spleen and more importantly the kidneys (Figure 4.2.6).

Figure 4.2.6: Tissue histology of mice injected with ‘cold’ GlcNAcβ-NaI@SWNT dispersed in BSA/saline. Hematoxylin and eosin-stained sections of lung, liver, spleen and kidney at 24 hrs and 30 days post-injection with ‘cold’ GlcNAcβ-NaI@SWNT (50µg) or vehicle alone (0.5% BSA in PBS). Black arrows indicate GlcNAcβ-NaI@SWNT either big aggregates in the lungs or smaller ones in liver, spleen and kidneys. All photomicrographs were captured at 40x magnification.
4.2.5. DISCUSSION

CNT biodistribution studies have been based on the tracking of the radio-labelling attached to the CNT via chelating molecules like DTPA and DOPA [28, 31, 41]. While the attachment of chelating molecules to CNT via the functional groups generated through chemical functionalisation was achieved, other groups have attempted the attachment of chelating molecules just to the non-covalent coating wrapping the CNT [13]. The latter is a risky approach as the detachment of the coating is thought to occur once in vivo and hence the actual biodistribution observed can become of the coating molecule instead of that of CNT. In addition, other groups have attempted the radio-labelling of the carbon skeleton with carbon \(^{13}\)C isotope and quantitatively measured the \(^{13}\)C signal in organs of accumulation using isotope ratio mass spectrometry (Isotope-MS) [26, 35]. While this is a very interesting method for tracking the biodistribution of CNT in vivo, it cannot be widely used as it depends on the initial production of CNT and most groups who are studying CNT biodistribution do not have this facility but instead use commercially available CNT. In addition, imaging cannot be possible using this technique since it is an analytical method. Herein, a novel technique of radiolabelling was achieved by filling SWNT with the radiometal iodine \(^{125}\)I and imaging and biodistribution were attained using SPECT/CT and gamma scintigraphy.

SWNT were previously shown to be filled with a variety of compounds including organic [152] and inorganic materials [153]. Initially the metal halide NaI (radioactive \(^{125}\)I or cold I) was successfully entrapped inside SWNT as described by Hong et al.[121] and the surface of the filled SWNT was then covalently functionalised with carbohydrates [149] in an attempt to enhance their aqueous dispersibility and improves their biocompatibility. Glycoconjugates are highly
expressed in nature and play important roles in many biological processes such as
cell signalling and immune response [149, 151]. For instance, glycoproteins and
glycolipids which are key constituents of cell membrane and extracellular matrix
play an essential role in cell-protein interactions and molecular recognition of
antibodies and hormones.

Unfortunately, the dispersibility of the GlcNAc$^D$-Na$^{125I}$ @SWNT construct
was not greatly improved and BSA was required for their dispersion before
biological studies were performed. It should be mentioned that SWNT are usually
much harder to functionalise due to their initial existence in bundle forms and this
can be one of the reasons behind the dispersibility issue in here. Moreover, the
GlcNAc$^D$-Na$^{125I}$ @SWNT construct was found to be non-cytotoxic to lung
carcinoma A459 cells as assessed by the modified LDH assay which was developed
to avoid the interference that CNT suffers from with the established cytotoxicity
assays and it will be further discussed in Chapter V.

Interestingly, ultrasensitive imaging of the lungs was achieved using this
construct as observed by the strong lung signal observed with SPECT/CT after 30
min, 4 hrs, 24 hrs and even 7 days post-injection (Figure 4.2.4A and Figure
4.2.5A). Moreover, no leakage of the encapsulated iodine from SWNT was observed
as confirmed by the absence of thyroid and stomach signals which are considered the
organs of highest affinity to free iodine (Figure 4.2.4B). This meant that this
technique of encapsulation and radiolabelling is very efficient as stability of the
construct in vivo was proven. It should be mentioned however that once the
radiometal is encapsulated, oxidation of SWNT is not permitted as it opens up the
ends of the SWNT and can lead to the release of the encapsulated material. Other
chemical functionalisation chemistries as that performed in here (1, 3 dipolar cycloaddition) can be performed as long as it doesn’t open the closed ends of SWNT. Iodine radionuclides are considered the most effective treatment for thyroid cancer [154] due to the presence of thyroid iodide transport proteins; however this limits their use for other disease sites. Iodine was used in this study as an illustration that SWNT can be used as nanocapsules for radioisotopes as well as due to its 60 day half life. It is highly thought that other radioisotopes can be also encapsulated and the constructs can be used for other types of cancer either as imaging tools or for therapeutic purposes.

Interestingly, no dose dependent biodistribution was observed with the construct as the highest dose (250 µg) showed a similar lung uptake. Moreover, the merely lung accumulation of the construct was found as a striking result as none of the previous studies done with functionalised SWNT showed lung uptake only. Instead the biodistribution spectrum ranged from liver alone with SWNT:F106 [25] to lung, liver and spleen with $^{13}$C-enriched backbone SWNT coated with Tween 80 [26]. While, urine excretion was observed with ammonium functionalised SWNT and SWNT-COOH (hydroxylated) [28, 33]. While it is assumed that this selective lung uptake cannot be due to poor dispersibility as previously coated SWNT with surfactants showed different biodistribution profiles, it is thought that the carbohydrate attached to the surface of SWNT could have lead to this selective lung accumulation. In addition, the effect of the filling should not be ignored in here as previous biodistribution studies were performed only with empty functionalised SWNT. Although non-quantitative, histological examination of tissues after the injection of non-filled and sugar functionalised SWNT were found in the lungs as
well as smaller aggregates in the liver and spleen (APPENDIX II), however more work is needed in this front especially with filled material.

Lastly, from an imaging point of view the radioactivity doses used with the encapsulated iodine (0.2-0.8 MBq) are thought to be much lower than what is used for imaging purposes with SPECT/CT [41]. This means that ultrasensitive non-invasive imaging was achieved with a much lower radioactivity due to the condensed filling of iodine inside SWNT. Interestingly, gadolinium; the most commonly used contrast agent for magnetic resonance imaging (MRI), was also encapsulated inside SWNT leading to the formation of gadonanotubes [155]. The latter showed a 40-90 times better efficacy than other existing gadolinium based contrast agents in use in the clinical settings. This study along ours confirms that by taken advantage of the inner volume of CNT, these can be further developed for molecular imaging and diagnostics.
4.3. CONCLUSION

This part of the project attempted to determine some of the critical factors that can contribute to the pharmacokinetic profile of injected CNT. It was found that aryl functionalisation led to the formation of different MWNT dimensions ranging from fully individualised to large aggregates which behaved differently in the body after intravenous administration. Early urinary excretion of CNT was observed of what it is suggested as fully individualised MWNT and accumulation of CNT aggregates of different dimensions in lungs and RES organs. Moreover, the lung uptake showed to be transient as dissociated aggregates translocate to the spleen at later time point. The individualisation and the aggregation of the CNT dispersion which are factors of the functionalisation chemistry, play a detrimental role in determining the \textit{in vivo} fate of the CNT and to a larger extent can provide an answer to the currently observed variability in the CNT \textit{in vivo} biodistribution data.

While the tracing of the biodistribution of aryl functionalised MWNT was based on further functionalising the MWNT with a chelating molecule (DTPA) which caged the radioisotope indium (\(^{111}\)In), the second section of this chapter discussed the development of a new tool for the biodistribution and imaging of CNT. The development of GlcNAc\(^{2}\)-Na\(^{125}\)I @SWNT construct was achieved based on the encapsulation of SWNT with low doses of the radioisotope \(^{125}\)I and their subsequent functionalisation with glycoconjugates. Interestingly, high stability of the developed construct was achieved and proven by the complete absence of iodine signals in the high affinity organs for iodine (thyroid and stomach). For the first time, only lung uptake of CNT is described which can be due to the carbohydrate attached to the surface of the SWNT. However, the filling is also speculated to have affected the biodistribution to some degree but more work is required in this area since this is the
first biodistribution study done using filled SWNT instead of their empty counterparts. This construct can be therefore used for imaging as well as therapeutic purposes if iodine 125 is exchanged by the therapeutic iodine 131.
CHAPTER V

THE TOXICOLOGICAL ASSESSMENT OF FUNCTIONALISED CARBON NANOTUBES IN VITRO AND IN VIVO
5.1. Development of accurate and reliable cytotoxicity assay to determine carbon nanotubes toxicity

This section describes the toxicological assessment of chemically functionalised and copolymer coated MWNT using traditional cytotoxicity assays such as the MTT, Annexin V/ PI and the LDH assays. The cellular uptake and internalisation of those two types of MWNT were investigated. Moreover, a valid toxicity assay for the accurate and reliable *in vitro* cytotoxicity assessment of CNT was developed.
5.1.1. CNT characteristics and functionalisation

In an attempt to validate the traditionally used cytotoxicity assays and their reliability for use with CNT, two types of materials that are highly utilised in biomedical application, were used. Those two types were either prepared by chemical functionalisation via the conversion of the carboxylic groups into amino-functionalities leading to ammonium functionalised MWNT (MWNT-NH$_3^+$) or by non-covalently coating the MWNT with the copolymer Pluronic F127 which exhibit surfactant-like properties and hence the generation of MWNT: F127. Both types of MWNT showed to be very well dispersed in aqueous media as can be seen in Figure 5.1.1B. It should also be noted that both methodologies achieved a concentration of 0.5-1 mg/ml of MWNT in aqueous media which is a reasonably high concentration that can be achieved in general with both chemically and coated CNT. No MWNT precipitation was observed over time with both types of MWNT dispersions. In addition, transmission electron microscopy (TEM) images depict the individualisation of both MWNT-NH$_3^+$ and MWNT: F127 which further indicated the good colloidal state of MWNT (Figure 5.1.1C). The similar characteristics observed with both types of MWNT is important for an accurate comparison between the two materials especially that lack of similarities in the physicochemical properties of the used CNT in the current published toxicity data makes the generalised conclusions about CNT inaccurate. The number of amino groups at the surface of the MWNT-NH$_3^+$ was determined to be 0.320 mmol per gram of material by the quantitative Kaiser test. Figure 5.1.1 shows a schematic representation of the two types of MWNT and their dispersion and TEM images.
Figure 5.1.1: A) Schematic of the structures of the ammonium functionalised MWNT (MWNT-NH$_3^+$) and Pluronic F127 coated MWNT respectively. B) Dispersion of MWNT-NH$_3^+$ and MWNT-F127 in aqueous media showing a good dispersibility of MWNT. C) Transmission Electron Microscopy (TEM) images of MWNT-NH$_3^+$ and MWNT-F127 respectively. Scale bar 100 nm.
5.1.2. The cellular uptake and internalisation functionalised MWNT

First we looked at the internalisation of both types of MWNT after 24 hrs incubation with human lung epithelial carcinoma (A549) cells. Light microscopy images (Figure 5.1.2, Top Panel) show that MWNT-NH$_3^+$ were uptaken en masse and they took up the shape and morphology of the epithelial cells especially at the highest concentration of 125µg/ml. In contrast, the MWNT: F127 seemed to have minimal interaction with the cells as shown by only few black MWNT on the cells as observed at the highest concentration of 125 µg/ml (Figure 5.1.2, Bottom Panel).

![Figure 5.1.2: Analysis of cellular uptake of f-MWNT by light microscopy. Photomicrographs of A549 cells treated with increasing concentration of MWNT-NH$_3^+$ (Top Panel) and MWNT: F127 (Bottom Panel) ranging from 0-125 µg/ml in complete media for 24 hrs. Higher cellular internalisation of MWNT-NH$_3^+$ compared to MWNT: F127. The images were representative of 3 wells. (Photomicrographs were taken at 20 x magnification).](image-url)
Flow cytometry was used to determine the uptake of the two different types of MWNT semi-quantitatively. Flow cytometry-based assays have been proposed to assess the interaction between nanoparticles and cells but the difference between surface binding and cellular uptake is difficult to determine. Internalisation of nanoparticles was previously assessed either quantitatively by measuring the absolute number of fluorescently labelled nanoparticles associated with cells or qualitatively based merely on increased sideward scattering of cells incubated with non-fluorescent nanoparticles [156, 157]. Side scatter was used to assess whether there is any cellular association of MWNT-NH$_3^+$ and MWNT: F127 with A549 cells as it is a measure of granularity and complexity of cells. The increase in side scatter of cells incubated with CNT was previously found to be a reliable measure for assessing the interaction between CNT and cells [158, 159]. As shown in Figure 5.1.3 (Top Panel), there is a dose dependent increase in side scatter as the concentration of MWNT-NH$_3^+$ is increased up to 125 μg/ml. However, there was a minimal change in side scatter with the co-polymer coated MWNT even at the highest concentration which suggests a low change in cellular granularity and hence low intracellular accumulation of MWNT: F127 (Figure 5.1.3, Bottom Panel). This is in complete agreement with the light microscopy images shown in Figure 5.1.2. In addition, the CNT do not interfere with the granularity data as they appear to be excluded from the gate of events (cells) and instead emerge in the left corner of the histogram (near the excluded area of the cell debris).
Further clarification of the intracellular trafficking of the different MWNT was studied using transmission electron microscopy (TEM). Both ammonium functionalised MWNT and polymer coated MWNT at concentration of 50µg/ml were incubated with A549 cells at 37 °C for 24 hrs. Ultra-thin sections of A549 cells were then prepared and examined by TEM. The MWNT-NH₃⁺ seemed to be more abundant in the cytoplasm than the MWNT: F127 (Figure 5.1.4). Staining with uranyl and lead made it difficult to visualise the polymer coated MWNT which led to the use of unstained ultra-thin sections (Figure 5.1.4). While, MWNT-NH₃⁺ were observed in mass quantities compared to MWNT: F127 which affirmed the light microscopy and flow cytometry data, the MWNT: F127 were identified inside cells but to a lesser degree (Figure 5.1.4B-D). It can be observed that MWNT-NH₃⁺ enter the cells and pass through the plasma membrane as individualised MWNT in a perpendicular manner (Figure 5.1.4A), however once inside the cytoplasm they are
re-arranged in aggregates which are sometimes observed enclosed within membraneous intracellular compartments (Figure 5.1.4B).

Figure 5.1.4: Cellular internalisation of MWNT inside human lung carcinoma A549 cell section observed by transmission electron microscopy. (A) The interaction of MWNT-NH$_2$ with plasma membrane and the piercing in a perpendicular manner. (B) MWNT-NH$_2$ observed inside intracellular vesicles. (C) MWNT: F127 inside A549 unstained cell sections. (D) High magnification TEM image of the highlighted section in (C).
5.1.3. Toxicological assessment of both types of MWNT using established assays

The cytotoxicity of ammonium functionalised MWNT and polymer coated MWNT was assessed using established assays (MTT, LDH and Annexin V-FITC/PI).

5.1.3.1. MTT assay:

The MTT assay showed false negative cell viability with MWNT-NH$_3^+$ which was indicated by the dose dependent increase in cell viability as the concentration of MWNT-NH3+ was increased from 1.9 to 125 μg/ml (Figure 5.1.5A).

![MTT Assay Graph](image)

**Figure 5.1.5**: Cell viability of human lung carcinoma A549 cells after 24 hrs incubation with f-MWNT. Percentage Cell viability of A549 assessed by the MTT assay at 0-125μg/ml final concentrations of (A) MWNT: F127 and the equivalent Pluronic F127 concentration (0-1250 μg/ml) and (B) MWNT-NH$_3^+$. DMSO 10% was used as a positive control. Cell viability was calculated as a percentage of media-only controls. MWNT: F127 showed a dose dependent toxicity after 24hr exposure while the MWNT-NH$_3^+$ showed a dose dependent increase in the cell viability.
However, the polymer-coated MWNT showed a dose-dependent reduction in A549 cell viability (Figure 5.1.5B). Due to the reported false positive results with the MTT assay, the interaction between formazan and MWNT was mimicked. As the formazan was spiked with increasing concentrations of MWNT, an increase in the absorbance at 570 nm was observed reaching a saturation limit at 62.5µg/ml as shown in Figure 5.1.6. This indicated that the intrinsic absorbance of MWNT was a contributing factor in the absorbance readings and it is not simply due to formazan absorbance at 570 nm which should have stayed as low as the control if no interference was occurring between the formazan and MWNT.

![Figure 5.1.6: Formazan-MWNT interaction by spiking the formazan with f-MWNT dispersions at 0-250 µg/ml final concentrations. A dose dependent increase in the absorbance at 570 nm is observed with increasing MWNT concentrations up to 250µg/ml final concentration.](image)

5.1.3.2. Annexin V-FITC/PI assay: a second commonly used assay was performed which is based on flow cytometry using Annexin V-FITC/PI staining kit. This is a well established *in vitro* cytotoxic assay in which the early and late stages of apoptosis/necrosis can be determined following exposure to the test material. In the early stages of apoptosis, phosphatidylserine flip flop to the outerlayer of the plasma membrane, to which the Annexin V-FITC; a calcium dependent phospholipid
binding protein, bind to it giving rise to FITC signal. At later stages of necrotic state the membrane impermeable dyes such as propidium iodide cross defective plasma membranes and reach the nucleus. **APPENDIX III** shows flow cytometry data as indicated by the four quadrons representing live (R5, FITC-/PI-), early apoptotic (R6, FITC+/PI-) and late necrotic (R4, FITC+/PI+) and cell debris (R3, FITC-/PI+). There was no shift of cell population from healthy population R5 (FITC-/PI-) to apoptotic populations (R6, R3, R4) for the different concentrations used after a 24 hr treatment period with both types of MWNT and the F127 control except for the positive control used cationic liposome (**APPENDIX III**). When flow cytometry data is expressed as percentage cell survival (percentage of healthy cells in R5 relative to control); no significant difference between the treated cells with MWNT: F127 and F127 alone, nor with that of MWNT-NH$_3^+$ and the untreated cells was observed and with approximately 80% cell survival obtained for all conditions (Figure 5.1.7). Therefore, the Annexin V/PI assay indicated no considerable toxicity with MWNT-NH$_3^+$, MWNT: F127 and F127 alone (Figure 5.1.7A-C). Although this assay may be more accurate to describe cellular uptake of MWNT as it is not based on fluorophores but depends on the complexity of the cells, it is difficult to judge its validity in the presence of CNT especially that CNT are known quenchers of fluorescent probes [14, 160] in addition to the possibility of underestimating the apoptotic -cell population due to the washing up step performed after the removal of CNT containing media.
Figure 5.1.7: Cell viability of human lung carcinoma A549 cells after 24hrs incubation with MWNT assessed by Annexin V-FITC/PI staining and quantified by flow cytometry after 24 hrs incubation with (A) MWNT-NH$_4^+$, (B) MWNT: F127 at a concentration of 0-125 μg/ml and (C) Pluronic F127 alone at a concentration of 0-1250 μg/ml. No cytotoxicity was observed except with the cationic liposomes at 0.48mM used as positive control of toxicity.
5.1.3.3. Original LDH assay: Next, the lactate dehydrogenase (LDH) assay was performed. This assay is considered to be safe for use with carbon nanotubes as suggested by Worle-Knirsch and co-workers [67]. This assay is based on the indirect assessment of the number of lysed cells as indicated by the amount of released LDH following cell damage and lysis. Figure 5.1.8 shows that with the original LDH assay, there is an increased release of LDH with both types of MWNT. However, this release is not genuine but it is primarily due to the intrinsic absorbance of MWNT alone at 470 nm as can be seen in Figure 5.1.8. Surprisingly, the positive control DMSO 10 % did not cause any increase in LDH release but instead caused a decrease which can be due to the inhibition of the enzyme activity by this organic solvent used (DMSO). It should also be noted that the time points assessed by the original LDH assay cannot be very long because of the half life of LDH is only 9 hrs [71].

Figure 5.1.8: Cytotoxicity of human lung carcinoma A549 cells after 24hrs incubation with f-MWNT as assessed by the original LDH assay. The percentage LDH release (Absorbance at 490 nm) after treatment of cells with different concentrations of MWNT: F127 and MWNT-NH$_2$ ranging from 0-125µg/ml and equivalent Pluronic F127 concentrations (0-1250 µg/ml). The absorbance of MWNT alone dispersions were used as control. DMSO 10 % was used as positive control of cytotoxicity.
Figure 5.1.9: Validation of the modified LDH assay by comparing it to the MTT and Annexin V/PI (FACS) assays using cationic liposomes and DMSO as positive controls. Different concentrations of cationic liposomes ranging from 0.0075-0.48 mM and DMSO at 5 and 20 % v/v in complete media were incubated with A549 cells for 24 hrs.

The cytotoxicity of two types of MWNT using the modified LDH assay was then carried out. As can be seen in Figure 5.1.10, the ammonium functionalised MWNT are non-toxic throughout the used concentration range (0-125 μg/ml). However, the polymer coated F127 MWNT, showed a dose dependent toxicity with a maximum toxicity of 60% reached at 125 μg/ml. However, when evaluating the toxicity of the polymer alone, this showed 100 % of cell survival i.e. without any detrimental toxicity at the equivalent concentrations (0-1250 μg/ml) used with the MWNT. After 48 hrs, the MWNT-NH₃⁺ were still non-toxic at the highest concentration of 125 μg/ml, while the MWNT: F127 showed 40 % cell death part of which can be attributed to the polymer F127 alone as by itself showed a reduced cell survival to 60%.
The cytotoxicity observed with MWNT: F127 was further explored and the mechanistic reasons behind it were investigated using transmission electron microscopy to check for any cellular morphology changes. A549 cells were treated with 50\(\mu\)g/ml of both types of MWNT for 24 hrs after which the cells were ultra-thin sectioned for TEM images. It can be clearly observed that only the MWNT: F127 treated cultures have damaged mitochondria which are shown to be swollen with the loss of their cristae which are the inner invaginations and folds of the mitochondrial membrane responsible for ATP synthesis (Figure 5.1.10B). Interestingly, the polymer alone at the equivalent concentration used with MWNT: F127 (500\(\mu\)g/ml) did not cause any mitochondrial damage or any other characteristic abnormalities. Furthermore, the MWNT-NH\(_3^+\) did not show any mitochondrial damage.
Figure 5.1.10: Cell survival of human lung carcinoma A549 cells after 24hrs incubation with MWNT-NH₃⁺, MWNT-F127 and Pluronic F127 alone as assessed by the modified LDH assay. (A) The percentage cell survival after treatment of cells with different concentrations of MWNT-F127 ranging from 0-125μg/ml and equivalent 0-1250 μg/ml of Pluronic F127. DMSO 10 % was used as a positive control. (B) TEM of ultra-thin cell sections shown mitochondrial damage with cells treated with MWNT-F127 but not with Pluronic F127 alone.
5.1.4. DISCUSSION

Carbon nanotubes have proven to be of great importance as delivery vectors for a variety of diagnostics and therapeutics modalities. While the use of CNT in nanomedicine is expanding, a comparison between the published studies poses problems due to differences in every measured parameter. On one hand mainly non-functionalised CNT are being tested while their dispersibility is considered poor for use in biomedical applications. On the other hand, different assays, tissue cultures, incubation times have been assessed. Hence, it was thought that a systematic comparison of the cellular uptake and cytotoxicity of CNT can only be made if materials have similar physical properties in terms of dispersibility and are compared using the same assays under similar conditions. In view of this, two types of functionalisation methodologies that render the CNT water dispersible were explored. The first was based on the chemical functionalisation of pristine MWNT leading to the creation of carboxylic groups and their subsequent conversion into ammonium functionalities, while the second was based on the non-covalent coating of MWNT with the block copolymer Pluronic F127.

Cellular internalisation and uptake of CNT

Previously, researchers have studied the cellular internalisation of CNT based on fluorescence and confocal microscopy and used different types of fluorescently labelled CNT constructs [161-163]. Others have based their internalisation studies on the labelling of the macromolecules coating the CNT instead of the direct labelling of the nanotubes [164, 165]. It should be mentioned that the presence of macromolecules like DNA and protein can alter the surface characteristics of the CNT and hence their subsequent interaction with the plasma membrane. Since both approaches can alter the trafficking pathway of the CNT, one should be more careful
when interpreting the data obtained from them. The needs for other techniques which are not based on modifying the CNT surface with fluorescent molecules are therefore prerequisite.

Taking this into consideration, the cellular uptake of MWNT-NH$_3^+$ and MWNT: F127 was investigated based on changes in the side-scatter (complexity) of cells in addition to light microscopy and more mechanistically with TEM of cell sections. It was found that while MWNT-NH$_3^+$ are uptaken in mass quantities; they are not cytotoxic to A549 cells. In contrast, the less internalised polymer coated MWNT (MWNT: F127) were found highly cytotoxic according to the modified LDH assay and confirmed by mitochondrial damage (Figure 5.1.4 and Figure 5.1.10). This technique which is based on cellular side-scatter can be used as a screening tool to determine the uptake of CNT and can be more accurate than using fluorescently labelled CNT since the surface chemistry of CNT is not altered. In addition, there is evidence that CNT can act as quenchers [160] and hence there will always be risk that the fluorescence of the attached probes can be quenched with CNT. This also means that the avoidance of fluorescence based techniques is highly desirable with CNT.

Since cellular side-scatter does not differentiate between simple membrane association and uptake; neither indicates the exact location of CNT within the cells the use of TEM of cell sections was attempted for this purpose. CNT are clearly observed by TEM as shown in Figure 5.1.1C, therefore TEM of ultra-thin cell sections has been explored by many groups in an attempt to visualise the exact location of CNT inside cells. For instance, Cheng et al.[166] have recently investigated the cellular uptake of purified and non-purified MWNT on human
monocyte derived macrophages using 3D Dark Field Scanning TEM. It was found that unpurified MWNT are uptaken both actively via phagocytosis and passively through direct cytoplasmic translocation. In addition, the intracellular trafficking of CNT has been studied using other high resolution microscopy techniques such as those performed by Porter et al. [167, 168] namely Raman spectroscopy scanning transmission electron microscopy combined with electron energy-loss spectroscopy (STEM-EELS) and high angle annular dark field (HAADF)-STEM. Authors found that acid treated MWNT were localised inside lysosomes but also found them in the cytoplasm [168]. In this work, MWNT-NH$_3^+$ were found abundant in the cytoplasm of A549 cells as individualised nanotubes but also in aggregate forms inside membraneous vesicles which have been hypothesised to be formed after their initial internalisation as individual nanotubes (Figure 5.1.4). Similar observations were described by Simon-Deckers et al. [63] in which MWNT dispersed in Arabic gum were found in the cytoplasm and surrounded by a membrane. Moreover, Chithrani et al. [169, 170] observed rod-shaped gold nanoparticles inside similar cytoplasmic membrane-structures to that where the MWNT-NH$_3^+$ were found as indicated by TEM. In addition, our copolymer coated MWNT were only observed individualised inside the cytoplasm but their uptake was to a much lower degree than the MWNT-NH$_3^+$.

The cytotoxicity profile of chemically functionalised and copolymer coated MWNT

**MTT assay:** The dose dependent toxicity observed with MWNT: F127 using the MTT assay (Figure 5.1.5) can be attributed either to a real cytotoxic activity of the MWNT: F127 or a strong interaction between the MWNT and the insoluble formazan formed after the reduction of the MTT by viable cells (Scheme 3.1.5,
Materials and Methods). The latter can cause the non-solubilisation of the formazan crystals leading to low viability as has been suggested by Krug et al. [67]. Authors demonstrated that there is an interaction between CNT and most cell viability markers like MTT, Neutral Red, Commassie blue and even the more soluble tetrazolium derivative WST-1 [67, 68]. Casey et al. [68] have also studied the interaction between the different cell viability markers and SWNT by spectroscopy and concluded that the interaction is a factor of physisorption of the marker molecules onto the CNT surface through Van der Waals forces leading to an overall decrease in the absorbance.

It should be also noted that false (high) cell viability can be seen due to the intrinsic absorbance of CNT mainly when high cellular uptake of CNT occurs as was observed with the MWNT-NH₃⁺ (Figure 5.1.2). The high viability was also described by Monteiro-Riviere et al. [50] stating the possibility of false indications in cell viability when the toxicity of carbon black (CB) was studied using the Neutral Red assay and they have also reported that the use of CB can affect viability markers used in MTT and Interleukin release assays due to strong Van der Waals forces. Since it is impossible to predict the amount of CNT uptaken by cells or that of formazan formed by viable cells, this can cause variability and non-reproducibility in the MTT data. The amount of CNT uptaken versus that bound to the formazan can vary making any interpretation regarding cell viability very hard to make if not impossible using the MTT assay. Hence, the avoidance of the MTT assay is highly recommended especially when a massive uptake of CNT is taken place.

Annexin V-FITC/ PI assay: Moreover assays like the Annexin V-FITC/ PI, although very useful in providing a detailed description of the time scale of
apoptosis, should be avoided for use with CNT for two reasons. The first being the presence of fluorescent probes (FITC and PI) which can be readily quenched with the CNT and secondly due the technicality of the assay in which the apoptotic population can be easily underestimated due to the washing off step performed during the assay procedure as also mentioned by Monteiro-Riviere et al.[71].

**The modified LDH assay:** A different cytotoxicity assay known as the lactate dehydrogenase assay (LDH) was used in parallel. This assay is based on the indirect assessment of the number of lysed cells indicated by the amount of released LDH following cellular damage as the LDH enzyme is cytoplasmic in nature (Scheme 3.1.7 & 3.1.8, Materials and Methods). This assay was modified however because it is colorimetric and gives rise to false negatives (high viability) due to the intrinsic CNT absorbance (Figure 5.1.8). Therefore, cells were artificially lysed and the cell lysate centrifuged in order to precipitate the CNT and avoid subsequent interference with the assay reagents (Scheme 3.1.9, Materials and Methods). Therefore, by assessing the LDH in the cell lysate, we are indirectly looking at the number of viable cells that survived after the MWNT treatment. Based on this assay, it was found that MWNT: F127 are cytotoxic compared to MWNT-NH$_2$ which show high viability even at the highest concentrations (Figure 5.1.10). The copolymer F127 alone was found to be non-cytotoxic at the used equivalent concentrations that are present with the MWNT as shown by the modified LDH assay and the other assays (Figure 5.1.5- Figure 5.1.9). However, we are speculating that more copolymer is uptaken by the A549 cells once bound to CNT. Hence, the observed toxicity with MWNT: F127 can be attributed to higher concentrations of copolymer inside cells than expected. Interestingly, Kabanov et al. [171] have suggested that block copolymers can act as biological modulators despite having always been regarded as
biologically inert entities. It is also thought that Pluronic copolymers can affect membrane microviscosity as they incorporate into the membrane and induce a dramatic reduction in ATP levels. In addition, they can induce the release of cytochrome C and increase the reactive oxygen species [171]. It was also described that Pluronic copolymer can incorporate into the mitochondria leading to the inhibition of the cellular respiration which is mainly due to monomers of the copolymer. We can therefore speculate that the mitochondrial damage is not a secondary effect due to necrosis but instead it is probably due to the direct exposure of cells to higher concentrations of Pluronic copolymer through their uptake with the CNT. However, we cannot completely exclude any direct damage to mitochondria caused by MWNT especially that Narita et al.[172] have observed MWNT inside the mitochondria.

Overall, the modified LDH assay has became the primary tool for assessing the cytotoxicity of MWNT in our laboratory. The MTT assay is to be avoided because of the uncontrolled two-level interactions that occur with CNT. Moreover, the Annexin V-FITC / PI assay, although very effective with other delivery vectors like liposomes, was found to be non-sensitive for use with CNT due its technicality issues and since it is a fluorescence-based assay.
5.2. Can chemical functionalisation alleviate the asbestos-like pathogenicity-risk-associated with long pristine MWNT?

The effect of chemical functionalisation on the carcinogenic risk associated with the long and rigid pristine MWNT was evaluated in vivo. A structure-activity paradigm of toxicity based on the direct exposure of the abdominal cavity of mice to MWNT was used. The inflammatory reaction of the chemically functionalised MWNT was assessed by total polymorphonuclear leucocytes and protein exudation. In addition, structural changes in the diaphragms surface and its morphology were observed using SEM and Haematoxylin and Eosin staining respectively.
5.2.1. CNT characteristics and functionalisation

In order to investigate the effect of chemical functionalisation on the carcinogenic risk associated with the long pristine MWNT which was described by Poland et al. [85], the same long pristine MWNT (denoted as \textbf{NT}_2 \text{Pristine}) were chemically functionalised using two different reactions. As shown in \textbf{Scheme 5.2.1}, the long \textbf{NT}_2 \text{Pristine} were functionalised using octyl-iodide (octyl functionalisation) [130, 131] generating \textbf{NT}_2 \text{-Alkyl} as shown by \textbf{reaction 1} or through the 1, 3 dipolar cyclo-addition [20, 132] generating \textbf{NT}_2 \text{-TEG} (\textbf{reaction 2}).

\begin{center}
\textbf{Scheme 5.2.1}: Chemical functionalisation of long pristine MWNT (\textbf{NT}_2 \text{Pristine}). \textbf{Reaction 1} depicts the introduction of an Alkyl group onto the surface of the \textbf{NT}_2. \textbf{Reaction 2} represents the functionalisation of \textbf{NT}_2 through the 1, 3 dipolar cycloaddition and the introduction of TEG moieties onto the \textbf{NT}_2 surface.
\end{center}
All types of MWNT were then characterised using transmission electron microscopy (TEM) and atomic force microscopy (AFM) as shown in Figure 5.2.1A. In addition, the long-fiber amosite asbestos (LFA); which is known as a positive control of carcinogenicity, was also used in this study. TEM and AFM images showed that the NT\textsubscript{2} Pristine are the longest (after LFA) followed by NT\textsubscript{2}-Alkyl and the shortest being the NT\textsubscript{2}-TEG. This was further confirmed by length distribution data obtained using the Image J software and low magnification TEM for length measurements as shown in Figure 5.2.1B. Since we used a structure-toxicity paradigm model that relates the length and the bio-persistence of fibres to the development of granuloma formation, the length distribution was further represented as percentage of different length ranges as shown in Table 5.2.1. It was observed that the NT\textsubscript{2} Pristine and NT\textsubscript{2}-Alkyl had a similar length distribution with around 70% of the nanotubes above 5 μm in length (Table 5.2.1, shown in red), while the majority ( > 65%) of the NT\textsubscript{2}-TEG are below the 5 μm and with just 13% of the NT\textsubscript{2}-TEG longer than this range (Table 5.2.1, shown in green).

Table 5.2.1: Length distribution of NT\textsubscript{2} samples and LFA

<table>
<thead>
<tr>
<th>Percentage of NT in the range</th>
<th>LFA</th>
<th>NT\textsubscript{2} Pristine</th>
<th>NT\textsubscript{2} - Alkyl</th>
<th>NT\textsubscript{2} - TEG</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 1 μm</td>
<td>0 %</td>
<td>4.35 %</td>
<td>0 %</td>
<td>20.51 %</td>
</tr>
<tr>
<td>1-5 μm</td>
<td>0 %</td>
<td>26.09 %</td>
<td>30.26 %</td>
<td>66.67 %</td>
</tr>
<tr>
<td>&gt; 5 μm</td>
<td>100 %</td>
<td>69.56 %</td>
<td>70.06 %</td>
<td>12.82 %</td>
</tr>
</tbody>
</table>

163
Figure 5.2.1: Fibre characterisation using Transmission electron microscopy (TEM) and atomic force microscopy (AFM). Samples were dispersed in 0.5% BSA/saline prior to visualisation with TEM and AFM. NT\textsubscript{2} Pristine and NT\textsubscript{2}-Alkyl samples contained longer MWNT compared to NT\textsubscript{2}-TEG as shown by TEM and AFM images (A) and confirmed by the size distribution analysis (B) that was based on TEM images at low magnifications. * Size distribution of LFA samples was hard to measure since the LFA fibres were longer than the TEM grid; hence the current size distribution is underestimating the exact length of the fibres.
5.2.2. Toxicological effects of intraperitoneally injected NT$_2$ samples

**Inflammatory reaction after 24 hrs and 7 days post-injection:** In an attempt to determine the sensitivity of the mesothelium to the functionalised NT$_2$ in comparison to the long NT$_2$ Pristine and the long-fibre amosite (LFA) used as positive control; 50 µg of each type of NT$_2$ (dispersed in bovine serum albumin (BSA) /saline) were injected into the peritoneal (abdominal) cavity of C57/BL6 mice. The abdominal cavity was then lavaged with physiological saline, 24 hr and 7 days post-injections as previously described by Poland *et al.*[85]. The inflammatory response was examined by the change in the protein levels and the polymorphonuclear leucocytes (PMN) in the peritoneal lavate. 24 hrs post injection, the NT$_2$ Pristine and NT$_2$-Alkyl caused a significant increase in the PMN and protein levels similar to the inflammatory response observed with the asbestos positive control (*Figure 5.2.2*). Interestingly, the 1, 3 dipolar cycloaddition functionalised MWNT (NT$_2$-TEG) werer safer after 24 hrs without causing any surge in both inflammatory markers (PMN and protein). However, a clear decrease in the inflammatory reaction was observed after 7 days with the NT$_2$ Pristine, NT$_2$-Alkyl and LFA. *Figure 5.2.2* depicts the dramatic decrease in the total PMN and the protein levels observed after 7 days.
Figure 5.2.2: Inflammatory reaction in the peritoneal cavity 24 hrs post injection with fibres. Female C57B1/6 mice were intraperitoneally injected with 50μg of vehicle control (0.5% BSA/saline), NT2 pristine, NT2-Alkyl and NT2-TEG and long-libre amosite (LFA) as positive control. Inflammatory response was evaluated by (A) the total polymorphonuclear leukocytes (PMN) and (B) total protein (protein exudation) after 24 hrs. Data represent the mean of 4 animals ± STDV. ***P value <0.005 versus vehicle control.
Figure 5.2.3: Inflammatory reaction in the peritoneal cavity 7 days post injection with fibres. Female C57Bl/6 mice were intraperitoneally injected with 50µg of vehicle control (0.5% BSA/saline), NT₂ Pristine, NT₂-Alkyl and NT₂-TEG and long-fibre amosite (LFA) as positive control. Inflammatory response was evaluated by (A) the total polymorphonuclear leukocytes (PMN) and (B) total protein (protein exudation) after 7 days. Data represent the mean of 4 animals ± STDV.
Granuloma formation after 7 days: Since the common pathogenic response to long fibres like asbestos is abnormalities and changes in the mesothelial surfaces as previously described by Poland et al.[85] Haematoxylin and Eosin (H&E) histological staining of the diaphragms was conducted together with SEM of the surface of the diaphragms (Figure 5.2.4). The surface of the diaphragms after treatment with NT₂ Pristine and NT₂-Alkyl show a granular appearance observed at low magnification (Figure 5.2.4A) which is absent with the BSA/Saline vehicle and the NT₂-TEG, both of which showed smooth surfaces. Figure 5.2.4B shows high magnifications of the diaphragm surfaces treated with the NT₂ Pristine and NT₂-Alkyl associated with a collection of immune cells that have probably attempted the elimination of the foreign NT₂ samples. This immune reaction is also observed with the positive control LFA (Figure 5.2.4A-B) and is thought to be a granuloma formation as clearly identified with the H & E staining (Figure 5.2.4C). The H & E histology clearly shows the development of granuloma lesions seen as purplish lesions with the NT₂-Alkyl similar to that observed with the long NT₂ Pristine and LFA. No such lesions were observed with NT₂-TEG and the vehicle control.
Figure 5.2.4: The effect of fibre on the diaphragms after 7 days. Female C57Bl/6 mice were intraperitoneally injected with 50µg of vehicle control (0.5% BSA/saline), NT2 Pristine, NT2-Alkyl and NT2-TEG and long-fibre amosites (LFA) as positive control. The mice were killed after 7 days and the diaphragms excised, fixed and prepared for visualisation. (A) SEM images of the diaphragm surface at low magnification and at high magnification (B) and (C) the histology using H & E staining showing the presence of granulomatous inflammation with NT2 Pristine, LFA and NT2-Alkyl but not with NT2-TEG.
5.2.3. DISCUSSION

CNT are increasingly being used in nanomedicine ranging from nanosensors to implants and as drug delivery vectors. However, these applications of CNT are still viewed with apprehension due to their unknown toxicological profile and their posed environmental and health risks especially that they resemble the needle like carcinogenic asbestos fibres [44, 85, 97, 173]. Several research groups have therefore explored the carcinogenic risks of CNT using several in vivo models and paradigms [85-87, 174]. One study that highlighted potential safety issues with pristine MWNT was done by Poland et al. [85] using a structure-toxicity paradigm which was originally validated with asbestos fibres [175] and relates the length, dose and bio-persistence of asbestos fibres to the development of mesothelioma (cancer of the pleura). This is a fibre paradigm and identifies the length of fibres as the most determinant factor in toxicology in addition to bio-persistence as both dictate the clearance kinetics of the fibres from the lungs. Therefore, this paradigm describes a pathogenic fibre as one being long, thin and bio-persistent. Based on this paradigm, non-functionalised and rigid MWNT (NT$_2$ Pristine) longer than 20 μm were found to cause granuloma formation 7 days after intra-peritoneal injection (i.p) similar to what is observed with long fibres amosites (LFA) used as positive control [85]. Similar observations regarding the carcinogenic potential of pristine MWNT were earlier described by Takagi et al.[86] but no mechanistic explanation was given as that provided by Poland et al.[85] but instead the mice used were more susceptible to mesothelioma formation due to the fact that they are heterozygous of p53. In a longer term study, Muller et al. [87] have investigated the effect of MWNT with and without structural defects over 24 months and found no mesothelioma formation with both types which is thought to be due to their short length of about 1 μm. While
all those studies speculate the carcinogenic potential of pristine MWNT if inhaled, none of them have verified this hypothesis by direct inhalation of mice to CNT due to the difficulty in setting this model but most of them based their conclusions on well established paradigms of toxicity. Recently Rayman-Rasmussen et al.[176] have observed the translocation of CNT into the subpleural cavity after direct inhalation of CNT and subsequently described the formation of subpleural fibrosis after 2-6 weeks following inhalation.

Taking a step further, herein we attempted to investigate whether chemical functionalisation of long MWNT would alleviate their associated granuloma risks especially that chemical functionalisation which ensures a stable dispersion of individualised MWNT in physiological media has previously shown to cause no detrimental effects to the injected mice but also lead to urinary excretion of these non-biodegradable nanomaterials [36, 37, 177] compared to its pristine counterparts.

Two different chemistries (Scheme 5.2.1) were therefore employed to study the effect of functionalisation on the carcinogenic risks associated with NT₂ Pristine. It was observed that a functionalisation that shortened the length of the CNT (NT₂-TEG) alleviated the asbestos like pathogenicity observed with their pristine counterparts (NT₂ Pristine). In fact whenever functionalisation did not have an impact on the length of the original pristine MWNT (NT₂ Pristine) as observed with NT₂-Alkyl, the inflammatory response was similar to what was seen with the long NT₂ Pristine and LFA fibres (Figure 5.2.2) together with a clear granuloma formation observed 7 days post-injection (Figure 5.2.4). This is thought to be due to frustrated phagocytosis in which macrophages were unable to phagocytose fibres that are longer than the length that they can completely engulf which will lead to
continuous release of inflammatory mediators [85]. Scheme 5.2.2 below shows this effect in greater detail [173]. This phenomenon of frustrated phagocytosis was also shown by histology in the Poland study [85] with the \textit{NT}$_2$\textit{Pristine}.

Scheme 5.2.2: The effect of CNT length on their clearance by phagocytosis. While short CNT are easily engulfed by macrophages and cleared out (A), long CNT cannot be phagocytosed and accumulate in tissues where they can promote carcinogenesis (B). (Adapted from reference [173]).

Interestingly, the 1,3 dipolar cycloaddition reaction shortened the length of the \textit{NT}$_2$\textit{Pristine} leading to a much higher fraction of shortened MWNT and no pathological changes were observed since macrophages were able to clear out the short foreign \textit{NT}$_2$-\textit{TEG}. These observations are in good agreement with the long-short paradigm of toxicity in which the \textit{NT}$_2$-\textit{Alkyl} behaved like long fibres while \textit{NT}$_2$-\textit{TEG} behaved as short fibres. The functional group did not seem to be an influential factor since the positively charged \textit{NT}$_2$-\textit{TEG} did not cause any toxicological pathologies, however their enhanced hydrophilicity and individulisation compared to the \textit{NT}$_2$-\textit{Alkyl} could also be a reason for their safety profile. However, this cannot be confirmed using this paradigm which is only based
on length distribution and do not take into consideration the change in surface chemistry. In addition, the contribution of soluble contaminants was also excluded since both NT2-Alkyl and NT2-TEG were functionalised from the same precursor NT 2 Pristine which have previously shown to be negative to soluble contaminants [85].

Moreover, the more commercially available MWNT (from Nanostructured and Amorphous materials.Inc) and that are highly investigated by our group were found non-pathogenic (APPENDIX IV). In addition, their further functionalisation with carboxylic groups was also found to be non-pathogenic. So we can conclude that the 1,3 dipolar cycloaddition functionalised MWNT (NT2-TEG) and commercially used NanoAmorph Pristine behaved as the short tangled MWNT described by Poland et al.[85] and also as the short MWNT used by Muller et al. [87] and are therefore environmentally and therapeutically safer to use for biomedical applications.

Overall, chemical functionalisation can alleviate the carcinogenicity risk associated with long pristine MWNT; however the choice of functionalisation is prerequisite. For instance, the 1, 3 dipolar cycloaddition functionalisation was found to improve on the dispersibility of MWNT and shortens the length of the pristine precursor without causing any inflammatory or granuloma formation.
5.3. CONCLUSION

This chapter described the *in vitro* and *in vivo* toxicological assessments of carbon nanotubes. The development of an effective and reliable *in vitro* toxicological assay was achieved based on the pitfalls and drawbacks of a few traditionally used cytotoxicity assays. Interestingly, it was found that the chemically functionalised MWNT-NH$_3^+$ are non-cytotoxic to lung carcinoma cells compared to the non-chemically functionalised (MWNT: F127). TEM of ultra-thin cells sections provided valuable information regarding the cellular internalisation of MWNT and further explanations regarding the cytotoxicity observed with MWNT: F127 which was attributed to mitochondrial damage. MWNT-NH$_3^+$ can therefore provide valuable platforms for further biomedical applications as delivery systems.

In addition, the effect of chemical functionalisation on alleviating the pathogenic risks previously described with long and rigid pristine MWNT was verified *in vivo*. A known structure-activity paradigm of toxicity that is based on the direct exposure of the abdominal cavity of mice to MWNT was used. It could be clearly concluded that a functionalisation that shortens the length of the MWNT is desirable more than another that keeps their length similar to the pristine precursor. No inflammatory reaction or granuloma formation was observed with short MWNT of less than 5 µm in length (either functionalised with TEG or the commercially available pristine from Nanostructured and Amorphous Materials,Inc). Since these CNT types are not associated with pathogenicity, they can be further developed for biomedical applications and cancer therapy.
CHAPTER VI

CANCER THERAPY USING CARBON NANOTUBES AS DELIVERY VECTORS
6.1. CNT: Doxorubicin supramolecular complexes for cancer therapeutics

The development of CNT- doxorubicin complexes through non-covalent $\pi$-$\pi$ stacking is described. The formation of the CNT: Doxorubicin complexes was investigated using fluorescence spectroscopy and transmission electron microscopy. In addition, the biological activity of those complexes was assessed in \textit{in vitro} cell culture and animal (\textit{in vivo}) tumour models.
6.1.1. The formation and characterisation of CNT: Doxorubicin complexes

The discovery that CNT are capable of traversing the plasma membrane [24] and promoting the cellular uptake of small molecules and macromolecules (e.g. nucleic acids and peptides) has offered new opportunities for various biological applications [23, 98, 99]. Functionalised CNT have shown great promise as novel delivery systems especially based on their ability to cross biological barriers independently of the cell type they interact with and the functional group at their surface [24]. In addition, the high aspect ratio of CNT offers great advantages over existing delivery vectors, as the high surface area provides multiple attachment sites for drugs. Interestingly, the surface of CNT was found to be a good platform for supramolecular complex formation with molecules such as porphyrin macrocycles [178] and pyrene molecules [179] due to π-π stacking; which are non-covalent interactions that occur between compounds containing aromatic rings [180, 181]. In this regard, the formation of this type of non-covalent complexes with biologically relevant molecules was investigated using the anticancer drug; doxorubicin.

Doxorubicin (Dox) belongs to the family of antracyclines that have been clinically used. Dox is a fluorescent molecule with a chromophore composed of three planar and aromatic hydroxyanthraquinonic rings and hence its supramolecular complexation with MWNT can be studied through changes in its fluorescence intensity. After the initial dispersion of pristine MWNT in Pluronic F127 as described previously, the coated MWNT were allowed to interact with Dox by mixing equal volumes of doxorubicin hydrochloride (20μg/ml) with increasing the concentration of MWNT aqueous dispersion (10, 20 and 40 μg/ml). The complexes formed contained from 0.5x10^{18} to 2x10^{18} molecules of Dox per mg of MWNT which is also represented for simplicity as MWNT: Dox mass ratio of 2:1 to 0.5:1.
respectively. The interaction between Dox and MWNT was studied by monitoring the emission spectrum of Dox using fluorescence spectrophotometry. As can be seen from Figure 6.1.1, the fluorescence intensity of Dox was dramatically decreased as the final concentration of MWNT was increased from 5 to 20 μg/ml. Maximum quenching was occurring at 0.5x10^18 Dox molecules per mg MWNT (MWNT: Dox 2:1 mass ratio) indicating that optimum interaction between the drug and the MWNT occurs at this mass ratio. In a control experiment, when the same number of Dox molecules were mixed with the equivalent Pluronic F127 concentration in the absence of MWNT, no decrease in fluorescence intensity compared to that of Dox in water (Figure 6.1.1) was observed, suggesting that no interaction was taking place between the drug and the copolymer molecules.

Figure 6.1.1: Normalised fluorescence intensity of MWNT: Dox complexes. Final concentration of doxorubicin was fixed to 10 μg/ml while MWNT final concentration was increased (5, 10 and 20 μg/ml) which is equivalent to MWNT: Dox mass ratio of 0.5:1, 1:1 and 2:1. Pluronic F127 alone and Pluronic: Dox are also shown as controls.
Transmission electron microscopy (TEM) was used to study the structural characteristics of the MWNT: Dox complexes. Figure 6.1.2A shows copolymer-wrapped MWNT as well-individualised and dispersed nanotubes, clean from any impurities, and confirming the ability of the polymer molecules to disperse the CNT effectively. Dox alone showed crystal-like structures at low magnification (Figure 6.1.2B). Overall, the TEM images revealed that MWNT were strongly interacting with Dox via the formation of supramolecular clusters. These MWNT: Dox clusters were very well visualised as the number of Dox molecules was decreased from $2 \times 10^{18}$ to $0.5 \times 10^{18}$ molecules per mg MWNT (Figure 6.1.2 C, D & E, F). The observed clustering between the MWNT and Dox also correlated well with the maximum fluorescence quenching of Dox (Figure 6.1.1). These experiments indicated that Dox can form non-covalent complexes with MWNT through $\pi-\pi$ stacking.
Figure 6.1.2: TEM images of MWNT: Dox complexes. (A) MWNT alone (scale bar 100 nm), (B) Dox alone (scale bar 2 microns), (C) $2 \times 10^{-13}$, (D) $1 \times 10^{-13}$, (E) $0.5 \times 10^{-13}$ Dox molecules per mg MWNT with scale bar of 500 nm and (F) $0.5 \times 10^{-13}$ Dox molecules per mg MWNT at higher magnification (scale bar corresponds to 100 nm). The final MWNT concentration was kept constant at 0.5 mg/ml.
6.1.2. Cytotoxicity assessment of MWNT: Dox complexes in vitro

Next, the biological activity of the MWNT: Dox complexes was initially studied in vitro using the MTT assay. It was thought that no interference would occur between the MTT and MWNT since a lower MWNT concentration was used compared to what was used in Section 5.1. The epithelial breast cancer derived MCF-7 cells were seeded and the cell viability following interaction for 24 hrs with the MWNT: Dox complexes was assessed. The MWNT: Dox mass ratio of 2:1 was used since maximum quenching (Figure 6.1.1) and supramolecular complexes (Figure 6.1.2) were obtained at this ratio.

Figure 6.1.3 depicts that Dox was still active even after complexation with MWNT as shown by the statistically significant enhancement in the cytotoxic capability of the MWNT: Dox complexes compared to that of Dox alone (p < 0.01) and the equivalent Pluronic: Dox (p < 0.005) after 24 hrs. Interestingly, cells treated with the equivalent Pluronic-coated MWNT and Pluronic alone demonstrated no difference compared to untreated cells, exhibiting 100 % cell viability. Importantly, the copolymer does not seem to be contributing to this improved cytotoxic capacity, since mixing Dox with pluronic alone showed no significant difference as compared to Dox alone (p=0.493). This correlated well with the fluorescence spectroscopy data that indicated no complex formation between Dox and the block copolymer molecules (Figure 6.1.1).

In addition, when the biological activity of the MWNT: Dox complexes were assessed by the modified LDH assay developed in this thesis and described in Section 5.1; a similar activity to the drug alone was observed (APPENDIX V).
Overall, the activity of doxorubicin molecules was maintained even when they were complexed with MWNT.

Figure 6.1.3: The percentage cell viability of MCF-7 cells after 24 hr incubation at a final concentration of Dox (600 nM); MWNT:F127 (651.5 ng/ml); Pluronic (6.5 μg/ml) and Pluronic: Dox and MWNT: Dox at a mass ratio of 2:1. Untreated cells were used as control. Statistical significance was observed between the MWNT: Dox complexes and † Dox (p<0.01) and the *** Pluronic: Dox groups (p<0.005) respectively.

6.1.3 Therapeutic efficacy of MWNT: Dox complexes in vivo

The in vivo therapeutic efficacy of the MWNT: Dox complexes was then investigated using C57BL6 mice bearing B16 melanoma tumours; an aggressive syngeneic tumour model. In addition, a chemically functionalised type of MWNT (MWNT-NH$_3^+$) which do not require the Pluronic coating for dispersion but still allow for π-π stacking with Dox, was also used for the therapeutic experiment. Tumours were treated by intra-tumoural injections in a trice weekly regimen for a total of 5 doses. The tumours in the control 5% dextrose group rapidly increased in volume in this aggressive tumour model. Nearly 50 % of the control groups were
culled by day 12; therefore the tumour volume measurements were carried out until that time point. Tumours treated with MWNT alone increased similarly to the naive controls which was considered as an indication that MWNT alone did not cause any cytotoxicity. Interestingly, both types of MWNT: Dox complexes (Pluronic coated and MWNT-NH\(_3^+\)) showed a similar increase in tumour volume as that of the drug alone with no statistically significant difference (p > 0.01) (Figure 6.1.4A-B).

On day 28 after tumour implantation the therapeutic outcome following a total of 5 doses (on day 7, 9, 11, 13, 15) was analysed in terms of survival for the treated groups. Animals treated with the MWNT: Dox complexes lived longer than their naive counterparts (Figure 6.1.5). However, the Dox alone treated group showed the best survival rate with 50 % of the mice surviving until day 28, followed by the Dox complexed with the chemically functionalised MWNT and then the Pluronic coated MWNT. Overall, the therapeutic activity of the MWNT: Dox complexes was not improved compared to the drug alone, however the chemically functionalised MWNT seems more advantageous than the Pluronic coated MWNT.
Figure 6.1.4: Growth curves after intratumoral administration of MWNT: Dox complexes in B16F10 melanoma tumours. B16F10 cells were inoculated under the skin of nude mice and when the tumour volume reached 200 mm$^3$, tumours were intratumorally injected with (A) Dox alone, MWNT:F127, MWNT: Dox and (B) MWNT-NH$_3^+$ and [MWNT-NH$_3^+$]: Dox. Arrows indicates injections.
Figure 6.1.5: Survival curves after intratumoral administration of MWNT: Dox complexes in B16F10 melanoma tumors. B16F10 cells were inoculated under the skin of nude mice and when the tumor volume reached 200 mm³, tumors were intratumorally injected with (A) Dox alone, MWNT:F127, MWNT: Dox and (B) MWNT-NH₃⁺ and [MWNT-NH₃⁺]: Dox.
6.1.4. Tumour histology following administration of MWNT: Dox complexes

The mechanism behind the observed reduction in tumour volume with the Dox alone and the MWNT: Dox complexes was further investigated histologically. Tumours from all treated groups were excised when the maximum allowed tumour volume (800-1000 mm$^3$) was reached and then sectioned for Hematoxylin and Eosin (H &E) staining. Extended necrosis was observed in the tumour tissue treated with MWNT: Dox , [MWNT-NH$_3$]: Dox around the areas containing the nanotubes but also extended to distant regions from the injection sites as observed in Figure 6.1.6 (light pink patches). As expected the Dox alone treated tumours also showed great regions of tissue necrosis. Interestingly, mainly viable tissue was observed in all other tumour sections treated with MWNT alone (coated and covalently functionalised) indicating that the MWNT per se are not cytotoxic.

In addition, other organs (liver, spleen and heart) were also examined histologically and no tissue necrosis or CNT accumulation was observed as shown in APPENDIX VI.
Figure 6.1.6: Hematoxylin and eosin-stained sections of tumour lesions. Tumour tissues from naive mice (A), Dox alone (B), MWNT: F127 (C) and MWNT: Dox (D) MWNT-NH$_3^+$ (E), [MWNT-NH$_3^+$]: Dox (F). Tumours with distinct tumour necrosis (light pink areas) are shown in Dox alone (B), MWNT: Dox (D) and [MWNT-NH$_3^+$]: Dox treated tumour sections (F). The black f-MWNT in both MWNT: Dox (D) and [MWNT-NH$_3^+$]: Dox (F) treated groups are associated with tumour necrosis and are more obvious due to an increased hydrophobicity with the MWNT: Dox complexes. No black stain from the f-MWNT are observed in C and E due to high dispersibility of both f-MWNT types prior complexation with Dox. All photomicrographs were captured at 20 x magnification.
6.1.5. DISCUSSION

The development of efficient delivery systems with the ability to enhance cellular uptake of existing potent drugs is needed. CNT may offer great advantages compared to other drug delivery systems especially based on their intrinsic ability to cross the plasma membranes irrespective of the cell type and the functional group at their surface [24]. In addition, their high surface area and aspect ratio offers numerous attachment sites for drug targeting. Pastorin et al. [22] were the first to develop CNT-methotrexate conjugates via covalent linkage for use as cancer therapeutics. Though such conjugates seem promising, the efficacy of the drug activity is dependent on the nature of the covalent bond between the CNT and the small molecule. In this work a simpler approach was adopted via studying the possibility of non-covalent interactions between the anticancer drug doxorubicin and MWNT.

It was demonstrated in this thesis that that block copolymer (Pluronic) coated MWNT can form non-covalent supramolecular complexes with Dox. The formation of such complexes was evidenced by a sharp decrease in the fluorescence intensity of Dox and took place presumably via π-π interactions with the MWNT backbone. Dox exhibits a unique fluorescence emission spectrum that is susceptible to changes in its microenvironment [182]. Interaction with MWNT resulted in changes in its molecular conformation leading to complete quenching of the fluorescence signal at the 2:1 MWNT: Dox mass ratio (Figure 6.1.1), similar to that described when Dox binds to human α-1 glycoprotein [182]. The affinity of the Dox chromophore for self- and hetero-association with various compounds with planar aromatic ring systems has also been described before [183, 184]. Moreover, the binding and intercalation of Dox between the aromatic bases of nucleic acids is well established,
also known to cause considerable reduction in Dox fluorescence intensity [182]. The possibility of electrostatic or hydrogen bond formation between the Dox molecule and the non-ionic block copolymer may also be enhancing the interaction between Dox and MWNT, however is not dominant as shown in Figure 6.1.1 when Dox interacted with block copolymer alone. This indicates that the structure of the CNT backbone can act as a platform for the formation of supramolecular complexes with small drug molecules, similar to what has been described before with the aromatic bases of nucleic acids [16] and other molecules [178, 179].

The biological activity of MWNT: Dox complexes were initially evaluated in vitro using MCF7 cell cultures. Dox was still active once complexed with MWNT and showed an enhancement in its biological activity as shown by the MTT assay (Figure 6.1.3). This was surprising especially that Pluronic coated MWNT showed limited cellular uptake as observed by TEM of cell sections shown in Section 5.1. The issue of interference was ruled out since the concentration used was low to cause this significant enhancement in the cytotoxicity (p < 0.01 compared to Dox alone). The cytotoxicity of the MWNT: Dox complexes was also assessed using the modified LDH assay and showed similar activity to Dox alone. This suggests that the MWNT: Dox complexes are as active as the drug alone. Interestingly, the formation of CNT: Dox complexes was reported by other research groups. For instance, almost simultaneously with the work carried out in this thesis, Lui et al. [101] reported pH and diameter dependent loading and release of Dox from SWNT coated with phospholipid–polyethylene glycol (PL-PEG). No improvement in the cell killing capacity of the SWNT-PEG: Dox complexes compared to the drug alone was however observed. Therefore, both SWNT and MWNT seem to offer available surface area for π-π interactions with the aromatic rings of doxorubicin.
Since the publication of this work [14] and that of Liu et al.[101], more studies with the CNT:Dox complexes was conducted by others. For instance, chemically functionalised CNT have also shown the ability to strongly bind Dox as was described by Heister et al. [103] who used carboxylated and bovine serum album (BSA) functionalised SWNT. Monoclonal antibodies against the carcinoembryonic antigen (CEA) were also attached to the SWNT in an attempt to improve their targeting capabilities. Confocal laser microscopy showed that the targeted SWNT: Dox constructs were efficiently uptaken by human colon WiDr cancer cells that overexpress CEA antigens, however no comparison with the non-targeted construct was given in this study. In a different study [104], carboxylated and polysaccharide coated SWNT were complexed with doxorubicin at pH 7.4 in contrast to what was suggested by the Liu et al. [101] (stating that the binding occurs at basic pH (9.5)). Folic acid (FA) was also used as a targeting moiety and the FA-SWNT: Dox complexes were found to enhance the uptake in human cervical HeLa cells compared with the non-targeted complexes. In addition, cytotoxicity studies showed a dose and time dependence cellular killing with the targeted complexes. The latest study using CNT : Dox complexes was conducted by Chaudhuri et al. [105] who described the use of Pegylated SWNT complexed to pyrene Dox. The use of a carbamate linker between the pyrene and Dox allowed the controlled release of the drug in vitro. There was a time-dependent cytotoxic activity reported with a maximum efficacy occurring after 72 hrs of incubation with the construct.

The therapeutic efficacy of the MWNT: Dox complexes was then studied in vivo using B16F10 melanoma tumour model to which the complexes were intratumorally injected. B16F10 tumour model was used as it is an aggressive model at which control tumours reaches a volume of 800 mm$^3$ by day 12 (Figure 6.1.4), so
the suppression of the tumour growth and the enhancement in survival could be apparent in this tumour model with therapeutically active molecules. In addition, B16F10 cells showed to be responsive to Dox treatment as shown in Appendix VII. It should be mentioned that intralesional injections were chosen since the systemic administration of CNT is still in problematic due to RES uptake of CNT once intravenously injected as discussed previously in Chapter IV. In addition, intratumoral administration can provide high doses of biologically active substances locally into the tumour site. Moreover, the intratumoral injection of MWNT-NH$_3^+$: siRNA complexes had proven to be very successful and led to therapeutic efficacy in vivo [43]. Herein, no improvement in the therapeutic efficacy of Dox was observed when complexed with MWNT. Although, there was a statistically significant suppression of tumour volume followed by the prolongation of survival of the B16F10 tumour bearing animals with the MWNT: Dox complexes when compared to naive untreated tumours, a similar therapeutic activity to the drug alone was achieved (Figure 6.1.4-5). The chemically functionalised (MWNT-NH$_3^+$) without the Pluronic coating showed a better survival of treated mice compared to the Pluronic coated MWNT (Figure 6.1.5) which is thought to be due to their better internalisation profile as discussed in Section 5.1. However, the general non-improvement on the therapeutic activity of Dox once conjugated to MWNT could be due to release issues of Dox from the surface of MWNT as π-π stacking are one of the strongest type of non-covalent interactions. Although, Liu et al.[101] described a pH dependent complexation and release of Dox from SWNT; this was not observed with our complexes as shown in Appendix VIII. In addition, similar in vivo results were observed by others [102, 105] with no enhancement on the therapeutic activity of CNT: Dox complexes even when intravenously injected.
Overall, this work established that Dox delivery into tumour cells \textit{in vitro} and \textit{in vivo} can be achieved using non-covalent complex formation with MWNT and its therapeutic activity was maintained \textit{in vitro} and \textit{in vivo}. Although, the chemically functionalised MWNT improved the survival of mice better than the Pluronic coated MWNT, the release of the drug from both types of MWNT is still an issue and can only be resolved by the introduction of cleavable linkers which requires chemically functionalised MWNT. This will be discussed in Section 6.2 but with the use of a different anticancer drug that does not $\pi$-$\pi$ stack onto the MWNT surface.
6.2. CNT-Methotrexate conjugates for cancer therapeutics

The covalent attachment of the anticancer drug methotrexate (MTX) to CNT using a variety of cleavable linkers is described. The CNT-MTX conjugates were formed and characterised and their cytotoxic efficacy was investigated on human carcinoma cell cultures. In addition, the therapeutic efficacy of those conjugates was studied in tumour models in vivo.
6.2.1. The preparation and characterisation of CNT-Methotrexate conjugates

Methotrexate (MTX) belongs to the clinically used family of folate antagonists and is commonly used as a first line treatment for many types of cancer. As any potent anticancer drug, MTX suffers from the drawback of low cellular uptake [185] and hence its conjugation to a carrier system is critical. Therefore, MTX was chemically linked to MWNT using different cleavable spacers. This work was a continuation of what was previously suggested by Pastorin et al. [22] who described the chemical conjugation of MTX to MWNT with no improvement in the cytotoxic potency of MTX achieved. This was thought to be due to the immobilisation of the drug once bound to the CNT by the non-cleavable linker and hence its inability to act on the target enzyme (dihydrofolate reductase). In this work, the introduction of cleavable linkers to conjugate MTX molecules onto MWNT was investigated in an attempt to improve on the therapeutic efficacy of the constructs.

After the initial oxidation, MWNT were further functionalised with amine moieties through the 1, 3 dipolar cycloaddition reaction of azomethine ylides [133, 186]. The amount of amine functional groups was assessed using the Kaiser test as shown in Table 6.2.1. The amine functionalities were then used for the coupling of the MTX onto the nanotubes. As can be observed in Scheme 6.2.1, the non-cleavable MWNT-MTX constructs were obtained by the direct coupling of MTX with the amine functional groups [187]. Two different types of linkers were explored for the cleavable MWNT-MTX conjugates. A tetrapeptide (Gly-Leu-Phe-Gly) linker that is sensitive to proteases over-expressed in tumour cells [135-137] and a 6-hydroxyhexanoic ester as an esterase sensitive linker were used [108, 134]. Hence the names MWNT-Peptidase Linker-MTX and MWNT-Esterase Linker-MTX conjugates are given respectively to the conjugates (Scheme 6.2.1). As shown in
Scheme 6.2.1, the MWNT-Esterase Linker-MTX was further derivatised at its carboxylic sides with the fluorescent probe rhodamine B and hence the use of this fluorescent construct in cellular uptake studies.

Scheme 6.2.1: Chemical synthesis of the different MWNT-MTX conjugates. Constructs synthesis was carried out by Dr. Cristian Samori (CNRS, Strasbourg, France). Adapted from reference [112].

The different conjugates were further characterised with the Kaiser test to indicate the loading of the MTX onto the nanotubes as shown in Table 6.2.1. In addition, a dramatic improvement in the dispersion properties of the MWNT-MTX conjugates was observed (Figure 6.2.1). The nanotubes were nicely dispersed in 5 % using bath sonication. Transmission electron microscopy images also depict the individualisation of the MWNT-MTX which confirms the macroscopic observation.
of the MWNT dispersions. It can also be observed that the different conjugates are structurally similar with an average length around 300 nm and a diameter of 9.5 nm.

Figure 6.2.1: Dispersion photographs and TEM images of the different MWNT-MTX conjugates. (A) Macroscopic photographs of the dispersion of MWNT alone and the different MWNT-MTX conjugates in an aqueous solution (5 % dextrose in water). (B) TEM images of the dispersed MWNT-MTX conjugates showing similar structural properties of the conjugates in comparison to the MWNT alone.
Table 6.2.1: Kaiser test and loading of MTX onto the MWNT. a) Reaction was performed twice giving slightly different loading as indicated (Adapted from reference [112]).

<table>
<thead>
<tr>
<th></th>
<th>Loading NH$_2$ (μmol/g) from Kaiser test</th>
<th>Efficiency of MTX coupling from Kaiser test</th>
<th>Amount of bound MTX (μg/mg of conjugate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MWNT Alone</td>
<td>180 or 220$^a$</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>MWNT-MTX (No cleavable linker)</td>
<td>220</td>
<td>90%</td>
<td>89.9</td>
</tr>
<tr>
<td>MWNT-MTX (Peptidase sensitive linker)</td>
<td>100</td>
<td>80%</td>
<td>36.4</td>
</tr>
<tr>
<td>MWNT-MTX (Esterase sensitive linker)</td>
<td>180</td>
<td>100%</td>
<td>81.8</td>
</tr>
</tbody>
</table>

6.2.2. Cellular uptake and internalisation of the MWNT-MTX conjugates

In attempt to establish the capacity of the MWNT-MTX conjugates to translocate into mammalian cells, human breast carcinoma (MCF-7) cells were incubated with the rhodamine labelled MWNT-MTX conjugate at a low concentration (10 μg/ml) that does not affect cell viability and were left to interact with the cells for 24 hrs. The cells were also treated with MWNT devoid of the rhodamine as a control. It can be clearly seen that the nanotubes were efficiently uptaken by the cells as indicated by the diffuse red signals throughout the cytosol due to the conjugated rhodamine B (Figure 6.2.2). A similar internalisation pattern was also observed with rhodamine labelled MWNT without MTX (APPENDIX IX). In addition, the differential interference contrast (DIC) images show viable cell
morphology of MCF-7 cells and the presence of few black aggregates inside the cells (Figure 6.2.2).

Figure 6.2.2: Confocal microscopy images of MCF7 cells incubated in the absence (naive) and the presence of 10 μg/ml of rhodamine B labelled MWNT-MTX construct. MWNT devoid of the rhodamine B was used as a control. Top panels depict rhodamine B optics (Red channel) and bottom panels show differential interference contrast (DIC) images with MWNT shown as black dots. Scale bar is 20 μm in all images.

6.2.3. Cytotoxicity assessment of the MWNT-MTX conjugates in vitro

The cytotoxic activity of the MWNT-MTX constructs against breast carcinoma MCF-7 cells was then investigated using the modified LDH assay described in Section 5.1. The concentration of MTX was kept constant at 10 μM with all constructs. As shown in Figure 6.2.3, MWNT-MTX with the peptidase sensitive linker showed a time dependence cytotoxicity which was not observed with the other constructs. 3 hrs post-incubation, none of the MWNT-MTX constructs was active but after 6 hrs the MWNT-MTX with the peptidase sensitive linker caused 70
% cell death which become almost 90 % after 24 hrs. The MWNT-MTX conjugate with the esterase sensitive linker showed only 20 % cytotoxicity after 24 hrs similar to the MTX alone. As expected, the MWNT-MTX conjugate with the non cleavable linker showed no activity over the 24 hrs incubation period. The most active conjugate was the MWNT-MTX with the peptidase sensitive linker as shown by its early cytotoxicity activity after 6 hrs of incubation (Figure 6.2.3). Interestingly, the MWNT alone did not cause any cytotoxicity, and DMSO 10% was used as positive control.

Figure 6.2.3: Cell survival of human breast MCF7 cells after 3, 6 and 24 hrs incubation with the different MWNT-MTX conjugates. MTX concentration was kept constant at 10 μM with and without MWNT. 10 % DMSO was used as positive control of toxicity. Time dependence cytotoxicity was observed with the MWNT-MTX with the peptidase sensitive linker. Statistical significance (p<0.005) was found between the MTX alone and CNT-Peptidase-MTX as indicated by the stars (***).
Optical microscopy images of MCF7 cells confirmed the time dependence cytotoxicity of the MWNT-MTX conjugates as shown in Figure 6.2.4 (last 2 bottom panels). Cell death as indicated by the empty cell patches is clearly observed with the MWNT-MTX containing the peptidase sensitive linker after 6 and 24 hrs treatment.

Figure 6.2.4: Light microscopy images of human breast MCF7 cells after 3, 6 and 24 hrs incubation with the different MWNT-MTX conjugates. MTX concentration was kept constant at 10 μM with and without MWNT. 10 % DMSO was used as positive control of toxicity. Clear cell death with the MWNT-peptidase linker-MTX after 6 and 24 hrs was observed (last panel).
6.2.4. Therapeutic efficacy of the MWNT-MTX conjugates *in vivo*

The *in vivo* therapeutic efficacy of the MWNT-MTX constructs in comparison to MTX alone was investigated next. Only the most active MWNT-MTX with the peptidase sensitive linker was used and compared to the non-cleavable construct. Immuno-compromised nude mice were subcutaneously inoculated with prostate DU145 carcinoma cells and the MWNT-MTX constructs were intratumorally injected. Since the growth of MCF7 xenograft tumour model is difficult as it depends on estrogens, DU145 was used instead as these cells showed a similar responsiveness to MTX as that observed with MCF7 cells (APPENDIX X). In addition, B16F10 cells used previously with the MWNT: Dox complexes (Section 5.1), were found very responsive to MTX (APPENDIX X) which could possibly compromise any pronounced therapeutic effects with MWNT-MTX conjugates if the tumours would be very responsive to the drug alone.

*Figure 6.2.5* depicts tumour volume after treatment with 3 doses and after 55 days post tumour inoculation. The MWNT-MTX conjugate with the peptidase sensitive linker seemed more active than the non-cleavable linked MWNT-MTX conjugate, however no statistical significance was observed compared with the drug alone (p value =0.18). MTX treated groups showed a similar increase in the tumour volume compared to untreated and the MWNT alone treated groups. Overall, the observed tumour growth of the control groups never reached levels high enough to reveal any therapeutic activity from the MWNT-MTX groups. Therefore, inconclusive evidence was reached from these studies.
Figure 6.2.5: Growth curves after intratumoral administration of MWNT-MTX conjugates in DU145 prostate xenograft tumours. DU145 cells were inoculated under the skin of nude mice and intratumoral injection of MTX alone, CNT alone, CNT-MTX without a cleavable linker, CNT-MTX with a peptidase sensitive linker. Treatment began when the tumour volume reached 200 mm³ and injections were performed on days 34, 41 and 48 post tumour inoculation. Arrows indicate injections.
6.2.5. DISCUSSION

Carbon nanotubes have attracted particular attention in cancer chemotherapy due to their unique physicochemical and biological properties [12, 20]. Since the efficacy of most potent anticancer drugs is constrained by drug resistance and adverse effects, their attachment to drug delivery carriers such as CNT can overcome those problems. The high surface area and presence of diverse chemical groups on the surface of functionalised CNT offer multiple attachment sites for the chemical conjugation of anticancer drugs. The anti-metabolite drug MTX competitively inhibits the enzyme dihydrofolate reductase (DHFR) responsible for conversation of dihydrofolate to tetrahydrofolate. This is a very important step in folic acid metabolism and will impair the synthesis of purine nucleotides, thereby inhibiting DNA synthesis which leads to decreased proliferation [188].

Pastorin et al. [22] were the first to develop CNT-MTX conjugates via covalent linkage for the use as cancer therapeutics, however the efficacy of the drug was dependent on the nature of the covalent bond between the CNT and the small MTX molecule and no improvement in the cytotoxic efficacy was observed. As a continuation of this work, MWNT were chemically conjugated to MTX but this time exploring a variety of cleavable linkers sensitive to intracellular enzymes. The cytotoxic efficacy of the conjugates was then investigated in *in vitro* cell culture and *in vivo* tumour models.

Even though such cleavable constructs have found limited attention, the introduction of cleavable linkers to trigger the controlled release of attached drug from CNT has been attempted by different groups. In this regard, Liu et al.[108] developed PL-PEG coated SWNT-Paclitaxel (SWNT-PTX) in which PTX was chemically conjugated via an ester bond to the distal end of the PL-PEG that coated
SWNT. The SWNT-PTX conjugates showed a significant tumour regression than the clinically used Taxol in a murine 4T1 breast cancer model. This therapeutic efficacy was attributed to the longer blood circulation and the 10 fold higher tumour PTX uptake once tethered to SWNT. As expected the systemic administration of the SWNT-PTX lead to RES organ uptake, however the authors claimed no tissue necrosis of liver and spleen due to the rapid release and excretion of the PTX. Cheng et al. [114] have built a targeted delivery system based on a taxoid prodrug model that becomes active only with biotin expressing cancer cells. The biotin targeted SWNT-Taxoid construct was designed based on disulfide linker chemistry in which the disulfide bonds are cleaved by endogenous thiols (e.g. glutathione and thioredoxin).

Initially the capacity of the designed MWNT-MTX conjugates to translocate into mammalian cells was investigated using fluorescently labelled MWNT-MTX constructs. Similar cellular uptake to MWNT alone was observed once the MTX was tethered to MWNT as shown by the diffuse rhodamine B signal throughout the cytoplasm (Figure 6.2.2). Previous studies had shown that MWNT were uptaken independently of the cell type and the functional group that they have on their surface [24]. It is well documented that MTX uptake alone is mediated via folate receptors and reduced folate carriers [189]. In addition, one of the reasons behind the reduced cellular uptake of MTX is attributed to its impaired transport and cellular resistance [185]. For this reason the conjugation of MTX to MWNT was thought to be a prerequisite in an attempt to divert the MTX receptor mediated cellular uptake to a non-receptor mediated described with CNT [24, 100, 161]. This work did not investigate in details the mechanism of uptake for these MWNT-MTX conjugates but this is warranted for future studies.
It was therefore demonstrated that the activity of the MWNT-MTX is dependent on the type of the linker used. As shown in Figure 6.2.3 the MWNT-MTX with the peptidase linker showed a clear time dependence activity with a cytotoxic killing activity starting 6 hrs after incubation. The high and statistically significant cell death with this construct is attributed to the efficient enzymatic hydrolysis of the drug tethered by cleavable peptide inside the cells in comparison with the stable ester bond [112]. Moreover, the spatial distance offered with the peptidase sensitive linker can further explain the ease in triggering the release of the drug compared to the presence of an ester bond. In addition, these results suggested that the attachment of MTX to MWNT offered an enhanced cellular internalisation of MTX by probably diverting it from the receptor mediated uptake into a direct cytoplasmic delivery once tethered to MWNT as suggested by the similar cellular uptake profiles with the rhodamine B labelled MWNT-MTX conjugates (Figure 6.2.2 and APPENDIX IX). Another reason for the efficient cellular uptake of the MWNT-MTX constructs may be their enhanced affinity to interact with the cell membrane via hydrophobic interactions due to the greater lipophilicity of these linkers. Interestingly the cytotoxic activity observed with the MWNT-MTX constructs is not attributed to the non-selective toxicity from the MWNT alone as cells treated with these were 100 % viable (Figure 6.2.3).

The therapeutic efficacy of the MWNT-MTX conjugates was then studied in vivo using intratumoral injections into prostate DU145 xenograft tumour model. Suppression of the tumour volume after treatment with the MWNT-MTX conjugates with the peptidase sensitive linker was observed which is thought to be due to the controlled release of MTX by enzymatic cleavage once uptaken by tumour cells. However, no statistical significance was observed with the CNT-MTX with the
peptidase sensitive linker compared to the CNT-MTX without a cleavable linker.

This can be due to the slow growth of the animal tumour model used which might have masked the therapeutic effect of the cleavable conjugates.
6.3. CONCLUSION

This chapter described the development of MWNT conjugates carrying anticancer carrying two anticancer drugs (Dox and MTX) based on two functionalisation methodologies. The first strategy was based on the non-covalent loading of Dox molecules to MWNT. The second methodology was based on the chemical conjugation of methotrexate to MWNT through cleavable linkers to facilitate the release of the drug once inside the cells.

It was found that doxorubicin form supramolecular complexes with MWNT: F127 due to π-π stacking. The biological activity of Dox was maintained in vitro and in vivo; however it was just similar to the drug alone. In addition, the chemically functionalised MWNT increased the survival of mice compared to its Pluronic coated counterpart. The effective release of Dox from MWNT seemed to be an issue which is thought to be one of the reasons behind the non-enhancement in the therapeutic activity observed with the MWNT: Dox complexes.

In a different strategy, the design of chemically functionalised MWNT – MTX constructs was achieved using cleavable linkers with the potential for controlled release of the drug. It was found that the activity of methotrexate strongly depended on the type of the cleavable linker used. While the non-cleavably linked MWNT-MTX construct didn’t show any cytotoxic activity against MCF7 cells as previously shown [22], the highest activity was observed using an enzyme sensitive peptide linker followed by the esterase sensitive construct. A diversion in the cellular uptake mechanism of MTX was expected once tethered to the CNT as seen by the similar diffuse signal between the MWNT and MWNT-MTX constructs. However the intratumoral injections of MWNT-MTX into DU145 tumour models did not
cause a statistically significant improvement in the therapeutic activity of Dox compared to the drug alone or the MWNT-MTX without cleavable linker.

Table 6.2.2 below shows a short comparison between the two strategies used for the attachment of anticancer drugs to carbon nanotubes.

<table>
<thead>
<tr>
<th></th>
<th>Non-Covalent</th>
<th>Covalent</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Preparation</strong></td>
<td>Straight forward complexation</td>
<td>Requires chemistry</td>
</tr>
<tr>
<td><strong>Dispersion</strong></td>
<td>Excellent</td>
<td>Good</td>
</tr>
<tr>
<td><strong>Type of drug</strong></td>
<td>Only for polycyclic molecules</td>
<td>Any type of molecule</td>
</tr>
<tr>
<td><strong>Controlled release</strong></td>
<td>No</td>
<td>Yes, prodrug system</td>
</tr>
<tr>
<td><strong>Intratumoral injections</strong></td>
<td>Ok</td>
<td>Ok</td>
</tr>
<tr>
<td><strong>Intravenous injections</strong></td>
<td>Not ready for it yet due RES organ uptake</td>
<td>Not ready for it yet due RES organ uptake</td>
</tr>
</tbody>
</table>

Table 6.2.6: Comparison between the non-covalent and covalent functionalisation methodologies used for the preparation of effective anticancer drug delivery systems.

The main advantage of using the first strategy is its simplest nature which is based on simple complexation between the MWNT and the anticancer drug as a result of non-covalent interactions. This approach suffers from the disadvantages that it can only be used with molecules containing aromatic rings in addition to the limited control one would have on the release of the drug from the MWNT. On the other hands, the second approach requires complicated chemistry but at the same time a wider spectrum of drugs can be used but the avoidance of polycyclic ring molecules
is required to circumvent any non-specific binding with the CNT surface due to \( \pi-\pi \) stacking. In addition, using this chemical approach the controlled release of the drug can be achieved by introducing cleavable linkers.
CHAPTER VII

SUMMARY AND FUTURE WORK

7.1. MAIN LESSONS AND MESSAGES FROM THIS THESIS

The work in this thesis explored key factors that affect the pharmacokinetics and toxicity of carbon nanotubes and attempted to develop CNT-based constructs as cancer therapeutics. Some key issues in those two important aspects of biomedical research were addressed and the main lessons and messages revealed from this work are highlighted below:

Lesson 1: Chemical functionalisation, the type and filling of CNT, all determine the dispersibility that plays an important role in CNT biodistribution and pharmacokinetics

Dispersibility is an important determinant factor in CNT biodistribution and was found to be highly dependent on the type of chemical functionalisation that provides well individualised CNT (Chapter IV). In this work we illustrate that aryl functionalisation led to the formation of CNT aggregates with different sizes which allowed their accumulation in the lungs and RES organs (Section 4.1). The filling of SWNT might have also affected the dispersibility properties whereby liver and spleen accumulation were observed with the non-filled SWNT compared to only lungs with the filled construct (Section 4.2). In addition, the type of CNT (SWNT or MWNT) might have also played role in the biodistribution profile. SWNT were found to be more difficult to functionalise compared to MWNT and they were found predominantly in bundles which could also affect their biodistribution and
pharmacokinetics profiles. Since the work described in Chapter IV (Section 4.2) is thought to be the first biodistribution study with filled nanotubes, more work is warranted in this area.

![Diagram](image)

Scheme 7.1: A representation of the factors affecting CNT dispersibility and individualisation and subsequently the biodistribution profile of CNT.

**Lesson 2:** The intravenous administration of CNT for therapeutic purposes should be avoided until the pharmacokinetics and the distribution profile of CNT vectors are elucidated

It was found that most CNT types studied in this thesis (aryl, sugar functionalised, amidated and F127 coated MWNT) that represent a broad spectrum of CNT types used in this field, accumulated at different levels in liver, spleen and also lungs (Chapter IV). Although no necrosis was observed in those tissues within the time frame of our experiments, the use of CNT as delivery vectors for systemic therapeutic and diagnostic purposes is hindered. CNT should be further engineered to achieve well-described biodistribution profiles and longer blood circulation half
lives before intravenous injections of CNT for therapeutic purposes can be carried out.

**Lesson 3: Chemically functionalised CNT should attain more attention for use in biomedical applications**

In terms of both biodistribution and cytotoxicity, chemical functionalisation of CNT has proven to be favourable to non-covalent functionalisation (coating with copolymers). The F127 copolymer coated MWNT caused significant cell death compared to ammonium functionalised MWNT (Chapter V, Section 5.1). MWNT-NH$_3^+$ can therefore provide a valuable platform for further biomedical applications as delivery systems.

In terms of asbestos like pathogenicity it was found that a chemical functionalisation that shortens the MWNT is desirable compared to a different one that maintains their length. No inflammatory reaction or granuloma formation was observed with short MWNT of less than 5 μm in length (either functionalised with TEG or commercially available pristine (Chapter V, Section 5.2). So chemical functionalisation can alleviate the pathogenicity risks associated with long pristine MWNT.

**Lesson 4: CNT interference with well established cytotoxicity assays is a two level interaction.**

The accurate assessment of CNT cytotoxicity *in vitro* has been difficult due to the interference of CNT with the well established cytotoxicity assays. The most likely interference is due to the physisorption of the marker molecules used in those assays onto the CNT surface through Van der Waals forces leading to an overall
decrease in the absorbance and hence false positive results as shown with the MTT assay (Chapter V, Section 5.1). However, false high cell viability can also occur due to the intrinsic capability of CNT to absorb light as observed with the original LDH assay. This phenomenon of false negatives can also be observed when high cellular uptake of CNT occurs as was seen with the MWNT-NH$_3^+$ in the MTT assay. Based on the pitfalls found with those assays for use with CNT, we have developed a reliable cytotoxicity assay was achieved by modifying the LDH assay through the introduction of centrifugation step to remove the uptaken CNT and therefore minimize the interaction between the marker molecules and the CNT.

Lesson 5: CNT can form supramolecular complexes with polycyclic aromatic molecules that maintain their biological activity

The aromatic backbone structure of CNT allowed for the formation of supramolecular complexes with doxorubicin chromophore which is known for its self- and hetero-association with various planar aromatic compounds (Chapter VI, Section 6.1). This simple methodology can also be implemented with other aromatic molecules; however the efficient release of the molecules from the CNT surface remains the main issue with this approach.

Scheme 7.2: A representation of doxorubicin stacking onto the surface of MWNT leading to star like complexes as shown by the TEM image. Adopted from reference [14].
Lesson 6: Chemically functionalised CNT can be derivatized with anticancer drugs for the use as cancer therapeutics.

In contrast to the non-covalent approach described previously which is only possible with poly-aromatic molecules, chemically functionalised CNT can be conjugated with a wider range of therapeutic molecules. Controlled release of the drugs can be achieved with this approach as cleavable linkers were introduced between the drug molecule and the CNT functional group as was observed with the CNT-MTX constructs described in Chapter VI, Section 6.2.

![Scheme 7.3: A representation of CNT-MTX construct with the presence of a cleavable linker and the fluorescent probe (Rhodamine B). The construct translocated throughout the cytoplasm of the cells as shown by the red rhodamine signal. Adapted from reference [112].](image)

Lesson 7: CNT can be filled with radiometals and used for therapeutic and diagnostic (theranostic) purposes.

The successful encapsulation of iodine inside SWNT was achieved and selective accumulation in the lungs was observed with sugar functionalised and filled SWNT construct (Chapter IV, Section 4.2). Interestingly, no leakage of the radiometal was observed even after 7 days post-injection. The same methodology can be used with other diagnostic and therapeutic molecules.
7.2. FUTURE WORK

One of the main advantages that CNT hold is their ability of escaping the endosomal compartment and allowing for direct cytoplasmic translocation which is considered of extreme importance when proteins and nucleic acids are to be delivered. In fact, CNT has already shown to be better than the existing lipid based drug delivery systems (liposomes) in the only in vivo published study that compared CNT to liposomes. The intratumoral injections of MWNT-NH$_3^+$ complexed with a cytotoxic siRNA sequence prolonged the survival of tumour bearing mice and dramatically reduced the size of the tumours compared to siRNA alone and cationic liposome: siRNA complexes [43].

It should be mentioned that although CNT hold great promises as diagnostic and therapeutic modalities, their real value could only be argued when direct comparative studies are conducted against existing nanoparticles (e.g. iron oxide, dendrimers and polymers) but also their toxicological profile should be carefully evaluated and the risk-to-benefit ratio cautiously weighed before they can translate into effective pharmaceutical products and before they can be considered better than other nanomaterials.

Currently, direct comparative studies against ‘benchmark’ and clinically used drug delivery systems are lacking because CNT are in their early stages of development; and hence direct comparison with other state-of-the-art delivery systems is hard to make without published data. However their physical properties such as absorbing NIR can be combined with the attachment and/or filling with therapeutic molecules and used for cancer therapy.
Therefore, future studies will aim to explore the fundamental competitive advantages offered by CNT over other delivery systems in terms of the large surface area available for conjugation with different functionalities (therapy, imaging, targeting) and the inner nanotube volume that can be also filled with molecules (e.g. radiometals for imaging or therapy) and this will be directly compared to other ‘benchmark’ nanomaterials.

While, the therapeutic efficacy of the MWNT-MTX is to be re-evaluated using different tumour models, further work will focus on the tailoring of CNT for systemic administration so the real therapeutic efficacy of CNT: Dox complexes and CNT-MTX constructs can be observed once they are intravenously injected. Focus will be on engineering CNT to avoid RES organ uptake and instead have long circulation in the bloodstream to reach their target cancer tissue. The PEGylation of CNT to provide long circulation in the blood will be explored, however careful design of PEGylated nanotubes should be considered especially that PEGylation would affect the cellular uptake of the CNT similar to what was observed with Pluronic coated CNT. Other future work will focus in exploring the interaction between CNT and blood component especially plasma proteins ex-vivo in order to add to the current knowledge about CNT biodistribution and to evaluate whether plasma protein coating of the CNT would affect the pharmacokinetics of CNT and change their surface characteristics especially that protein corona is thought to affect the biodistribution of other nanoparticles [190]. Once the careful engineering of CNT for systemic administration is achieved, the introduction of targeting ligands (monoclonal antibodies, folic acid) will be attempted. Active tumour targeting will play an important role for the effective delivery of therapeutic cargoes to their site of action.
In addition, to take the work with the iodine filled SWNT further, the use of SWNT filled with therapeutic iodine (I\(^{131}\)) will be investigated for the treatment of lung metastasis based on the fact that iodine filled SWNT showed selective deposition onto the lungs.

In addition, careful evaluation of the deeper penetration of CNT within avascular tumor models is deemed necessary for the engineering of effective CNT-based cancer therapeutics especially that anticancer drugs and even delivery systems exhibit poor therapeutic indices due to their limited diffusion in solid tumours as a result of the elevated interstitial pressure, hypoxia, cell packing and the extracellular matrix (ECM) [191-193]. Multicellular tumour spheroids represent a 3D model of avascular regions found in many solid tumour tissues. They have extensive cell-cell contacts, elevated interstitial pressure, hypoxia and the presence of quiescent cells [194]. Because of the essential knowledge that can be generated from the use of tumour spheroids, they will be used as a bridge between traditional planar cell studies and the \textit{in vivo} assessment of the delivered therapeutics. I have already conducted some preliminary work with tumour spheroids and CNT precursors (non-therapeutic) (\text{APPENDIX IX}) and MCF7 spheroids were prepared using the liquid overlay method [192, 195]. CNT-NH\(_3^+\) are shown to interact with the spheroids, however their exact localisation within the spheroid requires more specialised and powerful techniques other than optical microscopy and this will be explored further in attempt to deepen the knowledge about CNT interaction with cells and will be the bridge between the \textit{in vitro} and \textit{in vivo} work

Moreover, to have a better knowledge about the effect of functional groups on the pathogenicity associated with long pristine MWNT, another toxicological
paradigm should be tested that takes into consideration the effect of the surface chemistry. For instance, an ammonium functionalised MWNT will be compared to carboxylated nanotubes and a combination of ammonium and carboxylated MWNT in an attempt to conclude on the effect of surface functionalisation on the pathogenicity associated with long pristine MWNT; however the length distribution of the MWNT should be kept the same as the pristine counterparts to avoid multiple parameter effects.
APPENDIX I: Biodistribution profile of $^{[111m]}$In DTPA-amidated-MWNT

(A) SPECT/CT fused images of the whole mouse. Images were taken at 30 min, 4 hrs and 24hrs post-injection of 50μg of $^{[111m]}$In DTPA-MWNT (See inset for structure) with a scanning time of 40-60min. (B) The percentage injected dose per gram tissues (%ID/g) at 24 hrs after injection, quantified by gamma scintigraphy. Data were expressed as means ± SD (n=3-4).
**APPENDIX II:** Tissue histology after intravenous administration of non-filled GlcNAc^0@SWNT

Hematoxylin and eosin-stained sections of lung, liver, spleen at 24 hrs post-injection with non-filled GlcNAc^0@SWNT (50µg) or vehicle alone (0.5% BSA in PBS). All photomicrographs were captured at 40x magnification.
APPENDIX III: Dot plots analysis using the Annexin V-FITC /PI assay

A549 cells were treated with (A) MWNT-NH$_3$, (B) MWNT: F127 and (C) Pluronic F127 alone. Cationic liposomes were used as positive control of cytotoxicity and are shown in the 2nd dot plot in (A) and (B). The four-quadrant dot depicts live non-apoptotic cells in the R5 region (Annexin -/PI -), early apoptosis in the R6 region (Annexin +/PI -), late apoptosis (Annexin +/PI +), and cell debris (Annexin -/PI +).
**APPENDIX IV:** Assessment of the inflammatory reaction after intraperitoneal injection of the commercially available pristine MWNT and its carboxylated counterpart.

Female C57Bl/6 mice were intraperitoneally injected with 50µg of NanoAmorph pristine MWNT (from Nanostructured and amorphous materials.Inc) and their carboxylated counterparts; long-fibre amosites (LFA) as positive control and vehicle control (0.5% BSA/saline). Inflammatory response was evaluated by (A) the total polymorphonuclear leukocytes (PMN) and (B) total protein (protein exudation) after 24 hrs. Data represent the mean of 4 animals ± STDV.

![Chart A](image1.png)

![Chart B](image2.png)
APPENDIX V: The cytotoxicity assessment of MWNT: Dox complexes using the modified LDH assay.

A549 cells were treated with Dox alone (600 nM); MWNT (651.5 ng/ml); Pluronic (6.5 ng/ml) and Pluronic: Dox and MWNT: Dox at a mass ratio of 2:1. Untreated cells were used as control. Two types of MWNT were assessed; MWNT: F127 and MWNT-NH$_3^+$. 

![Graph showing percentage cell survival for different treatments.](image-url)
APPENDIX VI: Tissue histology after treatment with MWNT: Dox complexes

Hematoxylin and eosin-stained sections of lung, liver, spleen and heart of mice after the treatment with MWNT: Dox complexes. No accumulation of MWNT in tissue or apoptosis is observed. (Photomicrographs were taken at 10 x magnification).
APPENDIX VII: B16F10 cell line sensitivity to doxorubicin treatment

B16 F10 cells were treated with a range of doxorubicin concentrations (600-9600 nM) for 24 hrs and the percentage cell survival was assessed by the modified LDH assay. A dose dependent cytotoxicity was observed, which indicates that B16F10 cells are sensitive to doxorubicin.
APPENDIX VIII: The release profile of MWNT: Dox complexes at different pH

Buffers used are (A) PBS –pH 7.4; (B) Acetate buffer-pH 4; (C) Tris buffer-pH 9.

No pH dependent release of Dox from the MWNT: Dox complexes was observed.
APPENDIX IX: Cellular uptake of rhodamine B labelled MWNT with and without MTX

Confocal microscopy images of MCF7 cells incubated in the absence (naive) and the presence of 10 μg/ml of MWNT with and without MTX. Similar uptake pattern is observed with both MWNT alone and MWNT-MTX constructs after 3 hrs (top panel) and 24 hrs (bottom panel).
APPENDIX X: MCF-7, DU145 and B16 F10 cell line sensitivity to methotrexate treatment

MCF-7, DU145 and B16 F10 cells were treated with a range of MTX concentrations (0.1-1000 μM) for 24 hrs and the percentage cell survival was assessed by the modified LDH assay. B16F10 cells seemed very responsive to MTX, while DU145 and MCF7 cells showed no statistical significant difference (p > 0.05).
APPENDIX XI: The interaction of CNT-NH$_3^+$ with MCF7 tumour spheroids

Untreated MCF7 Spheroids

Treated with CNT-NH$_3^+$
## APPENDIX XII: Sources of the different CNT materials used in this thesis

<table>
<thead>
<tr>
<th>Section</th>
<th>CNT mononclature used</th>
<th>Functionalisation</th>
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BIBLIOGRAPHY


243


