New Functionalised Carbon Based Nanomaterials for Biomedical Imaging Applications

Haobo Ge

A thesis submitted for the degree of Doctor of Philosophy
University of Bath
Department of Chemistry
Jun 2015

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List of published articles during PhD


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Abstract

**Chapter one** includes the literature overview of research relevance to this project, consisting of three distinct parts: design and application of fluorescence sensors, purification, functionalisation and application of Single Walled carbon Nanotubes (SWNTs), and applications of functionalised SWNTs in cancer research.

**Chapter two** reveals the synthesis of different fluorescence sensors. This chapter also explains how different parts of the functional groups may be assembled together and the optimisations on the synthesis of fluorophores. Binding constant determination and quantum yield calculation for each fluorescence sensors are being discussed.

**Chapter three** presents the preparation of SWNTs used were for the synthesis of new SWNTs based hybrids. SWNTs are purified *via* a solvent oxidation method, which was adapted here from literature reports. SWNTs were then functionalised either on their external surface by β-D-glucan wrapping to form a scaffold structure for further SWNTs hybrids assembly, or inside their cavities, by filling with small molecules or metals, in order to establish a protocol to filling radiolabelled metals in the future. Finally, all SWNTs were characterised *via* TEM, SEM and Raman prior to further applications.

**Chapter four** describes *in vitro* cell cytotoxicity studies of fluorescence sensors synthesised from the chapter 2 and SWNTs prepared from the chapter 3. Several different cell lines (HeLa, PC-3and CHO) were incubated with each of compounds, which were available as synthesised from chapter 2 and SWNTs (SWNTs hybrids) assembled in chapter at concentrations for 48 hours. Finally MTT assays were carried out to understand the cytotoxicity effects in functionalised and non-functionalised system.

**Chapter five** examines the applications of fluorescent sensors built as discribed in chapter 2 and SWNTs hybrids (Fluorescent sensors@ β-D-glucan@SWNTs) from
chapter 3 under the confocal microscopy and FLIM with different environment conditions, including consideration of concentration, temperature, laser power, time as well as a variety of cell lines (HeLa, PC-3 and CHO).

**Chapter six** contains all experimental details of the work described in this thesis.

**Chapter seven** summaries current and future work emerging from this project.
Acknowledgements

I would like to thank my supervisor for giving me the opportunity to do my PhD in her group, and providing selfless support, guidance and inspiration throughout my PhD. Thanks must also be extended to my best friend, Dr Rory Arrowsmith, who has helped me to get started with cell culture and imaging techniques, my PhD would be much tougher without his assistance and inspiration. I also acknowledge Dr Zhiyuan Hu for initial training of some of the methods used widely by all those in Pascu’s group working in carbon nanotubes for the purification and functionalisation of SWNTs and which originated in the Oxford Nanotube Group. I also send my appreciation to my current group members, especially Dr Vincenzo Mirabello, who is an expert in synthetic chemistry and spectroscopic techniques. He offered me his invaluable support throughout my PhD. Dr David Gonzalez Calatayud and Mr Boyang Mao for their assistant in TEM, SEM and Raman spectroscopy acquisition and assignment.

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Last but not the least, special thanks goes to my family, who have always had confidence in me and provided me with selfless care. It is their support which has

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enabled me to pursue this PhD I am keen on. Completing a PhD is not easy. Because of these lovely people, I found it rewarding.
# Abbreviations

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<tbody>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>1D</td>
<td>one-dimensional</td>
</tr>
<tr>
<td>2D</td>
<td>two-dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>three-dimensional</td>
</tr>
<tr>
<td>A</td>
<td>absorbance</td>
</tr>
<tr>
<td>Å</td>
<td>Angstrom</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>Ar</td>
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</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BBB</td>
<td>blood brain barriers</td>
</tr>
<tr>
<td>Bodipy</td>
<td>boron-dipyrromethane</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CCD</td>
<td>charge-coupled device</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese Hamster Ovarian</td>
</tr>
<tr>
<td>CIS</td>
<td>carcinoma in situ</td>
</tr>
<tr>
<td>cm</td>
<td>centimetre</td>
</tr>
<tr>
<td>COSY</td>
<td>correlation spectroscopy</td>
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<td>CT</td>
<td>X-ray computed tomography</td>
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<tr>
<td>CVD</td>
<td>chemical vapour deposition</td>
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<tr>
<td>cyt c</td>
<td>cytochrome complex</td>
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<tr>
<td>DCC</td>
<td>N, N-Dicyclohexylcarbodiimide</td>
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<td>DCM</td>
<td>dichloromethane</td>
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<td>DDS</td>
<td>drug delivery system</td>
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<td>DIC</td>
<td>differential interference contrast</td>
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<tr>
<td>DMEM</td>
<td>whilst Dulbecco’s Modified Eagle’s Medium</td>
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<td>N,N’-dimethylformamide</td>
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<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>DWNTs</td>
<td>double walled carbon nanotube</td>
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<tr>
<td>EDS</td>
<td>Energy-dispersive X-ray spectroscopy</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<td>EMEM</td>
<td>Eagle’s Minimum Essential Medium</td>
</tr>
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<td>eq</td>
<td>equivalents</td>
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<td>Electrospray ionization</td>
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<td>FCS</td>
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<td>fluorescein isothiocyanate</td>
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<td>FLIM</td>
<td>Fluorescence-lifetime imaging microscopy</td>
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<td>f-SWNTs</td>
<td>functionalised SWNTs</td>
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<td>FWHM</td>
<td>full width at half maximum</td>
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<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>h</td>
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<td>HBSS</td>
<td>Hank’s solution</td>
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<td>High performance liquid chromatography</td>
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<td>Hz</td>
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<td>ICT</td>
<td>Intra-molecular Charge Transfer</td>
</tr>
<tr>
<td>K</td>
<td>Kelvin</td>
</tr>
<tr>
<td>k</td>
<td>kilo / equilibrium constant</td>
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<tr>
<td>K_b</td>
<td>binding constant</td>
</tr>
<tr>
<td>L</td>
<td>litre</td>
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<tr>
<td>LC</td>
<td>Liquid Chromatography</td>
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<tr>
<td>m</td>
<td>milli / meter</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>------------</td>
</tr>
<tr>
<td>M</td>
<td>molar / mega</td>
</tr>
<tr>
<td>m/z</td>
<td>mass-to-charge ratio</td>
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<tr>
<td>Me</td>
<td>methyl</td>
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<td>minute</td>
</tr>
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<td>mol</td>
<td>mole</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
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<tr>
<td>MTT</td>
<td>3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>MWNT</td>
<td>multi-walled carbon nanotube</td>
</tr>
<tr>
<td>n</td>
<td>nano</td>
</tr>
<tr>
<td>NADP</td>
<td>nicotineamido adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotineamido adenine dinucleotide phosphate reduced form</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>NIR</td>
<td>near infrared</td>
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<td>NMR</td>
<td>Nuclear magnetic resonance spectroscopy</td>
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<td>PA</td>
<td>photoacoustic imaging</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer saline</td>
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<td>PDT</td>
<td>Photodynamic therapy</td>
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<td>Polyethylene glycol</td>
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<td>photoluminescence spectroscopy</td>
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<td>PET</td>
<td>Photo-induced Electron Transfer</td>
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<td>-log10[H+]</td>
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<td>Ph</td>
<td>phenyl</td>
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<td>PMT</td>
<td>photomultiplier tube</td>
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<td>ppm</td>
<td>parts per million</td>
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<tr>
<td>PTP</td>
<td>permeability transition pore</td>
</tr>
<tr>
<td>QD</td>
<td>quantum dots</td>
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<tr>
<td>RBM</td>
<td>radial breathing mode</td>
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<tr>
<td>ROS</td>
<td>reactive oxidative species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>Rt</td>
<td>retention time</td>
</tr>
<tr>
<td>rt</td>
<td>room temperature</td>
</tr>
<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscope</td>
</tr>
<tr>
<td>SFM</td>
<td>serum free medium</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutases</td>
</tr>
<tr>
<td>SPECT</td>
<td>Single-photon emission computed tomography</td>
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<tr>
<td>STM</td>
<td>Scanning tunnelling microscopy</td>
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<tr>
<td>SWNTs</td>
<td>Single Walled Carbon Nanotubes</td>
</tr>
<tr>
<td>t1/2</td>
<td>half life</td>
</tr>
<tr>
<td>t-Boc</td>
<td>tert-butyloxycarbonyl</td>
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<tr>
<td>TCSPC</td>
<td>Time-correlated single-photon counting</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscope</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
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<tr>
<td>TGA/DTG</td>
<td>Thermal analysis</td>
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<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
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<td>TLC</td>
<td>Thin layer Chromatography</td>
</tr>
<tr>
<td>US</td>
<td>ultrasound</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>Ultraviolet visible spectroscopy</td>
</tr>
<tr>
<td>XPS</td>
<td>X-ray photoelectron spectroscopy</td>
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</tbody>
</table>
XRD X-ray diffraction
δ chemical shift
τ fluorescence lifetime
τ₁ major component of fluorescence lifetime
τ₂ minor component of fluorescence lifetime
τₘ weighted average of fluorescence lifetime components
Φ quantum yield
ε molar absorption coefficient
λ wavelength
λₑₓ excitation wavelength
λₘₐₓ maximum wavelength of excitation
μ micro
Comprehensive list

Compounds synthesised in this PhD

1. 2,5-dioxopyrrolidin-1-yl 5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanoate
   Chemical Formula: C_{14}H_{19}N_{3}O_{5}S
   Molecular Weight: 341.38

2. N-(6-aminohexyl)-5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide
   Chemical Formula: C_{18}H_{30}N_{4}O_{2}S
   Molecular Weight: 342.50

3. 5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-N-(6-((4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)amino)hexyl)pentanamide
   Chemical Formula: C_{29}H_{47}BN_{4}O_{3}S
   Molecular Weight: 558.59
Compounds list

2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-4-(3-(6-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)hexyl)-3-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)thiocarbamoyl)benzoic acid
Chemical Formula: C_{59}H_{58}BN_{7}O_{9}S_{2}
Molecular Weight: 947.97

tert-butyl (4-((5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)methyl)benzyl)carbamate
Chemical Formula: C_{23}H_{34}N_{4}O_{4}S
Molecular Weight: 462.61

5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-N-((4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)amino)methyl)benzyl)pentanamide
Chemical Formula: C_{31}H_{48}BN_{4}O_{4}S
Molecular Weight: 578.58

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Compounds list

2-(6-hydroxy-3-oxo-3H-xanthene-9-yl)-4-(3-(4-((5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)methyl)benzyl)-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)thioureido)benzoic acid
Chemical Formula: C_{52}H_{56}BN_{5}O_{9}S_{2}
Molecular Weight: 967.96

4-(3-(4-((tert-butoxycarbonyl)amino)methyl)benzyl)thioureido)-2-(6-hydroxy-3-oxo-3H-xanthene-9-yl)benzoic acid
Chemical Formula: C_{34}H_{37}N_{3}O_{5}S
Molecular Weight: 625.70

XVII
Compounds list

2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-4-(3-(3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)methyl)benzyl)thioureido)benzoic acid
Chemical Formula: C₃₉H₃₇N₅O₇S₂
Molecular Weight: 751.87

4-(tetramethyl-1,3,2-dioxaborolan-2-yl)benzaldehyde
Chemical Formula: C₁₃H₁₇BO₃
Molecular Weight: 232.08600

tert-butyl N-(4-[[4-(tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl]methyl]amino)methyl[phenyl]methyl)carbamate
Chemical Formula: C₂₃H₂₇BN₂O₄
Molecular Weight: 452.40200
Compounds list

4-(((4-(((tert-buty lamino)methyl)phenyl)methyl)(4-(tetram ethyl-1,3,2-dioxaborolan-2-yl)phenyl)methyl)carbamothioyl)amino)-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid
Chemical Formula: C_{47}H_{48}BN_{3}O_{9}S
Molecular Weight: 841.78300

4-((5,5-difluoro-1,3,7,9-tetramethy l-5H-4,4',5,5',dipyrido[1,2-c:2',1':f][1,3,2]diazaborinin-10-yl)-N-(4-(5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)methyl)benzyl)-N-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)benzamide
Chemical Formula: C_{53}H_{60}B_{2}F_{2}N_{6}O_{5}S
Molecular Weight: 928.75

XIX
Compounds list

2-(6-(diethylamino)-3-(diethyliminio)-3H-xanthen-9-yl)-4-(3-(4-(6-(DNA)pentanamido)methyl)benzyl)-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzylthiourea benzoate
Chemical Formula: C₉₀H₇₂BN₂O₂S₂
Molecular Weight: 1078.21

2-oxo-N-(4-((5-((3aS,4S,6aR)-2-oxoexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)methyl)benzyl)-N-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)-2H-chromene-3-carboxamide
Chemical Formula: C₄₁H₅₁BN₄O₇S
Molecular Weight: 750.72
Library of known compounds available from SIP group and used to establish MTT assays protocols (compound 16-17 were provided by Prof. Golzar Hossain, from University of Dhaka in Bangladesh and compound 18-19 provided by Dr. Rory Arrowsmith (Pascu group, University of Bath))

16a
1,3-bis(pyridine-3-carbonyl)thiourea; 1-(pyridin-3-yl)-3-(pyridine-3-carbonyl)thiourea; 3-benzoyl-1-(2-chlorophenyl)thiourea; 3-benzoyl-1-(2-fluorophenyl)thiourea; 3-benzoyl-1-(pyridin-3-yl)thiourea

16b
1-(pyridin-3-yl)-3-(pyridine-3-carbonyl)thiourea; 3-benzoyl-1-(2-chlorophenyl)thiourea; 3-benzoyl-1-(2-fluorophenyl)thiourea; 3-benzoyl-1-(pyridin-3-yl)thiourea

16c
1-(pyridin-3-yl)-3-(pyridine-3-carbonyl)thiourea; 3-benzoyl-1-(2-chlorophenyl)thiourea; 3-benzoyl-1-(2-fluorophenyl)thiourea; 3-benzoyl-1-(pyridin-3-yl)thiourea

17a
1-(pyridin-3-yl)-3-(pyridine-3-carbonyl)thiourea; 3-benzoyl-1-(2-chlorophenyl)thiourea; 3-benzoyl-1-(2-fluorophenyl)thiourea; 3-benzoyl-1-(pyridin-3-yl)thiourea

17b
1-(pyridin-3-yl)-3-(pyridine-3-carbonyl)thiourea; 3-benzoyl-1-(2-chlorophenyl)thiourea; 3-benzoyl-1-(2-fluorophenyl)thiourea; 3-benzoyl-1-(pyridin-3-yl)thiourea
Compounds list

XXII

Zinc(II) bis(4-ethyl-3-thiosemicarbazone) acenaphthenequinone

Copper(II) bis(4-ethyl-3-thiosemicarbazone) acenaphthenequinone

Zinc(II) 4-ethyl-3-thiosemicarbazone-thiocarbohydrazide acenaphthenequinone

Zinc(II) bis(4-phenyl-3-thiosemicarbazone) acenaphthenequinone

Copper(II) bis(4-phenyl-3-thiosemicarbazone) acenaphthenequinone

Zinc(II) 4-phenyl-3-thiosemicarbazone-thiocarbohydrazide acenaphthenequinone
Known compounds (Dyes) commercially available from STFC Harwell and SIP group, used for the colocalisation work in confocal microscopy:

- **Hoechst 33342**
- **MitoTracker Red FM**
- **MitoTracker Green FM**
- **LysoHunt Red DND-99**
- **LysoSensor™ Yellow/Blue DND-160**
Known compounds (dyes) available from SIP and TDJ groups and used in the confocal imaging work to compare with newly synthesised probes as benchmarks: (Compound 24, 25 was provided by Dr. Sabrina Wang, compound 26 was provided by Dr. Stephen Flower, compound 27 was provided by Dr. Meng Li form Tony James group)
Compounds list

2-[6-(diethylamino)-3-(diethyliminiumylyl)-3H-xanthen-9-yl]-5-[[3-(dihydroxyboranyl)phenyl]carbamothioyl]amino]benzoate

8-(ethylamino)-3-[(E)-[2-(tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl]methylidene]amino]-3-azatricyclo[7.3.1.0^{3,13}]trideca-1(13),5,7,9,11-pentaene-2,4-dione

XXIV
Chapter 1. Introduction

1.1. Aims of the project and context

Cancer is one of the top ten leading causes of death in the world. According to a recent cancer statistics report from the World Health Organisation (WHO), 14.1 million adults in the world were diagnosed as cancer and 8.2 million (58.16%) deaths occurred as a result of cancer, in the world in 2012.¹ In the UK, cancer remains in the top three common causes of death. In 2011, there were 331,487 people diagnosed with cancer and 159,178 deaths (48.02%) from cancer in the UK (Cancer Research UK). Another statistic showed that 70% of cancer patients had survived in the first year after diagnosis. Then this number reduced to 54.3% in the period of five years after diagnosis. Finally, amongst these only 49.8% of cancer patients had survived in ten years time.²

Table 1. Five year survival rates in colorectal cancer by stage at diagnosis (1995-2005)³

<table>
<thead>
<tr>
<th>Stage at diagnosis</th>
<th>5-year survival rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>In early stage (local)</td>
<td>90</td>
</tr>
<tr>
<td>Spread to adjacent organ / lymph node</td>
<td>67</td>
</tr>
<tr>
<td>Spread to distant sites</td>
<td>10</td>
</tr>
</tbody>
</table>

This survival rate was improved by an early diagnosis: 90% of patients had survived in five years when cancer was detected at the early stage (Table 1.1). Once tumour cells spread to adjacent tissue, i.e. middle stage, this rate dropped to 67%. Finally, only 10% of cancer patients survived when diagnosed at the metastatic stages. This was particularly the case with the cancer of lung, and another report from SEER cancer statistics review showed that the earlier stage tumour was diagnosed, the higher survival rate for this it would be.⁴ As it can be seen in Figure 1.1, only 15% of lung cancer could be diagnosed at localised stage, but 54% of patients survived. When the incidence of cancer was discovered at the spreading stage (22%), the survival rate dropped to 26%. However, the majority of patients were diagnosed at late stages, hence,
only 4% survived at the five years’ milestone. Thus, it can be concluded that early cancer diagnosis as equally important as rapid, effected and targeted cancer treatment. An early and precise diagnosis of cancer can significantly improve patients’ survival rate.

**Figure 1.1.** Lung cancer diagnosis and survival by stage, 2003-2009

**Figure 1.2.** Stages of cancer development and range of techniques can be used (SPECT = Single-photon emission computed tomography, PET-CT= Positron emission tomography–computed tomography, CT= computed tomography, MRI= Magnetic resonance imaging)
However, unlike other common diseases, the early-stage cancer normally appears with initial lack of symptoms, which makes early diagnosis of cancer extremely challenging. In fact, the majority of cancer patients are diagnosed at the middle or late stages, when a tumour mass is already grown as a result of tumour angiogenesis. There are currently four methods which can identify cancer, namely medical imaging, endoscopy, biopsy and blood (other) sample tests.

Medical imaging techniques include X-ray imaging, X-ray computed tomography (CT scans), Magnetic Resonance Imaging (MRI scans), Positron Emission Tomography (PET scans) and Single-photon emission computed tomography (SPECT). All techniques use one source of energy (X-ray, magnetism or positron) to create a detailed view of the body to locate a tumour mass. The medical imaging techniques are matured techniques, which can be generally used to scan and diagnose all types of cancer. The quality of medical imaging methods rely heavily on radioisotopes applied as contrast agents. For example, positron emission tomography is a modern nuclear imaging method that uses positron (β+) – emitting radioisotopes of elements, including $^{11}$C, $^{15}$O, $^{13}$N and $^{18}$F, as well as some unconventional metallic radioisotopes of $^{64}$Cu, $^{63}$Zn, $^{38}$K, $^{82}$Rb, $^{32}$P, $^{59}$Fe, $^{68}$Ga and the halogen series. Some radionucides including these radio-metals are at the clinical research stage. In PET two gamma rays signals are emitted and detected, as they arrive in pairs (occurring at 180 degree angle). They are produced by a positron annihilation event by collision with an electron at the site of interest, with a well-defined image subsequently produced and interpreted computationally. Some radioisotopes and their half-lifes are listed in Table 1.2, recently, it was found that radioisotopes with a longer half-life are more appreciated for future diagnostic imaging, because of their potential to emit during prolonged circulation of tracer in vivo which can improve tumour/background ratio significantly. Therefore, those radioisotopes with only minutes of half-life available will not be preferred for most targeted delivery in vivo imaging experiment. However, a long lasting radioactivity can also damage living cells. Thus, those radioisotopes with greater than 10 days half-life will be considered as toxic to living organisms. Therefore, yttrium and gallium become popular radioisotopes in clinical research and recent work showed that, when $^{68}$Ga was anchored onto a suitable in vivo delivering vehicle able to target
tumours in precancerous stages, it is selectively accumulated around infected sites and areas of inflammation to report the site of rapid cell division. By contrast, $^{90}$Y and $^{86}$Y are found recently in many multimodal early diagnosis and treatment approaches.

**Table 1.2.** Lists of radioisotopes used in modern PET and their corresponding half-life

<table>
<thead>
<tr>
<th>Radio isotope</th>
<th>Decay type</th>
<th>Half-life</th>
<th>Radio isotope</th>
<th>Decay type</th>
<th>Half-life</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{14}$O</td>
<td>$\beta^+$</td>
<td>1 min</td>
<td>$^{62}$Zn</td>
<td>$\beta^+$</td>
<td>9.2 h</td>
</tr>
<tr>
<td>$^{15}$O</td>
<td>$\beta^+$</td>
<td>2.1 min</td>
<td>$^{65}$Zn</td>
<td>$\beta^+$</td>
<td>243.7 d</td>
</tr>
<tr>
<td>$^{13}$N</td>
<td>$\beta^+$</td>
<td>10.0 min</td>
<td>$^{38}$K</td>
<td>$\beta^+$</td>
<td>7.6 min</td>
</tr>
<tr>
<td>$^{11}$C</td>
<td>$\beta^+$</td>
<td>20.3 min</td>
<td>$^{82}$Rb</td>
<td>$\beta^+$</td>
<td>1.3 m</td>
</tr>
<tr>
<td>$^{18}$F</td>
<td>$\beta^+$</td>
<td>109.7 min</td>
<td>$^{59}$Fe</td>
<td>$\beta^-$</td>
<td>44.5 d</td>
</tr>
<tr>
<td>$^{32}$P</td>
<td>$\beta^-$</td>
<td>14.3 d</td>
<td>$^{64}$Ga</td>
<td>$\beta^+$</td>
<td>2.6 min</td>
</tr>
<tr>
<td>$^{61}$Cu</td>
<td>$\beta^+$</td>
<td>3.35 h</td>
<td>$^{60}$Ga</td>
<td>EC</td>
<td>3.26 d</td>
</tr>
<tr>
<td>$^{62}$Cu</td>
<td>$\beta^+$</td>
<td>4.7 h</td>
<td>$^{68}$Ga</td>
<td>$\beta^+$</td>
<td>68.1 min</td>
</tr>
<tr>
<td>$^{64}$Cu</td>
<td>$\beta^+$</td>
<td>12.7 h</td>
<td>$^{86}$Y</td>
<td>$\beta^+$</td>
<td>14.7 h</td>
</tr>
<tr>
<td>$^{67}$Cu</td>
<td>$\beta^-$</td>
<td>61.9 h</td>
<td>$^{90}$Y</td>
<td>$\beta^-$</td>
<td>64 h</td>
</tr>
</tbody>
</table>

Blood sample tests are also common methods that are applied to reveal cancer, as characteristic makers such as overexpressed molecules, including sugars, fats, proteins, RNA and DNA can be identified by the *in vitro* assays. However, there remains selectivity or sensitivity problems in the current testing reagents. Biopsy continues to be the most certain way to diagnose cancer; whereby a tissue sample is collected from the site of interest. Subsequently the morphology and gene status is examined. However, it is still the case that it is usually difficult to locate a site of a tumour. In this sense, molecular imaging has the potential to speed up the diagnosis in combination with blood sample testing to allow location of a tumour or early cancer detection, including more recently near IR imaging investigations using advanced endoscopy for colorectal cancer and breast imaging. Other methods, more commonly used in the diagnosis of tumours which are difficult to access, involve optical fluorescence imaging. These involve the use of fluorophores and, for enhanced imaging, quantum dots (QDs) which have recently been used as parts of endoscopes. Endoscopy involves a long, thin flexible tube equipped with a camera and a light source on the end, which can be guided to the interior of a hollow organ or cavity of...
the body allowing detailed visualisation of the probe’s environment. In general, endoscopy is applied at the middle or late stages of cancer diagnosis and is combined with other imaging tools to confirm a cancer diagnosis, or with biopsy to collect a tissue sample for further investigations. 22

In conclusion, medical imaging techniques are not suitable in early cancer diagnosis unless the sensitivity of tools can be further improved and radio-toxicity of the tracers in medical use is considerably reduced. Biopsy is a method applicable in early cancer research; but due to lack of precision, it could perhaps be more effective if used together with more sensitive techniques as a confirmation of a diagnosis. Hence, blood sample tests would be ideal for the early cancer diagnosis, and one long term aim of this project would be to assemble fluorescent probes that can target cancer cells selectively and which would induce minimum harm to normal cells.

The other interest of this project is how to deliver the diagnostic or therapeutic reagent precisely to a desired location in living cells and tissues. The design of a successful drug delivery system is determined by many factors, including the choice of the types of vehicle improved in the drug containment and delivery, size and shape, methods of functionalisation, ease of incorporation of the contrast agent (where this may be radioactive or fluorescent), issues of cellular and tumour microenvironment uptake as well as general side effects induced if the body identities as a “foreign objective”. A biocompatible nanoparticulate drug delivery agent would need careful selection as the first step, and this could play a crucial role in establishing a drug delivery system. It would directly, as well as indirectly, affect the subsequent steps in design, synthesis and imaging in vivo for a diagnostic nanomedicine. As vectors for drug delivery, nanoparticles offer a range of unique advantages, including their ability to modify drug pharmacokinetics in vivo and their capacity to be loaded with high concentrations of different drugs, for advanced combination of diagnostic and therapeutic (denoted “nano-theranostics”). A nanoparticles properties can also be modified to control drug release, alter blood circulation half-lives and improve bio-distribution profiles, increase tissue permeability and target specificity, as well as provide enhanced metabolic stability.26
Currently, there are at least nine different types of nanocarriers which are most widely used in biomedical research and have applications with relevance to tumour diagnosis and treatment (Figure 1.3).

**Figure 1.3.** General categories of nanocarriers and current development stages for molecular imaging of cancer

As it was shown in the figure above, most of the nanocarriers studied to date could be categorised into four groups: (a) lipid-based systems, (b) surfactant-based systems, (c) polymer-based systems and (d) inorganic-materials based systems. Liposomes were the first types of vesicle which were discovered in 1965. Phospholipids were one of the few solubilisers that could be well tolerated, and an
increase in the solubility of any drug trapped within was found to be hundred to ten thousand folds. Followed by nearly 50 years of intensive experiment and studies, the application of liposomes in the biomedical field was approved. Both micelle and polymeric vesicles were less widely studied than the lipid-based systems, hence, the development and in vivo testing of these two types of carriers proceeded slower than that of liposomes.

In recent years, inorganic nanocarriers have become intensely studied due to their sizes, shapes and ability to act as nanomedicines scaffolds, as well as their unique physicochemical properties, e.g. magnetism, emission/absorption spectroscopies, etc. Most inorganic carriers are currently at a preclinical and clinical trial stage. The most common of these, Quantum Dots, are intensely fluorescent and it was already demonstrated that these may be further bound to anticancer agents to make the resulting dual functional (imaging and therapeutic) composition, which is traceable in tissues. At the other end of the spectrum in terms of proximity to clinical use, single-walled carbon nanotubes (SWNTs) have a rigid framework and can absorb near infrared (NIR) light to generate heat, which could be used for photo-thermal therapy cancer treatment. There have been several other reviews with a focus on the development of single-walled nanotubes and their biocompatibility, which raise awareness of their inherent toxicity.

Furthermore, iron oxides are probably the most popular nanomaterials in clinical use: they are magnetic, can be rapidly derivatised and combined with fluorescent probes to generate a dual modality imaging and/or sensing complex or nanohybrid. For these, a core shell design in nano-theranostics (a nanoscale amalgamation of diagnostics and therapeutics) is a relatively new but promising field with multidisiplinary applications, potentially revolutionising the efficacy of diagnostic nanomedicines and in the long-term their use as combined therapeutics, for patient benefit. This offers a targeted approach on a cellular level with future applications on a molecular scale which still currently represents an unmet clinical need for improved diagnostic contrast agent design.

Small molecule-based, contrast agents can be incorporated in in vivo nano-diagnostics, thus greatly increasing the sensitivity and selectivity of otherwise undetectable pathologies. Nano-medicines advance further increase this diagnostic
potential by opening up new possibilities of imaging technologies and applications, such as those using fluorescence imaging techniques. There are many different types of nanomaterials currently under development – with some even at the clinical phase, such as gadolinium chelates and salts (Magnevist®), and iron oxide nanoparticles.\textsuperscript{35} Nanomedicine offers use of current imaging technologies, including new opportunities in fluorescent tagging, which until now has not been widely used due to poor signal strength, wide emission bands \textit{in vivo}, and poor tissue penetration.\textsuperscript{34} It offers even more insight into disease progression and can give information on tumour uptake mechanisms.\textsuperscript{36} This level of depth is required in order to probe diseased tissue on a molecular level and to gain a full understanding of the mechanisms involved. Hence, longer term, more precise patient diagnosis of treatment may become available through nanomedicine.

1.2. Introduction to SWNTs: from synthesis and characterisation techniques

1.2.1. Carbon allotropes

Carbon is one of the most abundant elements on earth; it widely exists in the atmosphere, as component of living organisms and in minerals. The majority of carbon occurring naturally forms a compound by combining with other elements. For example carbon and oxygen form carbon dioxide in the atmosphere; carbon and hydrogen form a hydrocarbon, such as alkanes, alkenes or aromatic rings, which represent the fundamentals of organic chemistry. However, elemental carbon is found as carbon allotropes, some of which have only recently been identified, e.g. fullerenes, nanotubes and graphene, and their discovery led to two Nobel prices so far (The Nobel Prize in Chemistry, 1996 Robert F. Curl Jr., Sir Harold W. Kroto and Richard E. Smalley, for their discovery of fullerenes. The Nobel Prize in Physics, 2010, Andre Geim and Konstantin Novoselov, for ground-breaking experiments regarding the two-dimensional material graphene.)

There are some well-known allotropes of carbon existing in the nature, namely graphite, diamond and amorphous carbon.\textsuperscript{37} The arrangement of carbon atoms in
amorphous carbon is a non-crystalline, irregular, giving a glassy state. The structure is similar to that of graphite but the atoms are not held in a crystalline macrostructure. As a result, amorphous carbon presents as a powder. Graphite is the most common carbon allotropes, formed at normal pressure. Each carbon atom is sp² hybridised and connected to three atoms to form a single layer sheet with a repetitive flat six-member ring structure, known as a hexagonal framework. A single layer of graphite can be isolated and the resulting material is known as graphene. Layers of graphene therefore stack together and constitute the 3-D structure of graphite. Delocalised \( \pi \)-electrons in the hexagonal framework give rise to strong light absorption abilities and electrical conductivity for this material. As a result, graphite has very good physical - chemical properties. Diamond is formed of sp³ hybridised carbon and emerges as a result of extremely high pressure. Each of the carbon atoms is attached to four other carbon atoms in a tetrahedral arrangement. Then, three other tetrahedral structures are joined to form 3-D six membered rings to give the extended structure. The repetition of these building blocks forms a network of covalent structures, which make diamond highly transparent, heat conductive and as such, one of the hardest natural materials in the world. (Figure 1.4)

![Figure 1.4](image)

**Figure 1.4.** Schematic representing fullerene and SWNTs from graphite

There are intermolecular and intramolecular forces in graphite. Each carbon atom in the hexagonal framework is connected by covalent intramolecular forces; and each graphite sheet is stacked *via* van der Waals intermolecular forces (non-covalent interacts). Therefore, it is possible to separate the graphite into individual sheets,
forming graphene without damaging the hexagonal framework. After the first exploration of the theory of graphene by P. R. Wallace in 1947, another allotrope of carbon, fullerene was revealed in 1985. Similar to graphene, fullerenes consist of carbon atoms also sp² hybridised. However, fullerenes (C₆₀ and higher fullerenes) contain not only hexagonal ring structures, but also pentagons, even heptagons of carbon atoms, which cause the closing of the sheet into spherical structures. The properties of fullerenes have not yet been fully analysed and represented an intense area of research in nanomaterials. As a result of a thorough investigation into fullerene, a tubular structure was discovered by a Japanese scientist Sumio Iijima in 1991, when he wanted to synthesise C₆₀ fullerene via the arc discharge method. However, multi-layer tubes of sp² hybridised carbons, which had 3 nm to 30 nm diameters and were formed to be closed at both ends, had been observed in the carbon soot of graphite electrodes. After two years of further studies of these structures, Sumio Iijima and Bethune discovered single-walled carbon nanotubes (SWNTs).

1.2.2. Structure of SWNTs

The shape of SWNTs, seen as a single layer of hexagonal carbon atom ring frameworks (or a graphene sheet), can be rolled into a seamless cylindrical structure which normally has spherical ends (analogous to hemi-fullerenes). Many studies had indicated that the properties of SWNTs were correlated to their structure (Figure 1.5). The structure of the SWNTs is determined by how the graphene sheet is rolled into a tubular structure. A chiral vector C is used to represent the overall chirality of the nanotube. This arrangement may be described from unit vectors a₁ and a₂ in the hexagonal framework via the equation (1) as follows:

\[ C = na₁ + ma₂ \] (1)

Where n and m are one pair of integers used to define the unit chiral vector. This pair of integers can be used to identify three different structures of nanotubes, namely zigzag, chiral and armchair. As it could be seen in the Figure 1.5, when the integer appeared as (n, 0), the structure is classified as zigzag. If the integer is (n, n), it is
Chapter 1. Introduction

armchair structure. Finally, once the integer is any number between 0 and n, i.e. (n, m), the structure is known as chiral.\textsuperscript{50, 51}

![Diagram of possible vectors arranged by pairs of integers (n, m) for a general structure of SWNTs](image)

**Figure 1.5.** Possible vectors arranged by pairs of integers (n, m) for a general structure of SWNTs (the alternating double bonds in the sp\(^2\) graphene layer have been omitted for clarity)\textsuperscript{52}

In the same way, the structure of the nanotube can also be defined using a chiral angle \(\theta\), via a different equation (2) as follows:

\[
\theta = \tan^{-1}\left[\frac{\sqrt{3}m}{m+2n}\right] \tag{2}
\]

In armchair structure, \(m = n\),

\[
\theta = \tan^{-1}\left[\frac{1}{\sqrt{3}}\right] = 30^\circ
\]

In zigzag structure, \(m = 0\), the equation describing the structure is:

\[
\theta = \tan^{-1}[0] = 0^\circ
\]

Where in the chiral SWNT structure, \(0 < m < n\), for example, \(0^\circ < \theta < 30^\circ\), Chirality of the tube structure affects many properties of the SWNTs, such as the electrical, optical, thermal, magnetic and mechanical properties of carbon nanotubes, particularly in
electrical properties. The general rules for the metallic character of SWNTs are given as follows:

1. Once \( n - m = 3q \), where \( q \) is an integer, the structure of SWNTs is metallic. Otherwise it would be semi-conductive.

2. In case of an armchair structure, because \( m = n \), \( n - m = n - n = q = 0 \), all armchair structured SWNTs are metallic.

3. In chiral and zigzag SWNTs, where \( q = (n - m) / 3 \) and \( q = n / 3 \) respectively, the majority of those SWNTs is semi-conductive in nature.\(^{53}\)

In practise, the separation of the three types of SWNTs remains extremely challenging, and most samples of SWNTs available commercially or synthesised in house constitute of a statistic mix of the tube (1) – (3).

1.2.3. Synthesis of SWNTs

There are many different ways to generate SWNTs, including (a) electric arc discharge, (b) laser ablation and (c) chemical vapour deposition (CVD).

The electric arc discharge was the first method developed for the carbon nanotube production. This method could be traced back to the early sixties, used by R. Bacon for the synthesis of carbon fibres.\(^{54}\) The same technique was applied to create fullerenes in good yields by Krätschmer and Huffman in 1990.\(^{55}\) This method was soon modified and improved for the synthesis of multi-walled (MWNT) and single-walled (SWNT) carbon nanotubes. At the same time, other methods such as the laser ablation and chemical vapour deposition (CVD) were developed and successfully adopted in the synthesis of single walled carbon nanotubes. The laser ablation is a synthetic technique similar to the arc discharge method. The difference between these two methods is mainly due to the fact that laser ablation gives a higher purity, but lower quality of the desired product. However, the arc discharge and the different types of CVD methods are the most promising and leading techniques in the large scale production of single walled carbon nanotubes.\(^{56, 57}\)
(a) **Electric arc discharge method**

For the electronic arc discharge methods, a high electric current (50-150 A) passes through both the carbon anode and cathode; as a result, a high temperature is generated at the carbon anode. The carbon sources on the carbon anode are evaporated. A constant gap between anode and cathode is maintained by adjusting the position of the anode. During the arc discharge, a plasma is formed between the electrodes. The plasma can be stabilised for a long reaction time (30 sec – 10 min) by controlling the distance between the electrodes by means of the voltage (25 – 40 V) control. The container is also filled with inert gas at low pressure, such as helium or argon. This avoids the risk that the vaporised carbon reacts with oxygen to form carbon dioxide. This also ensures the pressure at the inert - electrode zone remains low. When the reaction finishes, all vaporised carbon will be deposited on the cathode. Figure 1.6. shows the schematic representation of this process.

![Figure 1.6. Schematic representation of the synthesis of SWNTs by electronic arc discharge](image)

Figure 1.6. Schematic representation of the synthesis of SWNTs by electronic arc discharge
Table 1.3. Synthesis of different types of SWNTs via arc discharge under different conditions

<table>
<thead>
<tr>
<th>Product</th>
<th>Conditions</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>SWNTs, CNT Ribbons</td>
<td>Ho/Ni catalyst</td>
<td>High yield</td>
</tr>
<tr>
<td>SWNTs Fibre</td>
<td>Y/Ni catalyst, S</td>
<td>High purity</td>
</tr>
<tr>
<td>SWNTs, Nanohorns</td>
<td>Ni, Liquid N₂</td>
<td></td>
</tr>
<tr>
<td>SWNTs, Fullerenes, Metallofullerenes</td>
<td>Y/Ni and CaC₂/Ni catalyst, He</td>
<td>(d = 0.9 - 1.4 \text{ nm})</td>
</tr>
<tr>
<td>MWNTs, SWNTs, Carbon anocapsules</td>
<td>NaCl solution</td>
<td></td>
</tr>
</tbody>
</table>

Arc discharge can be used to synthesise both SWNTs and multi-walled carbon nanotubes (MCNTs) in the presence or absence of a metal catalyst, such as Ni, Y, Fe or Co etc. The quantity and quality of the nanotubes obtained this way depend mainly on the metal / carbon ratio, reaction environments and pressure of the container. Various conditions are listed in the Table 1.3. It was clearly shown that metal catalyst nickel (Ni) has a strong influence on the formation of the SWNTs.\textsuperscript{58} For instance, Eu / Ni catalysed synthesis produces very few SWNTs with nanoparticles; but replacing Eu with Y would improve the yield up to 60%.\textsuperscript{59} Additionally, Y / Ni catalyst with small amount of sulphur can produce a high purity SWNTs fibre.\textsuperscript{60} On the other hand, catalysts also affect the diameter of the SWNTs. For example, the use of CaC₂ / Ni leads to production of SWNTs with 0.9 – 1.1 nm diameters,\textsuperscript{61} whilst SWNTs created by Y / Ni have a diameter range between 1.3 – 1.4 nm. Liquid N₂, deionised water and NaCl can be used as a medium. Only a few SWNTs, with different unpurified by-products, have been find to form in the end.\textsuperscript{62, 63} Finally, pressure is another factor that determines the difference in diameter. An arc discharge reaction in He at 700 Torr have been shown to result in high density SWNT bundles \((d = 20 - 30 \text{ nm})\), whilst at 300 Torr, the main products are a mixture of fullerenes and metallofullerenes.\textsuperscript{61}
(b) **Laser ablation method**

There are two types of laser ablation methods: (i) pulsed laser vaporisation and (ii) continuous laser vaporisation. The major difference between the two protocols is that the former method uses a pulsed laser, whilst the later method adopts a continuous laser to heat the carbon sources. Because the pulsed laser can provide a much higher power density, pulsed laser vaporisation is more commonly used in carbon nanotubes synthesis than continuous laser vaporisation. Laser ablation utilises an intense laser pulsed at a carbon sources with 0.5% of catalyst (nickel or cobalt). A flow of inert gas is blown through the tube, carrying the vaporised carbon downstream to the water-cooled copper collector. Carbon nanotubes assemble and can be gathered from the collector at room temperature, as it is shown in figure 1.7. This method can produce higher yields SWNTs than standard arc discharge methods. However, the production yield is low and the pulsed laser vaporisation or laser ablation method is both infrastructure and energy intensive.56

![Figure 1.7. Schematic representation of the synthesis of SWNTs: pulsed laser ablation](image)

The pulsed laser-ablation designed for the synthesis of single walled carbon nanotubes was originally established by Guo *et al.* at Rice University.56 Then, the method was further developed by Smalley *et al.* in 1996, in order to produce SWNTs on a large scale.54 Simultaneously, a double beam laser method was applied by both Thess *et al.* and Rao *et al.* to improve the purity of SWNTs up to 90% with a better
tubular structure than those produced in the arc discharge method.\textsuperscript{65, 66} However, the amount of carbon deposit found at the end of process was found to be significantly decreased. The laser ablation technique was normally adopted to synthesise SWNTs. Whereas, MWNTs are only formed under special reaction conditions.

The work of Maser \textit{et al.} investigated the effect of metal concentration in the catalyst, gas flow and pressure on the synthesis of SWNTs.\textsuperscript{67-69} There were two types of catalyst were applied in the synthesis, one was mono-catalyst (Co or Ni) and the other one was catalyst mixture with two metals at equal (Ni/Co) or different concentration (Ni/Y). Individual SWNTs were produced in the mono-catalyst system, whereas in the mixed catalyst system, majority of SWNTs formed were SWNT bundles.\textsuperscript{69}

Varieties of gases were chosen as carrier gas, including Ar, N\textsubscript{2}, He, but a high yield of SWNTs could be achieved under Ar or N\textsubscript{2} environment and the pressure of 200-400 Torr.\textsuperscript{69} it has been shown that there were no SWNTs formed once the pressure was dropped below 100 Torr. Muñoz \textit{et al.} reported studies that revealed the synthesis on C/Ni/Y target in Ar and N\textsubscript{2} at 50–500 Torr of pressure.\textsuperscript{70} A high yield of filamentous soot with web-like structures were observed under 200 – 500 Torr pressure. SWNT bundles formed in this soot were about 10–20 nm in diameter and more than 1 μm in length.\textsuperscript{71} Gas flow rates (Ar and N\textsubscript{2}) have been found to be a less important parameter in the system: modifying this parameter has been found not to lead to remarkable changes in the quantity and quality of the final product.

There have also been observations regarding the effects varieties of laser conditions, including mode and type of laser, could be modified to change the quantities and qualities of the SWNTs formed in the end of the process. The choice of the laser mode would dramatically influence the final result. There were basically two laser modes: continuous- and pulsed- mode. In the continuous laser mode, the carbon source is evaporated at a rate of 200 mg/h and this leads to formation of high quality SWNTs at the end of the process. In the pulsed laser mode, the evaporation rate is ca. 4 mg/h and the majority of the product emerging has been found to be amorphous carbon. A gas-powder suspension method was developed by Bolshakov \textit{et al.} in order to optimise the absorption of the laser radiation for carbon source evaporation.\textsuperscript{72} In the end, 20% -
40% of the product find in the carbonaceous deposit was formed of SWNTs of 1.2-1.3 nm diameter. CO$_2$ laser and Nd:YAG laser are the two most common types of laser used in this research field.\textsuperscript{67, 73, 74} Both continuous and pulsed CO$_2$ lasers can produce SWNTs at room temperature, but many studies have reported that the continuous laser mode is more effective to produce a higher yield SWNTs than in the pulsed mode.\textsuperscript{75} A continuous mode CO$_2$ laser has been applied to vaporise a carbon source in an Ar atmosphere with laser power of 400 – 900 W. This resulted in the formation of bamboo-like strands of SWNTs with a wide diameter distribution in a range of 6 – 20 nm.\textsuperscript{76} When a pulsed CO$_2$ laser vaporisation was used, the majority of SWNTs formed have been found to have a uniform diameter, at around 1.3 nm.\textsuperscript{77} As for the Nd:YAG laser, two pulsed Nd:YAG laser mode were tested, one was Nd:YAG laser pulsed with intensity of $0.5 – 4.6 \times 10^9$ W/m$^2$, the other one was Nd:YAG laser double pulsed with intensity of $0.6 – 5.6 \times 10^9$ W/m$^2$. The reported furnace temperature has been in between 800 and 1150 $^\circ$C, under an Ar atmosphere.\textsuperscript{78} Both single- and double-pulsed lasers produced high quality SWNTs, but structure and diameter of SWNTs varied slightly depend on the choice of the conditions.\textsuperscript{73} The results reported have demonstrated that a larger SWNTs with a diameter around about 1.4 nm has been formed with a high intensity laser and furnace temperature. In a single-pulsed laser with a high furnace temperature 750 – 1150 $^\circ$C, the diameter of SWNTs was reported to fluctuate between 2 nm and 13 nm. In addition to CO$_2$ laser and Nd:YAG laser, XeCl excimer laser and UV laser KrF excimer have been also adopted to synthesise SWNTs. A web-like deposit has been observed in XeCl excimer laser ablation technique at 1000 $^\circ$C, and the soot contained SWNTs bundles with 20 nm in diameter and composed of individual SWNTs with a diameter in the range of 1.2 – 1.7 nm.\textsuperscript{71} When the UV laser KrF excimer has been used, a thicker SWNTs bundles with an average diameter of 70 nm have been reported to formed at 1150 $^\circ$C.\textsuperscript{79} The quantity and quality of the nanotubes depend on the laser type, carrier gas parameter and temperature. All the conditions were listed in table 1.4.
Table 1.4. A summary of the conditions reported for the synthesis of SWNTs via laser ablation in different conditions

<table>
<thead>
<tr>
<th>Method</th>
<th>Product</th>
<th>Conditions</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>XeCl excimer</td>
<td>SWNT bundles, fullerenes</td>
<td>Process temperature: 1000–1350 °C; C/Ni/Co; Ar</td>
<td>Web-like deposit</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>d = 20 nm</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>diameter range of 1.2 – 1.7 nm</td>
<td></td>
</tr>
<tr>
<td>CO₂ continuous wave</td>
<td>SWNT bundles, bamboo-like</td>
<td>Laser power: 400–900 W; C/Ni/Co, room T Ar: 200–400 Torr;</td>
<td>d = 6 – 20 nm</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>structures</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pulsed double Nd:YAG</td>
<td>SWNT bundles</td>
<td>Laser intensity: 532 nm, 1064 nm, double beam 532 and 1064 nm; C/Ni/Co; Ar; Furnace T: 800–1150 °C</td>
<td>Larger SWNTs with diameter at ca. 1.4 nm</td>
<td>78</td>
</tr>
<tr>
<td>CO₂ continuous wave</td>
<td>SWNT bundles</td>
<td>Gas-powder suspension catalyst; Ar, N₂; 1100 °C Gas nature Ar, He, N₂ 50–500 Torr; C/Ni/Y</td>
<td>d = 10 – 20 nm, their length was more than 1 μm in Ar and N₂</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CO₂ continuous and</td>
<td>SWNTs</td>
<td>Catalyst composition, gas conditions: Ar, He, N₂ 50–500 Torr, laser power density: 9 – 12 kW/cm² Configuration of laser wave</td>
<td>d = 5 – 25 nm Diameter of SWNTs was 1.3 – 1.4 nm</td>
<td>70</td>
</tr>
<tr>
<td>pulsed wave</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pulsed Nd:YAG laser</td>
<td>Thin SWNTs</td>
<td>Target composition, reaction T and gas flow velocity</td>
<td>d = 2 – 13 nm</td>
<td>70</td>
</tr>
<tr>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>CO₂ pulsed laser</td>
<td>SWNTs</td>
<td>Target composition Gas nature and its pressure</td>
<td>Diameter of SWNTs was 0.86 – 1.46 nm</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pulsed double beam</td>
<td>SWNTs</td>
<td>Effect of the laser intensity</td>
<td>Purity &gt; 70%</td>
<td>65</td>
</tr>
<tr>
<td>Nd:YAG</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Pulsed Nd:YAG</td>
<td>SWNTs</td>
<td>Effect of the laser intensity</td>
<td>Purity &gt; 70%</td>
<td>66</td>
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</tr>
<tr>
<td>KrF excimer</td>
<td>SWNTs</td>
<td>Furnace T = 550 °C</td>
<td>Purity &gt; 70%</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pulsed Nd:YAG</td>
<td>SWNTs</td>
<td>Laser parameters Target composition</td>
<td>d = 1.0 – 1.2 nm</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pulsed double beam</td>
<td>SWNTs</td>
<td>Gas pressure, flow</td>
<td>d ~ 1.0 nm</td>
<td>79</td>
</tr>
<tr>
<td>Nd:YAG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KrF excimer UV laser</td>
<td>SWNT bundles</td>
<td>Furnace temperature 25–1150 °C; Ar, C/Ni/Co</td>
<td>Bundles of SWNTs with average diameter of 70 nm</td>
<td>79</td>
</tr>
</tbody>
</table>
Chemical vapour deposition

Figure 1.8 shows chemical vapour deposition (CVD) route for SWNT production. For CVD production, carbon-rich gases, such as acetylene (C$_2$H$_2$), ethylene (C$_2$H$_4$), ethanol (C$_2$H$_5$O) or methane (CH$_4$), when mixed with ammonia, nitrogen or hydrogen, are converted into SWNTs.\textsuperscript{81} These gases are passed through a tube (shown in Figure 1.8), which contains a variety of catalysts on a support and which is placed, inside a furnace, to form carbon nanotubes. The whole system is normally heated up to ca. 700 °C, and then the product resulting is collected at room temperature.\textsuperscript{82}

![Figure 1.8. Schematic representation of the synthesis of SWNTs: chemical vapour deposition](image)

The mechanism of the CVD method involves the dissociation of hydrocarbon molecules, catalysed by a transition metal in the heterogeneous phase reaction. This step is followed by the dissolution and deposition of carbon atoms on the substrate. The precipitation from the saturated metal particles has been reported to induce the formation of a tubular structure for the SWNTs.\textsuperscript{81, 83} The type of nanotubes formed could be determined by the temperature and hydrocarbon gas employed. For example for multi-walled carbon nanotubes (MWNTs), the most common CVD processes employ ethylene and acetylene as the carbon source and the growth temperature was normally between 400 to 600 °C. For SWNTs, the temperature needed is usually higher than 700 °C. CVD constitutes an effective method to synthesise carbon nanotubes in
the high yield required for most industrial applications. Carbon nanotubes could also be directly grown on a desired substrate hence this reduces the risk of contamination or product loss during the transfer. As a result, CVD methods have recently emerged as a common method of synthesising carbon nanotubes in bulk.

Table 1.5. A summary of the conditions reported for the Synthesis of SWNTs via chemical vapour deposition in different conditions

<table>
<thead>
<tr>
<th>Method</th>
<th>Product</th>
<th>Conditions</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water-assisted</td>
<td>Vertically aligned SWNTs, DWNTs</td>
<td>With buffer layer</td>
<td>High yield</td>
<td>85-87</td>
</tr>
<tr>
<td>O₂-assisted plasma-enhanced</td>
<td>Vertically aligned SWNTs, DWNTs</td>
<td>With buffer layer</td>
<td>High yield</td>
<td>85-87</td>
</tr>
<tr>
<td>Microwave plasma-enhanced</td>
<td>Vertically aligned SWNTs, DWNTs</td>
<td>With buffer layer</td>
<td>High yield</td>
<td>85-87</td>
</tr>
<tr>
<td>Microwave plasma-enhanced</td>
<td>Vertically aligned SWNTs</td>
<td>Co-Ti/Si substrate without a buffer layer</td>
<td>High yield</td>
<td>88</td>
</tr>
<tr>
<td>Hot filament-enhanced</td>
<td>SWNTs, MWNTs</td>
<td>Fe-Co/SiO₂ with or without of Si support</td>
<td>Perpendicularly or vertically aligned</td>
<td>89</td>
</tr>
<tr>
<td>High power laser pulse alcohol CVD</td>
<td>SWNTs</td>
<td>Solid metal target</td>
<td>High purity</td>
<td>92</td>
</tr>
<tr>
<td>Alcohol CVD</td>
<td>SWNTs</td>
<td>Ferrocene-alcohol</td>
<td>High purity</td>
<td>90, 91</td>
</tr>
<tr>
<td>Simple CVD</td>
<td>SWNTs</td>
<td>Fe-Mo/Si substrate, methane</td>
<td>Individual and in bundles</td>
<td>84</td>
</tr>
<tr>
<td>Ultrasonic spray pyrolysis</td>
<td>SWNTs</td>
<td>Co-Mo/silicon substrate, ethanol</td>
<td>d = 0.8–1.2 nm</td>
<td>93</td>
</tr>
</tbody>
</table>

Table 1.5 showed SWNTs can be formed under different conditions, using varieties of carbon sources, inert gas, catalysts, support materials, substrates and different types of CVD furnaces. In general, SWNTs can be produced by a simple CVD at 900 °C using CH₄ as carbon sources and Fe/Mo as a catalyst, and their growth has been supported on the Si substrate. Individual nanotubes as well as tubes in bundles may be formed with a diameter of about 1.15 nm.⁸⁴ Water-assisted, O₂-assisted plasma-
enhanced and microwave plasma-enhanced CVD methods were able to produce high yields of SWNTs and double walled carbon nanotube (DWNTs) with the diameter range between 1 and 2 nm in a different buffer layer.\textsuperscript{85-87} However, if only Co-Ti catalyst and Si substrates are used in microwave plasma-enhanced CVD without a buffer layer, then only vertically aligned SWNTs with diameter at 2 nm have been observed in the end.\textsuperscript{88} Hot filament-enhanced CVD methods produce SWNTs with diameters in the range of 0.65 – 1.55 nm. The reaction is catalysed by Fe-Co solid phase catalyst and use SiO\textsubscript{2} as the mixtures substrate. The advantages of this method is that it required a relatively low substrate temperature of 660 °C and a low amount of carbon supply, i.e. low C\textsubscript{2}H\textsubscript{2} concentration and low reaction pressure. Because SWNTs synthesised by this method vary in diameter, nanotubes were found to be vertically aligned when they were large in diameter (0.65 – 1.55 nm). In contrast, when nanotubes were smaller than a certain size, they grew perpendicularly across the surface.\textsuperscript{89} The alcohol-based CVD technique was another common method which used a solution of ferrocene and ethanol with different ratios to achieve a high purity.\textsuperscript{90, 91} The ratio between ferrocene and ethanol was crucial to the reaction, a low concentration of ferrocene causes the mass production of amorphous carbon with only a small amount of nanoparticles, whilst too high concentrations of ferrocene has been found to lead to the formation of nanoparticles instead of SWNTs. After several experiments, the optimum ratio of ferrocene/ ethanol was established at about 1 – 1.5 wt %. The high power laser pulse alcohol-CVD method was an advanced method of alcohol-CVD and a high power laser pulse was used for the vaporisation of solid metal target. This was made up of ethanol and Co catalyst. SWNTs with diameter of 0.96–1.68 nm were synthesised in the end.\textsuperscript{92} Ultrasonic spray pyrolysis CVD used ethanol as carbon sources and Co-Mo as the catalyst and silicon as the substrate. SWNTs produced from this method mainly depended on the dipping time and catalyst concentration, the diameter of the SWNTs was in a range of 0.8 – 1.2 nm.\textsuperscript{93}
1.2.4. **Impurities typically found in as-made SWNT and their potential health and environment risks**

Regardless of the producing methods used, as synthesised SWNTs samples contain certain amount of impurities, including: amorphous carbon, fullerenes, metallic nanoparticles, multi-shell carbon nanoparticles, nanocrystalline graphite, various contaminations depend on the support, such as silicon substrate. These SWNTs cannot be used directly in most applications, because the presence of those impurities after synthesis will cause many problems, especially in biomedical applications. The major problems come from the metallic catalysts, which are highly reductive and lead to a significant rise of the reactive oxidative species (ROS) level in living systems.\textsuperscript{94}

ROS refers to free radicals and other oxygen-related reactive compounds, including singlet oxygen (${}^1\text{O}_2$), hydrogen peroxide (H$_2$O$_2$) and hydroperoxide (ROOH).\textsuperscript{95, 96} All these compounds are highly reactive to nearby environment, which makes ROS able to initiate free radical reaction to convert a non-radical molecule to a radical. In the beginning of ROS generation, molecular oxygen is reduced by a reducing reagent, such as metallic particles, to form a superoxide radical ion O$_2^\cdot$-. Then a series of free radical chain reactions will be initialled by this superoxide.\textsuperscript{97} Formation of hydrogen superoxide in cellular mitochondria may be described by the reaction scheme 1.1:

$$\text{O}_2^\cdot-+\text{O}_2^\cdot-+2\text{H}^+\rightarrow\text{H}_2\text{O}_2+\text{O}_2$$

**Scheme 1.1.** Formation of hydrogen superoxide in mitochondria

Scheme 1.1 showed a reaction to form a hydrogen peroxide (H$_2$O$_2$) from superoxide (O$_2^\cdot$-). In mitochondria, superoxide is further reduced to hydrogen peroxide and molecular oxygen, which is catalysed by superoxide dismutases (SOD). H$_2$O$_2$ is more kinetically and thermodynamically stable than superoxide ions and it can diffuse to the cytoplasm to enhance radical reactions, unless it has been eliminated by catalase or glutathione scavenges biomolecules. At the end of the radical chain reaction, the most reactive and harmful radical, hydroxyl radical (OH) is produced from H$_2$O$_2$ via
the Fenton reaction and Haber-Weiss reaction catalysed by transition metals, as shown in scheme 1.2. The hydroxyl radical has extremely short lifetimes (around 1 ns): this makes this radical species extraordinary reactive in living cells have the ability to damage all the biomolecules, including DNA, RNA, lipids, proteins and carbohydrates. In fact, hydroxyl radicals are normally consumed at the site of formation, hence, it makes hydroxyl radical impossible to eliminate by enzymatic reactions.

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \cdot\text{OH}^- \\
\text{or} \\
\text{O}_2^- + \text{H}_2\text{O}_2 \xrightarrow{\text{metal catalyst}} \text{O}_2 + \cdot\text{OH} + \cdot\text{OH}^-
\]

**Scheme 1.2.** Formation of hydroxyl radical from hydrogen peroxide

As a result of ROS level upregulation, there are a series of consequences outlined in the Figure 1.9. Both hydrogen peroxide and hydroxyl radical can damage DNA, and radicals can oxidise purine and pyrimidine bases to introduce strand breaks and mutations. Poly-ADP-ribose polymerase is subsequently activated to repair the damage via depleting malfunctioned DNA. Radicals, such as hydroxyl radicals can oxidise sulphhydryl groups and amino acids, leading to the formation of a defective protein. In the same way, hydroxyl radicals can also initiate a lipid peroxidation; it converts unsaturated lipids to lipid peroxide, and as a consequence, it leads to the disruption of the lipid phosphate bilayer structure, and a decrease in the membrane fluidity, whilst increasing the structure of membrane permeability and modifying the membrane proteins. All modification mentioned above will cause cell death by necrosis. On the other hand, ROS can also activate redox signalling, release protein cytochrome complex (cyt c) and open permeability transition pore (PTP) to initiate cell death via apoptosis. In general, both methods of cell death have been shown to lead to degenerative diseases or accelerated aging in living organisms.
Carbonaceous nanomaterials, such as amorphous carbon, fullerenes, multi-shell carbon nanoparticles and nanocrystalline graphite, are also known to cause problems and their presence limits the applications of SWNTs in nanomedicine. Although these carbonaceous nanomaterials will not cause direct damage to cells or tissues, they can attach to SWNTs (or self-aggregates) to encapsulate metal catalysts. As aggregates, they reduce the solubility of the nanomaterials, which makes functionalisation of SWNTs impossible. Many articles have pointed out that pristine SWNT strands in general will not cause drastic inflammatory or immune responses, until SWNTs are aggregated and have a significant larger diameter.\textsuperscript{108, 109}

In that case, SWNTs will be detected by immune system with the aim to eliminate these from the living organisms. In addition, impure SWNTs administrated \textit{in vivo}, can activate platelets in blood vessels to cause thrombosis.\textsuperscript{110} Finally, carbonaceous impurities also affect the functionalisation process of SWNTs, lead to a decrease in the degree of functionalisation and solubility of the functionalised SWNTs (f-SWNTs). Therefore, purification of SWNTs to remove metallic nanoparticles and carbonaceous impurities is an essential step before functionalisation and further biomedical applications of SWNTs can be considered.
1.2.5. Methods for the advanced purification of SWNTs for biomedical application

As-made SWNTs can be purified via chemical or physical methods. The former includes: liquid phase oxidation, gas phase oxidation and electrochemical oxidation. The latter method contains filtration, centrifugation, solubilisation with other functional groups and high temperature annealing. Many years of research have shown that only single purification method, especially physical purification techniques is to date, reliable enough to remove all the impurities at once. Therefore, more than two methods are typically combined together to enhance the effectiveness of the progress, whilst considering the yield of the purification process overall.\textsuperscript{111}

For the liquid phase oxidation methods, HNO\textsubscript{3} and H\textsubscript{2}O\textsubscript{2} have been used as oxidative reagents. Their action breaks sp\textsuperscript{2} bonds and creates defect sites on the hexagonal carbon framework.\textsuperscript{112} Due to the fact that carbonaceous materials presents on the surface of SWNTs are typically irregular and defective in shape, from the catalytic production process, they have larger surface areas than SWNTs strands when exposed to the oxidative reagents. Hence, they are vulnerable to be oxidised before the pure SWNTs surfaces.\textsuperscript{113, 114} As a result of this oxidative treatment, carboxyl or hydroxyl groups are introduced onto the carbonaceous impurities to make these carbonaceous impurities more soluble and dispersible in common organic solvents than SWNTs.\textsuperscript{115, 116} Microwave treatments are an effective method to enhance the liquid phase oxidation. This process heats up the metal catalysts encapsulated in the carbonaceous impurities that are attached to the surface of SWNTs. As a result, the thick carbon shell is cracked by microwave-heated metallic catalyst and this leaves the metal exposed to the outside atmosphere.\textsuperscript{117} Afterwards, an acid wash is subsequently applied to remove those resulting metal ions from SWNTs.\textsuperscript{118}

On the other hand, in gas phase oxidation methods,\textsuperscript{119-121} SWNTs can be purified via gaseous oxidants, including a mixture of Cl\textsubscript{2}, H\textsubscript{2}O, and HCl,\textsuperscript{122} H\textsubscript{2}S and O\textsubscript{2},\textsuperscript{123} hydrogen treatment,\textsuperscript{124} Air/H\textsubscript{2}O\textsuperscript{125}, fluorine\textsuperscript{126} and water vapour\textsuperscript{127}. Intact SWNTs ignite at higher temperature than amorphous carbon, therefore heating as-made SWNTs at around 500 °C in a wet stream can remove amorphous carbon before damaging the tubes.\textsuperscript{128} Ultrasonication of SWNTs in common solvents is normally used
to assist the previous two methods. Ultrasonication itself cannot purify the SWNTs, but it can conduct vibration forces through a water bath onto the SWNTs samples, providing a high local shear, particularly to the end of the nanotube bundles. This makes them temporarily better dispersed in the solvent, in another word, it increases the exposure of the surface of raw SWNTs to the oxidative reagent in order to facilitate the purification methods. However, prolonged ultrasonication will also damage the structure of carbon nanotubes, so it is important to manage the time of exposure.

Apart from the liquid phase and gas phase oxidation technique, electrochemical oxidation is another method to modify as-made SWNTs. Samples are immersed in an acid or a base solution and an electric potential is applied to the solution to etch both amorphous carbon and SWNTs. However, carbon materials with fewer defects normally show a lower corrosive rate in electrochemical oxidation. Hence, it has been assumed that intact SWNTs have a higher electrochemical oxidation resistance than amorphous carbon. In basic solutions, metal catalysts are exposed after amorphous carbons are etched. An acid wash is therefore necessary in the subsequent step to remove all metallic impurities. In contrast, in the acid solution, metal ions will be directly “dissolved” into the solution giving rise to MXₐ salts after amorphous carbons are etched. At the same time, the tips of SWNTs have been found to be opened in the acidic environment and there is no significant tip-open-effect in the basic solution. The advantage of this method has been considered to be very effective for SWNTs purification from amorphous carbon. The optimum time of the oxidation process for SWNT purification can be easily achieved by the redox potential monitoring. It was found that this can also open the tips during purification via choosing an acidic solution. However, this method cannot identify and remove carbon impurities with fewer defects such as polyhedral carbon, graphite particles and carbon onions. Most importantly, the amount of SWNTs purified in each batch is too small for many protocol purposes in many biomedical applications.
1.2.6. Functionalisation methods for SWNTs

For biomedical applications, it has been reported that functionalisation of SWNTs plays an equally important role as purification. After purification of the metallic, amorphous carbon and graphitic carbon impurities from the as-made SWNTs, functionalisation is used to modify the structure of SWNTs in order to improve the solubility and biocompatibility. It has been reported that the functionalisation of SWNTs enhances their dispensability into individual tubes in common solvents and further reduces the cytotoxicity of SWNTs in biomedical applications (figure 1.10).\textsuperscript{137}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{functionalisation_methods.png}
\caption{Schematic representations of different methods of functionalisation of SWNTs.\textsuperscript{53}}
\end{figure}
Figure 1.10 shows the most common methods of functionalisation. In general, it can be divided into two categories: covalent and non-covalent methods.

Covalent functionalisation involve breaking and forming new bonds on the defect and sidewall of SWNTs, as it can be seen in Figure 1.10 a and 1.10 b. There are ten common methods, described in Figure 1.11, e. g: acid-based treatment, ozonolysis, halogenation, reductive coupling, oxidative coupling, polymer grafting, hydrogenation, electrophilic addition, cycloaddition and nucleophilic addition to functionalise SWNTs.\textsuperscript{138}
The most commonly used covalent method is acid (oxidative) treatment, which introduces carboxyl groups at the sidewall, defect sites and open ends of SWNTs.\(^{139,140}\) Furthermore, carboxyl groups on SWNTs can be subsequently treated with hydroxyl or amino groups to form an ester bond or carboxamide bond. This allows the further modification by incorporating of other organic groups molecules onto the surface of SWNTs. Alternative ways to add functional groups on the side of SWNTs is for example through halogenation reactions. Once a halide is inserted onto the hexagonal framework surface of the carbon nanotubes, in subsequent steps this can be converted through standard organic chemistry, e.g. by carrying out substitution reactions onto halide-functionalised SWNTs.

As for non-covalent functionalisation, this is based upon supramolecular interactions between the surface of SWNTs and other intact molecules, such as polymers, polysaccharides, porphyrin oligomers etc. Instead of breaking and forming a new bond between the functional group and the \(sp^2\) carbon of the SWNTs (which causes the local disruption of the synthetic structure), the functional groups or molecules attach onto or “wrap-around” the SWNTs via van der Waals interactions or aromatic stacking. Polyethylene glycol (PEG) and pyrene have been commonly used for non-covalent functionalisation to achieve dispersions of SWNTs.\(^{141}\) Pyrene-based groups attach to SWNTs via \(\pi-\pi\) stacking. Pyrene can act as a scaffold molecule. Whilst further modifications can be performed to the attached pyrene to future functionalise the SWNTs constructs (figure 1.10 c).

PEGs are commercially available polymer chains that can wrap or coat SWNTs. PEGs have both hydrophobic and hydrophilic functionalities, thus it have been found that they are aiding the dispersion of SWNTs in aqueous media.\(^{142}\) Further functionalisation can be performed onto the PEG chains to introduce a variety of different molecules (1.10 d). The major advantage of the non-covalent method is that it does not damage the existing structure of SWNTs. Secondly, polymers can wrap the whole SWNTs, thus all of the surface area can be used for loading much larger molecules are than via the covalent method. Finally, the polymer layer not only acts as an anchor, but also as a protecting layer. This shields the SWNT surface from the environment and also may avoid further damage created by SWNTs to the surrounding
cells. Non-covalently wrapped-SWNTs have been shown reduce the cytotoxicity of functionalised SWNTs to cancer cells in the MTT assays.\textsuperscript{143} 

Apart from covalent and non-covalent functionalisation on the outer surface of SWNTs, an alternative possibility to functionalise them is the method known as internal functionalisation. This refers to the filling of SWNTs with ions or small molecules. The most important advantage is that the encapsulated molecules can be protected by the SWNT walls; hence it can lead to the stabilisation of reactive species inside the tubes, as well as to the prevention of contaminations or loss of molecule. However, this method is still under development regarding the precise control of the filling yield.\textsuperscript{144} There are still many problems, for example, the capacity of filling is limited and the mechanism of controlled release of particles trapped within carbon nanotubes is still under investigation.\textsuperscript{145, 146}

1.2.7. Developments and applications of SWNTs in the biomedical field

SWNTs is a relatively new inorganic material, however in the past two decades there were intensive studies and researches based on it to explore the properties and applications of this novel material at the interface with biological research fields.\textsuperscript{147-149}

![Figure 1.12. Properties and application of SWNTs in biomedical field](image-url)
Figure 1.12 highlights five basic properties having the potential applications in the biomedical field, namely: chemical properties, electrical properties, magnetic properties, optical properties and field emission properties. Because raw, as-made, even purified SWNTs are very difficult to dissolve into all ranges of the solvents, chemical properties were initially investigated aiming to improve its solubility. After chemical modification, the new bonds formed, for example, amide bonds, carboxyl or hydroxyl groups are introduced on the surface or on the side of the SWNTs via covalent functionalisation. On the other hand, polymer or oligomers can be functionalised on SWNTs by van der Waals intermolecular forces or $\pi-\pi$ stacking via non-covalent methods. Both ways not only improves the solubility of the SWNTs, but also extend the potential application of SWNTs towards biomedical areas.\textsuperscript{150} For example, a carboxyl group formed from covalent functionalisation can be reacted further with a hydroxyl group to form an ester bond, or with an amine group to form a peptide bond. As such, there are many different compounds or small molecules, (like radiolabelled probes, fluorescent molecules, DNAs and proteins), which can be introduced to process different biomedical tasks. For instance, SWNTs can be functionalised and act as a vector, in order to deliver drugs to desired site, or modified to act as a sensor to detect overexpressed growth factors in cancer cells.\textsuperscript{151}

Optical properties of the SWNTs are the other important factor which determines the application of SWNTs in the biomedical field. There are two main optical properties of SWNTs, (1) abilities to absorb all light in the visible region, hence, resulting in the colour of the SWNTs completely black. And (2) the absorbed light at wavelength between 700 and 1100 nm is emitted as fluorescent signals, or lead to convert the energy into heat. The former situation is applied as an imaging tool to track and imaging cells, whilst the latter their application in killing the targeted cells.\textsuperscript{152, 153}

Electrical properties of SWNTs have also been used to detect molecules, but rather than bind to target molecule and cause fluorescent intensity change, these lead to changes in their changes electrical potential instead.\textsuperscript{154, 155}

Magnetic properties of SWNTs can be normally introduced via internal cavity filling of metal: such as, metal ions of Ti, Fe, Co, Ni, Cu, Ga, In, Zn, Gd and Fe–Co alloys have been incorporated.\textsuperscript{156} The external coating of the SWNTs with magnetic
nanoparticles have also been reported. These magnetic CNTs are generally employed as a scaffold, in order to prepare more complex multifunctional vectors for biological applications. These vectors are able to carry fluorescent molecules, proteins, DNA, targeting ligands and therapeutic drugs, and these multifunctional vectors can also be used in cellular imaging, cell tracking, lymphatic targeting, cancer lymph node metastasis treatment and cancer gene therapy.

Field emission property of SWNTs is the last important properties of SWNTs. Electrons are readily emitted from the tips of the SWNTs, either due to oxidized tips or because of the curvature of the SWNTs. when a potential is applied between a carbon nanotube surface and an anode, this is generated by the continuous and pulsed X-rays, using a SWNTs-based field emission cathode. Miniaturized SWNTs-based X-ray device have been reported to have been inserted into the body by endoscopy to achieve a precise X-ray radiation therapy directly toward a desired area to be imaged and minimise damaging the surrounding healthy tissues.

1.2.8. Characterisation techniques for SWNTs

Characterisation of SWNTs is the final, yet most important stage of investigation prior to any application. Due to different methods of synthesis, as-synthesised batches of SWNTs samples contain tubes which are slightly different in diameter, length, chirality, purity, catalysts, impurity species and nature number of defects. After purification and functionalisation of SWNTs, and despite the standard removal of catalysts and impurities, one or both of the SWNT ends may be open, and the length of SWNTs strands are often reduced during the purification. Also, functional groups on the SWNTs are modified and some characterise defects are introduced onto the backbone of the SWNTs. Therefore, characterisation of SWNTs is essential to determine the quantity, quality and properties of each individual batch.

There are many techniques that have been reported and used to examine SWNTs, including, photoluminescence spectroscopy (PES), X-ray photoelectron spectroscopy (XPS), Electron microscopy (SEM & TEM), Scanning tunnelling microscopy (STM), X-ray diffraction (XRD), Neutron diffraction, Raman spectroscopy, Thermal analysis (TGA/DTG) and absorption spectroscopy (UV, Vis and IR). Electron
microscopy (SEM & TEM), absorption spectroscopy and Raman spectroscopy are the most widely used characterisation techniques due to accessibilities in standard labs and ability to scan bulk materials (especially SEM and Raman techniques).

Three most commonly-used electron microscopies are TEM, SEM and atomic force microscopy (AFM). In TEM (Figure 1.13), a beam of electrons is accelerated and projected onto an ultra-thin specimen. Subsequently electrons collide with the atoms in the sample and deflect into different directions in a process, known as three-dimensional angle scattering. The size of the scattering depends on the density and thickness of the sample (typically nanometre scale). The image is magnified and focused onto an imaging device, either a fluorescent screen, on a layer of photographic film, or to be detected by a sensor such as a charge-coupled device (CCD) camera. TEM

Figure 1.13. A schematic representation of transmission electron microscopy typically used to characterise SWNTs
Chapter 1. Introduction

consists of three parts: the electron gun and condenser system, imaging producing system and imaging recording system. An electron gun produces a beam of electrons, and then the beam is focused onto the object via the condenser system. Subsequently the beam is passed through the objective lens, intermediate and projector lenses, which focus the electrons onto the fluorescent screen to form a magnified image. Eventually, the image-recording system converts the electron image into an image visible to human eyes and additionally pictures can be captured and presented computationally. In addition, a vacuum system consists of a pump, gauges and valves are equipped to keep the whole system under vacuumed and with temperature control. Due to the extremely small wavelength of electrons, TEM (nm ~ µm) can produce a much more detailed image of an ultra-small objective than a light microscope (µm ~ mm) does. The range of image scale used in this project is usually in between 1 nm and 1 µm, which makes TEM a perfect tool to analyse the structure of the SWNTs functionalised hereby. In TEM, the shape and quality of SWNTs will be visualised, the average diameter and length of the carbon nanotube will be calculated and the impurities remaining on the tube will be revealed. The existence of impurities particularly in the heavier elements present, also can be confirmed via energy-dispersive X-ray spectroscopy (EDS) which is available in line with the TEM microscope at University of Bath.

![Diagram](image)

**Figure 1.14.** Scanning electron microscopy adopted to characterise SWNTs
SEM (Figure 1.14) also uses a beam of electrons to produce images, however in a significantly different way compared to TEM. In a typical SEM, a beam of electrons is generated from an electron gun, and then this electron beam carries energy (0.2 keV to 40 keV), which is focused onto an area about 0.4 to 5 nm in diameter. Subsequently, this electron beam will be deflected in the x and y axes through a deflection coil, in order to scan a rectangular area onto the sample surface. The electron beam loses energy during the scanning process due to repeated random scattering and absorption occurs in the interaction volume (Figure 1.15).

The interaction volume is a pear-shaped area created on the sample surface via the impact of the primary electron beam, the energy exchange between the electron beam and the sample results in the reflection of high-energy electrons. The secondary electrons are reflected from a 10 to 100 nm depth in the sample, which can then be used to determine the shape of the sample. Backscattered electrons come from 1 – 2 µm and provide information about the atomic number of the elements found in the sample. Characteristic X-rays from very deep areas of sample (2 – 5 µm) can be detected by EDS to identify element information. All signals from the sample will be collected by specific detectors and amplified via electronic amplifier to create images. Similar to TEM, SEM technology is used to characterise SWNTs by measuring the diameter and length of the tube, examining the shape and quality of the tube and also scanning impurities via EDS analysis.
Figure 1.15. Interaction volume of SEM

Figure 1.16. (a) Energy-dispersive X-ray spectroscopy adopted to characterise SWNTs
(b) Representation of the basic principle of EDS

Energy-dispersive X-ray spectroscopy is a common materials analysis technique, which coupled with TEM and SEM, is an extremely powerful tool used to study the elemental composition or chemical characterisation of SWNTs samples. The
basic principle of EDS is each element has a unique atomic structure leading to a unique X-ray emission spectrum after excitation (Figure 1.16 a). A standard EDS consists of two parts, electron gun and X-ray detector. The former generates a high energy electron beam, which has been focused on the sample in order to excite an electron in the inner shells; the excited electron is then ejected from the electron orbital and as a result it leaves an empty space in the inner shell. Subsequently, an electron from an outer orbital will be shifted into inner shell to fill the empty space. Energy will be lost when the electron shifts from the outer shell to the inner shell and converted into a characteristic X-ray. The emitted X-ray can be collected and measured by an X-ray detector. In this project EDS is used to examine the purity of SWNTs, especially the metal catalyst remaining on SWNTs (Figure 1.16 b).

![Diagram of Atomic Force Microscopy](image)

**Figure 1.17.** Atomic force microscopy adopted to characterise SWNTs

Atomic force microscopy (Figure 1.17) is a very sensitive and high-resolution type of scanning probe microscopy but more suitable for small samples of SWNTs, contains well dispersed strands. The information is collected via monitoring the force change between sample surface and probe tip. The AFM consists of three parts: a tip
and cantilever system, laser generator and photodiode system and detector and feedback control system. A silicon nitride tip is attached on a silicon nitride cantilever; the tip interacts with the sample surface at the atomic level and scans in a raster fashion over a rectangular area of the sample surface. A laser beam is pulsed on the back of the cantilever and reflected on a photodiode, in order to monitor the force difference and vibrations of the tip. The detector collects data to generate images and the feedback system sends feedback to the cantilever or platform, which moves the cantilever up and down across the surface or moves the platform up and down to avoid damage of tips or sample surface and keeps the force of the cantilever constant. AFM is commonly used to characterise nanoscale structures, including SWNTs, to study the surface condition, precise diameter and length.

![Raman microscopy diagram](image)

**Figure 1.18.** Raman microscopy applied to characterise SWNTs

Raman spectroscopy (Figure 1.18) is another spectroscopic technique which is widely accessible to study the structure of SWNTs in bulk, including the purity and
integrity of SWNTs batches. During the process, a monochromatic laser beam is pulsed on the sample, which absorbs and reemits the photons, whereby the photon scattering can be either elastic or inelastic. In fact, most of the photons will be reemitted in an elastic manual, which means the frequency of the photon remains the same. Those photons reemitted in a different frequency are known as inelastic scattering, which will be collected by spectrometers and used for Raman spectroscopy. Frequency of inelastic scattered photons can be shifted either lower or higher, which are known as “Stokes shift” and “anti-Stokes shift” respectively, both types of Raman shift reveal the vibration, rotation and frequency transitions in molecules.

**Figure 1.19.** A typical Raman spectrum of SWNTs$^{161}$

In a typical Raman spectrum of SWNTs, there are three characteristic peaks, namely, radial breathing mode (RBM), G band and D band. RBM determines the diameter of the tube, D band counts the defect site, the height of the D band depends on the degree of defects on the SWNTs. G band stands for the integrity of the tubular structure, similar to D band, the more intact the structure is, the higher the peak is.
However, the height of the peak can sometimes be relative due to the size of the SWNTs; therefore, $I_D/I_G$ ratio is normally used to determine the purity of samples.

**Figure 1.20.** UV-VIS spectroscopy method to characterise SWNTs in dispersed phase

Ultraviolet–visible spectroscopy (UV-Vis) (Figure 1.20) is a spectroscopic technique used to quantitatively measure the absorption of light in between ultraviolet and near-infrared spectral regions. Light energy is absorbed by molecules containing π-electrons or non-bonding electrons in order to excite these electrons to a higher energy state. In general, electrons with a lower energy gap will be easier to excite and can absorb light of a longer wavelength. The instrument of UV-Vis spectroscopy consists of five parts, namely: (a) light source, (b) monochromator, (c) sample chamber, (d) detector and (e) signal reader and readout. There are two different light sources in the machine, firstly a deuterium lamp produces the UV light via the following method (3):

$$D_2 + \text{electrical energy} \rightarrow D_2^* \rightarrow D^+ + D^- + h\nu$$  \hspace{1cm} (3)

40
UV light is generated in the range of 160 – 375 nm. Secondly, the tungsten lamp provides light in the visible range from 350 to 2500 nm. Two beams of light will be merged and refracted by a mirror, then the reflected light passes through filter and lands onto the monochromator, only the light with the desired wavelength will be selected to pass to the beam splitter. Subsequently, the light beam is split into two equal intensity beams, one passes through a transparent cuvette containing the sample solution, the other beam passes through the identical cuvette containing only the solvent for a reference reading. Afterwards, both beams are directed into data processor via a photodiode, where the information is analysed and readout. The Beer-Lambert law is the common method used to quantitatively determine the concentration of sample in solution or dispersed phase, is displayed in the following equation (4):

\[ A = \log_{10}(I_0/I) = \varepsilon c L \]  

Where \( A \) = absorbance (A. U.), \( I_0 \) = original wavelength of the light, \( I \) = transmitted wavelength of the light. \( L \) = length of the cuvette, \( c \) = concentration of the sample and \( \varepsilon \) = extinction coefficient, \( \varepsilon \) measures the molecular absorption properties of sample in a given solvent at a certain temperature and pressure. In SWNT dispersion characterisation, there are a characteristic peaks, which appear between 200 to 250 nm and depending on the quality of the SWNTs in the dispersing agent used.\(^{162}\)
1.3. Introduction to fluorescence imaging and design of boronic acid-based fluorescence probes

1.3.1. Basics of fluorescence spectroscopies

The majority of light emission can be categorised into two classes due to their sources, one is known as incandescence, which is generated by thermal radiation, i.e. heat. The other one is called luminescence emission, during which there is no heat generated. In luminescence, sources of light can be further divided into three kinds, namely, biological luminescence, chemical luminescence and physical luminescence, in which light is emitted by an organism, a chemical reaction and physical process, respectively. In physical luminescence, there are seven subtypes of luminescence due to different physical activities, for example, photon, mechanical, phonon, crystallisation, heat, electro and radioactive. Fluorescence belongs to one type of the photoluminescence and is different from phosphorescence (Figure 1.21).\footnote{163}

The primary observation of fluorescence could be traced back to the 17\textsuperscript{th} and 18\textsuperscript{th} centuries, but it was the British scientist Sir George G. Stokes who described this phenomena in a paper entitled “On the change of Refrangibility of Light” in 1852.\footnote{164} this paper describes for the first time, a beam of spectrum from a prism was passed through a test tube contained quinine solution, and then observed that when crossed into the ultraviolet region, the colour of the solution turned from colourless into blue, whereas no emissions observed in visible region. Stoke subsequently concluded that the wavelength of the transmitted light was longer than that of the incident light, wherefore it could be observed.\footnote{165} Stoke named this phenomenon as fluorescence in his second paper.\footnote{166}

There was a remarkable growth in the application of fluorescence spectroscopy in the past 20 years in many different fields of life science, such as biotechnology, flow cytometry, medical diagnostics, DNA sequencing and genetic analysis. The first fluorescence microscopes were developed between 1911 and 1913 by German physicists Otto Heimstädt and Heinrich Lehmann as a by-product from the ultraviolet instrument.\footnote{167}
In the beginning, these microscopes were used to observe bacteria, animal and plant tissues which were auto-fluorescent. Stanislav Von Provazek (1914) applied fluorescence microscopy to study tissues and living cells labelled with fluorescent dye. Finally, in the early 1940s, Albert Coons developed a technique for labelling antibodies with fluorescent dyes, which give rise to the field known as immunofluorescence nowadays. In twenty-first century, fluorescence microscopy is widely used in biological and pharmaceutical areas, due to the high specificity selectivity and sensitivity of fluorescent sensors to their target. The labelled molecules can provide information on many different types of processes on the molecular or nanoscale level. For example, interactions between solvent and fluorescent molecules, distribution of fluorescent biomolecules, distance between different sites of fluorescent molecules, conformational changes of a fluorescent molecule and binding of fluorescent molecules. In addition, most fluorescent probes are cheap and non-radioactive; and hence minimise
the cost of the experiment and damage to the living organism. Therefore, fluorescence is often applied to cellular imaging and single molecule detection. Furthermore, fluorescent sensors can be easily conjugated to other functional groups, such as amides, carboxyl groups and boronic acids, to induce more specificity and selectivity and make the whole device or sensor multifunctional.

1.3.2. Jablonski energy diagram

Fluorescence is a physical chemical process and one form of photoluminescence; which occurs as molecules emit light from their electronically excited states formed by absorption of light. Figure 1.22 represents Jablonski diagram, a well-known diagram to describe the energy state change of fluorescent molecules between light absorption and emission during fluorescence. When a high energy beam, such as UV light or laser beam hit a fluorescent molecule, an interaction occurs between the photon and oscillating electric field of electrons in the molecule. During the process, energy is transferred from the photon to the fluorophore, known as absorption. Absorption only occurs when there is enough energy transferred. In the most cases, a collision occurs between molecules and photons generate more energy than required to promote an electronic transition, the excess energy will be converted into vibrational energy. Therefore, following on light absorption by a fluorophore, it is subsequently excited to a higher vibrational state as it is shown in the Figure 1.22.

From the Jablonski diagram, it can be clearly revealed that the fluorescence process consists of three essential parts, each stage occurs at an extremely short timescale, in the range of microsecond ($10^{-6}$ second) and femtosecond ($10^{-15}$ second). Excitation of a fluorescent molecule from the ground state ($S_0$) to the excited singlet state ($S_1$) takes place immediately ($10^{-15}$ sec) via an incoming photon. Excited molecules are subsequently relaxed to the lowest energy level at excited singlet states via internal conversion in between $10^{-10}$ and $10^{-14}$ seconds. Finally, a longer wavelength of light is emitted and returns the fluorescent molecule to the ground state, this process occurs in the relatively long time period of nanoseconds ($10^{-9}$ s). Although the entire process of fluorescence lifetime is extraordinary short, it clearly inspects the interaction
between light and matter, in order to collect the information for the development of steady state and time-resolved fluorescence spectroscopy and microscopy, which have extremely sensitive emission profiles, spatial resolution, and high specificity of fluorescence investigations, the technique is rapidly becoming an important tool in genetics and cell biology.\textsuperscript{171}

Figure 1.22. Jablonski energy diagram

The energy absorbed from a photon strongly influences the excitation of fluorophores from ground state to excited state, and also directly affect the emission of light in fluorescence or phosphorescence. This amount of energy is measured in quantum terms, which is expressed by the equation (5):

\[
E = h\nu = \frac{hc}{\lambda}
\]  

(5)
This equation (8) known as Planck's Law, represents the relationship between energy (E) and frequency (ν), h is Planck’s constant, c is the speed of light and λ is the wavelength of the incoming photon. According to Planck's Law, the energy absorbed from a photon is either directly proportional to the frequency or inversely proportional to the wavelength of incoming photon, in another words, light at shorter wavelength contain more energy, which can excite more fluorescent molecules to an state and eventually more light can be emitted from those molecules.172

Immediately after excitation of a fluorophore (6), most molecules relax, for example, from $S_2=3$ or $S_1=4$ to $S_1=0$, i.e. the lowest vibrational energy level of the first excited singlet state without emission of light, and a subsequent return to the ground state. This process is known as internal conversion or vibrational relaxation, where energy loss in the absence of light emission, the amount of lost energy is converted into heat, which is then absorbed by surrounding solvent molecules. This process can be expressed in the following equation (7):

$$\text{Excitation: } S_0 + h\nu_{ex} \rightarrow S_1$$  \hspace{1cm} (6)

$$\text{Emission: } S_1 \rightarrow S_0 + h\nu_{em} + \text{heat}$$  \hspace{1cm} (7)

Phosphorescence differs from fluorescence in that excited molecules can also undergo a spin conversion to a triplet state $T_1$, in which the electron in the excited orbital has the same spin orientation as the ground-state electron. Transitions to the ground state are forbidden, in another words, moving from $T_1$ to $S_0$ state is kinetically unfavourable. Although it still takes place in the long term, the emission rates are significantly slower than fluorescence ($10^{-3} - 10^{3}$ second). The progress of phosphorescence is expressed below (8):

$$S_0 + h\nu \rightarrow S_1 \rightarrow T_1 \rightarrow S_0 + h\nu'$$  \hspace{1cm} (8)
Conversion from $S_1$ to $T_1$ is called intersystem conversion (IC). Emission from $T_1$ to $S_0$ is known as phosphorescence. As a result of a slow emission rate, phosphorescence typically have longer excitation lifetimes and generally shift to longer wavelengths, i.e. lower energy, compared to fluorescence.\(^{173}\)

1.3.3. Important parameters in fluorescence: extinction coefficient, quantum yield and fluorescence lifetime

Extinction coefficient ($\varepsilon$), quantum yield ($\phi$), and fluorescence lifetime ($\tau$) are three fundamental parameters commonly used in the measurement of fluorescence to identify different fluorophores. The extinction coefficient represents the ability of a fluorescent molecule to absorb light at a certain wavelength in a given solvent, whereas the quantum yield indicates the efficiency of excited fluorescent molecule to produce an emitted photon. Fluorescence lifetime describes the average time a fluorescent molecule spends in the excited state before returning to the ground state.

The extinction coefficient is emerges from measurements, it is carried out on a UV-vis spectrophotometer, \textit{via} the Beer-Lambert law, which is expressed as (9):

$$A = \varepsilon \cdot c \cdot l \rightarrow \varepsilon = \frac{A}{c \cdot l} \quad (9)$$

Where $A$ stands for absorbance, which is the result collected from the UV-vis spectrum. $c$ refers to the concentration of the fluorescent molecules in a certain solvent and $l$ represents the path length of the light, normally it is the diameter of the quartz cuvette. As it can be seen in the equation, the extinction coefficient is determined by measuring the absorbance of light at a certain wavelength for a given concentration of the chemical in a cuvette with a one-centimetre path length. The chosen wavelength is normally the wavelength that causes the maximum absorption in the ultraviolet to the visible light spectrum. Hence, the extinction coefficient is the parameter that can directly measure the strength of a fluorophore to absorb light.\(^{174}\)

The relative quantum yield is the parameter calculated from a UV-vis and fluorescence spectrum. The quantum yield equation (10) is expressed as follow:
\[ \phi = \frac{\text{the number of photons emitted}}{\text{the number of photons absorbed}} \]

\[ \phi = Q_R \times \frac{D_S}{D_R} \times \frac{A_R}{A_S} \times \frac{I_R}{I_S} \times \left( \frac{n_S}{n_R} \right)^2 \]  

\( \phi \) stands for the quantum yield of a fluorophore molecule, \( D \) is the area of the emission spectrum, \( A \) refers to the intensity reading from a UV-vis spectrophotometer, \( I \) represents the intensity of the fluorescent spectrum and \( n \) determines the average refractive index value of the solvent. Quantum yields normally range between zero and one, the higher the quantum yield, the greater the likelihood that excitation will result in emission. In fact, most imaging applications favour a fluorophore with a high quantum yield, however, the quantum yield of a fluorophore is usually sensitive to the environmental factors, such as pH, concentration, temperature and solvent polarity.\(^{175}\)

Fluorescence lifetimes are the parameter measured via fluorescence-lifetime imaging microscopy (FLIM), which is normally expressed in the following equation (11):

\[ I_t = I_0 \times e^{(-t/\tau)} \]  

Where \( I_t \) stands for fluorescence intensity measured at time \( t \), \( I_0 \) is the initial intensity observed immediately after excitation, and \( \tau \) is the fluorescence lifetime. In general, the fluorescence lifetimes are near 10 ns. However, lifetimes of fluorescence vary between \( 10^{-6} \) and \( 10^{-9} \) second. Several conditions may lead to a difference in lifetime, of which solvent can have a dominant effect. For example, the fluorescence of a single fluorophore will behave completely differently in a uniform or in a mixed solvent.

As can be seen in Figure 1.23 (a) and (b), uniform solvents will have a mono-exponential trend. However, in a mixed solvent, such as in a tissue fluid or inside living cells, there is a multi-exponential curve (Figure 1.23 (c)) detected. In addition, several other processes can compete with fluorescence emission for return of excited state electrons to the ground state, including internal conversion, phosphorescence (intersystem crossing), quenching and photobleaching, by which lifetime of the fluorescent molecules can also be changed.\(^{176}\)
Quenching is another route that leads excited electrons returning back to the ground state via a non-radioactive relaxation, i.e. without the photon emission pathway. Molecules which can act as fluorescent quenchers usually compete with fluorophores in the interaction of light with a higher affinity. As a result, fluorescent quenchers can easily absorb energy from light or the fluorescent molecule, and cause significant reduction or elimination of fluorescence emission. In turn this leads to a reduction in the excited state lifetime and intensity of fluorophores in this work, due to extended conjugated network and π-electrons, SWNTs are considered the quencher system. (Figure 1.24)\textsuperscript{177}

Fluorescent quenchers typically studied are usually non-fluorescent molecules, such as oxygen, halogens, amines, and many electron-deficient organic molecules such as acrylamide, etc. These collide with fluorophores in excited states. Energy is lost as a result of electron transfer, spin-orbit coupling and intersystem crossing to the excited triplet state finally to return to the fluorophores’ ground state.\textsuperscript{178}
In addition to quenching via collision, there are two other types of quenching methods (Figure 1.25). One is known as static quenching, whilst the other is called dipolar resonance energy transfer (dynamic quenching). The former type of quenching occurs when the fluorophores form a non-fluorescent and reversible complex with the quencher molecule in the ground state. This mechanism does not rely on diffusion or molecular collisions. Therefore, the whole process aims to limit the absorption by reducing the population of active and excitable molecules without altering the fluorescence lifetime of the molecule.\(^{178}\) In the excited state, fluorophores can also be quenched by a dipolar resonance energy transfer mechanism. The direct contact is not generally required to transfer energy, but, depending on the requirements of the sensing device, the distance between fluorescent molecule and fluorescent quencher need to be shortened to a certain range in order to transfer the excited state energy from the fluorescent molecule to the quencher non-radiative manner. As a result, fluorophores can lose energy and return to the ground state.\(^{179}\)
Photobleaching is a phenomenon when a fluorophore permanently loses the function to be fluorescent due to prolonged excitation. This in turn leads to irreversible chemical damage and structural modifications. Photobleaching is normally due to an intersystem crossing from excited singlet state to the excited triplet state. The triplet state is longer-lived compared to the excited singlet state, therefore a triplet excited fluorophore has a much longer period of time available to undergo chemical reactions with other molecules whilst in the excited state. This can lead to irreversible damage to the fluorophore, which can accumulate and eventually cause irreversible photobleaching. Each fluorophore has different average numbers of excitation and emission cycles, which is determined by its molecular structure and the environment, e.g. the solvent system used.

In fluorescence sensing research, only those fluorophores that are robust and can undergo millions of cycles before bleaching are sought. In addition, there is another type of photobleaching mechanism, which is known as photodynamic. This particular photobleaching process includes all fluorophores and is whereby the fluorophore can react with both light and oxygen to produce singlet oxygen species (also known as reactive oxygen species, ROS). As mentioned previously, ROS are very reactive and
can chemically modify other molecules in living cells. As a result of photodynamic photobleaching, the structure of the fluorophores is destroyed. Cell death occurs due to apoptosis and necrosis, and in the most severe cases, some cells undergo transformations and become tumourgenic, due to DNA damage induced by ROS. Since more and more fluorescent sensors are employed into biological and medicinal research, photobleaching, especially photodynamic photobleaching has become a matter of concern. Hence, there are currently three methods in use to minimise the photodynamic effect. The first and simplest way is to reduce the time period of exposure to the light source or to lower the excitation energy. However, this method reduces the fluorescent signal as well. Alternatively, deoxygenated solutions of fluorophores or cell suspensions are induced, but this method is not feasible for use with living cells and tissues. Finally, an approach is induced that limits the exposure of the fluorophores to intense light source by using neutral density filters. These are coupled with anti-photobleach reagents which can be added to the solution and cell culture media.

1.3.4. Current design for fluorescence based sensor

There are two main systems under current investigation as mono-fluorophore fluorescent sensor. One type is based on the theory of Photo-induced Electron Transfer (PET), the other one is based on a method known as Intramolecular Charge Transfer (ICT).

Photo-induced electron transfer is the most commonly used principle in the design of fluorescent molecular sensors due to the “off-on” system to control the activation of fluorescence of the sensor. A typical photoinduced electron transfer sensor consists of three parts: (a) fluorophore, (b) spacer and (c) receptors. The fluorophore part can absorb light and excite electrons to give a fluorescence emission. The receptor is the core part for the molecule recognition and activation of fluorescence. The spacer is an organic or inorganic functionality which connects the fluorophore with a receptor to form a stable and functional system. The spacer normally contains a lone pair of electrons which can be filled either into the empty electrical orbital of the excited fluorophore or the receptor. In case of filling into the fluorophore, the excited electron from the fluorophore is unable to return to the original orbital and prolonged excitation
of the electron leads to the quenching of fluorescence emission. This state is known as the “off” state of the Photo-induced electron transfer system. (Figure 1.26 a)\textsuperscript{186}

\textbf{Figure 1.26.} Schematic representation of a PET fluorescent sensor: (a) Photo-induced electron transfer sensor without the target molecule; (b) Photo-induced electron transfer sensor incorporating the target molecule.

Once receptors are attached to target molecules, the binding constant of receptors will be changed; hence, the lone pair of electrons from the spacer will be donated preferentially to the receptor instead of to the fluorophore. As a result, the
excited electron of the fluorophore returns to the ground state to complete a fluorescence cycle and a fluorescent signal will be again detected. This is called “on” state of the Photo-induced electron transfer system. (Figure 1.26 b)\textsuperscript{187}

![Diagram](image)

**Figure 1.27.** Schematic representation of an ICT fluorescent sensor, (a.) Without a target molecule (b.) With target molecules binding

Intra-molecular Charge Transfer (ICT) systems are also widely used in this research area due to its simplicity. ICT type fluorescent sensor consists of two parts, i.e. a fluorophore and a receptor; there is no spacer in between. As a result, charge transfer from fluorophore to receptor plays an important role in the molecule recognition and fluorescence emission. In the resting state, (Figure 1.27 a) ICT-based systems normally emit no fluorescent signals. Once receptors bind onto the target molecules, the binding
complexes will affect the electron density in the fluorophore, leading to a change in the fluorescence emission. (Figure 1.27 b)\textsuperscript{188}

![Figure 1.28. Schematic representation of a fluorescent excimer sensor](image)

Figure 1.28 shows the schemes of excimer fluorescent sensor. This is a multi-centre fluorophore sensor. This contains more than one fluorophore in its structure and when fluorescent sensors bind to target molecules; the conformation of the sensor will be altered. As such the distance between two fluorophores will be shortened and an intermolecular excimer will be formed. The excimer carries a broader and stronger wavelength emission than their individual monomers, as a result, the intensity of the fluorescence is increased. The distance between the fluorophores plays an important role in the formation of the excimer, if it is too large, the excimer will not be formed.\textsuperscript{189} If the distance is too short, the fluorophores have been found to stack and quench each other’s fluorescence. Therefore, the distance between two fluorophores need to be carefully adjusted in the synthetic design in order to get an ideal excimer fluorescent sensor.\textsuperscript{190, 191}
1.3.5. Design of biocompatible fluorescent sensors

A fluorophore is itself normally non-selective, non-specific and non-sensitive to meet a given biomedical purpose, and also as mentioned previously, many fluorophores can introduce ROS during the process of photodynamic photobleaching. Hence, a further modification of the fluorophore is required in order to make fluorescent molecules more biocompatible.\textsuperscript{192}

![Diagram of fluorescent sensors]

\textbf{Figure 1.29.} Schematic representation of a typical fluorescent sensor developed

As it can be seen in figure 1.29, a typical fluorescent sensor designed recently in James and Pascu typically groups consists of three parts including: a fluorescence group, a spacer and a targeting group. The fluorescence group is the core part of the fluorescent sensor, which absorbs the light energy, excited to give a fluorescent signal, the wavelength of the light emission depends on the chosen fluorophore.\textsuperscript{192-194}
There are six commonly used commercial fluorophores that absorb and emit light that cross the whole visible spectrum range (Figure 1.30). These fluorophores can absorb light at individual excitation wavelength and emit at characteristic wavelength. For instance, in the case of fluorescein, an excited state is obtained by absorbing light at 488 nm. Subsequently, a fluorescent emission takes place at 524 nm in an aqueous environment. In general, the majority of the fluorophores used in research are excited between 300 ~ 450 nm, which gives a refined fluorescent signal. However, this is not perfect for biomedical imaging applications, because the light source is high energy and will not only bleach fluorophores, but also cause damage to the tissue of a living organism. As mentioned above, high power lasers increase oxidative stress levels in living cells and tissues, leading to mutations in cells and resulting in cell apoptosis or tumours. Hence, near inferred fluorophores are preferred for the labelling of biological
molecules. Whereas a low energy light source raises another problem, most fluorescent molecules will not be excited, as a result, the intensity peak of the spectrum will be significantly lower than those of the UV side fluorophores. This also explains why a fluorescent sensor structure is developed, because anchoring a fluorophore to a spacer not only increases the distance between the fluorescent molecule and receptor molecule to avoid the interruption of the receptor molecule to the fluorophore, but also the signal of the fluorescence can be vary depend on the length of the spacer. The targeting group on the sensor is applied to recognise a molecule of interest, for example, in Figure 1.31, the molecule attached is a boronic acid group, which has a considerably high affinity for di-ols. Therefore, this type of molecular sensor will have sensitivity to detect and target monosaccharides, polysaccharides and glycoproteins in biological experiments. Binding to these target molecules has been shown to lead to an electrochemical change of the receptor molecule. This alteration of the electronic state is conducted via the spacer to the fluorescent molecule and initiates either enhancement or quenching of the fluorescence, which can in turn be monitored by fluorescence microscopy.

In addition, there has been some recent work involving the use of an extra functional group, which was applied onto an earlier sensor design from the Pascu group. This is a biomarker and enables the fluorescent sensor to apply for the biological purpose, for example, to detect and label cancer cells. Biomarkers can vary dependent on the cancer type and typically have a very high affinity to bind to those cancer cells. Unlike the common sensor based on photo-induced electron transfers, a sensor incorporate a biomarker is a dual targeting sensor. Therefore, the fluorescence cannot only be switched on and off, but also be enhanced or quenched once the sensor binds to the selected biological target.

1.3.6. Applications of boronic acids as receptor molecules

The receptor section of the fluorescent sensor takes responsibility for molecular recognition and activation, and a variety of receptors can be utilised dependent on the purpose and the guest molecule that it is interested in. As for the applications in a
biocompatible fluorescent sensor, boronic acid-related receptors are becoming the focus of increased attention due to several distinct advantages: Firstly, boronic acids have a significantly high affinity to react with a hydroxyl group to form an ester in aqueous solutions. As can be seen in Figure 1.31, boronic acids covalently react with 1, 2-diols (or 1, 3-diols) to form five (or six) membered cyclic esters in aqueous solution. Therefore, binding to hydroxyl-rich compounds, such as sugar, will considerably increase the solubility of the fluorescent sensor and also improve the biocompatibility at the same time. On the other hand, this reaction is reversible, which means that the equilibrium can be affected by changing the pH of the reaction environment; as a result, the binding rate to the target molecules can be essentially controlled in solution.\textsuperscript{197}

![Equilibrium reactions between a boronic acid and dihydroxyl groups in a functional diol](image)

*Figure 1. 31. Equilibrium reactions between a boronic acid and dihydroxyl groups in a functional diol*

Regardless of the ability of boronic acids to improve the biocompatibility of a fluorescent sensor, there are two other properties of boronic acids, which have been shown to draw the interest of researchers. One is their solubility and the other is the “off-on” system introduced by boronic acids in a PET system. Boronic acids are Lewis acids and can be easily be dissolved in water and form two different conformations.

![Behaviour or a boronic acid functionality in aqueous media](image)

*Figure 1. 32. Behaviour or a boronic acid functionality in aqueous media*
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Figure 1.33. Different possible routes for the boronic acid binding to diols

As shown in Figure 1.32, the original geometry of the boronic acid is trigonal plane, but it can also form a tetrahedral form in water by receiving a hydroxyl group from a water molecule. Both two conformations coexist in the system and can react with diol groups to form cyclic ester ring structures, as shown in Figure 1.33. As a result, there is more than one binding constant in the system, i.e. $K_2$, $K_3$ and $K_{eq}$. $K_2$ stands for the binding constant of the triangular boronic acid to the trigonal ester. $K_3$ refers to the binding constant of tetrahedral boronate to its ester form. However, both forms of the boronic acids coexist randomly in the solvent. Therefore, both $K_2$ and $K_3$ cannot represent the overall binding constant. As a result, the overall binding constant $K_{eq}$ is introduced, regardless of the conformation of the boronic acid groups in the solvent. All binding constants used in this project are $K_{eq}$.198

Figure 1.34. Electron transfers of lone pair electrons in photoinduced electron transfer fluorescent sensor: Case A. PET sensor not bind to any target molecule. Case B. PET sensor bind to target molecule
The “off-on” response is another characteristic feature of a boronic acid PET fluorescent sensor. The response is introduced by a boronic acid and controlled by the pH level of the solvent system. The basic principle for the “off-on” system is shown in Figure 1.34, at the “off” state (A), a lone pair is donated from the amide group to the fluorophore and so quenches the fluorescent signal.\(^{198}\) The optimum pH for boronic acid receptor binding to target molecules is 8.3, whereby the boronic acid receptor can easily bind to sugar molecules. The dissociation constant of boron centre then drops from 8.8 to 7 and as a result, lone pair from amide groups will more appreciated to be donated into boron ester group.\(^{199}\) Therefore the fluorescent signals are no longer be quenched (Figure 1.34). A boronic acid PET sensor with “off-on” system is sensitive to both target molecule binding and pH change.\(^{200,201}\) At the “off” state there is no fluorescent cycle and therefore no photo-bleaching. Hence, such a system is believed to have a rather low toxicity to living organisms and thus, could be considered as an ideal tools for both in vivo and in vitro sensing and imaging experiments.\(^{202}\)

1.3.7. Characterisation of fluorescence sensors

Confocal laser-scanning microscopy is an optical imaging technique, which involves a laser light excitation. An intense, narrow wavelength beam of light is generated from the laser generator and focused through the microscope lens to a single spot in the focal plane (see Fig. 1.35). The emission generated in the sample at the focal position is collected by a fast detector, usually a photomultiplier tube (PMT) and the laser beam is consecutively shifted by scan mirrors to a large number of raster positions. An image of the sample at a certain focal plane can be reconstructed from the time course of the recorded signal and the predefined path of the laser focus. Three-dimensional reconstructions can be formed by stacks of images from different focal planes. The main difference between conventional fluorescence microscopy and confocal microscopy is there is a confocal pinhole in front of the detector in the confocal microscopy. This pinhole prevents light reflected from above or below the focal plane in the sample from reaching the detector. As a result, out-of-focus emission is
eliminated, leading to an excellent spatial resolution and enables acquisition of refined optical sections from within thick biological samples.

However, there are also some drawbacks of confocal microscopy when applied to live imaging. First of all, with the existence of the pinhole, the sensitivity of confocal microscopy is significantly reduced due to the majority of light is blocked by the pinhole. In order to improve the resolution of images, the strength of light intensity will be increased simultaneously. As a result, photo damage introduced to the tissue as well as the fluorescent dye was bleached. Therefore, the ratio between excitation intensity and signal photons may become unacceptable in deep tissue layers, particularly in strongly scattering specimens. Confocal microscopy is a useful methodology in this project for investigating the fluorescence distribution of the sensor and the morphology of cells.

![Confocal microscopy diagram](image)

**Figure 1.35.** Schematic presentation of confocal microscopy

Fluorescence-lifetime imaging microscopy (FLIM) is an imaging technique which can produce an image based on the differences in the exponential decay rate of the fluorescence from a fluorescent sample. The lifetime decay of the fluorophore signal, (rather than its intensity), is used to create the image in FLIM. An average
fluorescence lifetime of a fluorophore is calculated at an individually spatially resolvable element of a microscope image. The nanosecond excited-state lifetime is independent of the probe concentration or light path length but dependent upon excited-state reactions. Hence, it minimises the effect of photon scattering in thick layers of the sample.

The FLIM consists of four parts, namely, pulse laser generator, scanner, detection PMT and Time-correlated single-photon counting (TCSPC) card. A beam of laser light is generated and pulsed onto a scanner, the scanner redirects the laser and scans the sample in a raster manner. Subsequently, the light is reflected and directed towards detection PMT via a dichroic mirror. The fluorescent signal is enhanced by the PMT and analysed by the computer, the data is recorded in a computer. In this project, FLIM is applied as a reliable method for determining the lifetime of a fluorescent sensor in a given environment, and also the lifetime change when a fluorescent sensor binds to its target, and when subtle changes of its environment occur as a result.

Figure 1. Schematic representation of FLIM microscopy setup
1.4. Applications of SWNTs and their decoration with fluorescent tags in cancer research

1.4.1. Introduction to cancer

Cancer is a group of various diseases involving unregulated cellular growth. In general, cancer cells can divide and grow rapidly and unlimitedly to form malignant tumours, and as a result, cancerous cells invade nearby tissues and parts of the body. Cancer is normally caused by genetic combined with environmental factors. In fact, only 5 – 10% of cancer is due to genetic defects alone. Most cancers are caused by environmental factors, such as smoking, obesity, life-style, certain infections, exposure to ionizing radiation and pollutants, all of which can alter the gene expression of healthy body cells, to form cancerous cells. In the early stage, the majority of the cancer cells possess similar characteristics to the other cells in the body, which makes early diagnosis of cancer extremely challenging.\textsuperscript{203}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{cancer_stages.png}
\caption{Stages of cancer progression}
\end{figure}

As seen in the Figure 1.37, tumour genesis is initiated with a cell mutation to form an oncogenic cell, which can replicate itself to increase the number and size of the oncogenic cell, this progress is known as hyperplasia. As the mutation of the oncogenic cell further accumulates, dysplasia subsequently occurs, which converts the oncogenic cell into a primary tumour cell. A group of primary tumour cells will form a carcinoma in situ (CIS). In the clinic, CIS is categorised as stage 0, all cancer cells are located in the place and replicate themselves, stage 0 and stage 1 cancer do not have abilities to
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Invasion of cancerous cells involves them spreading to nearby tissues. This stage of cancer is often highly curable, usually by removing the entire tumour mass with surgery. However, as mentioned above, these cells also are the most difficult stages to detect. As the cancerous cells keep growing, tumour mass becomes enlarged in size and grown more deeply into nearby tissue and subsequently it can spread to neighbouring lymph nodes. However, the number of cancer cells at this stage are not large enough to spread to other parts of the body. When cancer develops further to stage 4, the cancer has invaded to other organs or parts of the body, which is known as metastatic cancer. Stage 4 cancer is the last stage, at which cancer cells breach the nearby tissue and enter the circulatory system, as a result, cancer cells are transferred to all parts of the body making it impossible to be cured at present.\textsuperscript{203, 204}

1.4.2. Applications of SWNTs in therapeutic drug delivery

Nanomedicines, including those based on functionalised SWNTs in recent work, have been considered as excellent tools for cancer research due to their quasi-one-dimensional nanostructure, unique optical, mechanical and electronic properties, ultrahigh surface area, remarkable cell membrane penetrability and facile functionalisation by different methods. Hence, they are highly biocompatible and have been used in biomedical detection and imaging and CNT based therapeutics, including drug delivery, thermal therapy and gene therapy.\textsuperscript{205}

Fully functionalised SWNTs are ideal vectors in many research applications, such as sensors, probes, actuators, composites, nano-electronics devices and drug delivery systems within biomedical applications.\textsuperscript{206} In recent research SWNTs have been applied as a sensor synthetic scaffold and drug carriers or nanocapsules for biomedical research. In terms of the molecular vectors aspect of SWNTs, their behaviour depends on the method of functionalisation. Functionalised SWNTs have the potential to carry small molecules, peptides, proteins, genes, and DNA across cell membranes often with little cytotoxicity.\textsuperscript{207}
Epidermal growth factor (EGF) and EGF receptors, for example, are normally very low on healthy cell surfaces, however, they are often over expressed on cancer cells, and hence, it is an ideal target site for cancer recognition. Table 1.6 listed a series of cancer receptors that may be overexpressed in cancerous tissues and their corresponding biomarker and anticancer drugs. Figure 1.38 shows a recent research report concerns how a SWNT-based device can be designed as a drug delivery system (DDS) towards cancer cell targeting. First of all, SWNTs can be functionalised via a covalent method, carboxylic groups can be subsequently introduced onto the open ends and sidewalls of carbon nanotubes. Then an epidermal growth factor (EGF) was added to SWNTs via formation of carboxyl-amide bonds: this functionalised SWNT including its biomarker complex is known as an immunosensor. This can help in targeting of the recognition molecules on the surface of cancer cells.

Cis-platin, as well as the other anticancer drugs showed in the table 1.6 can be tagged onto SWNTs and delivered to the cancer cells. It is attached onto the defect site of carbon nanotubes via ester bonds. The whole drug delivery system (DDS) is modified in order to improve the water solubility as well as to selectively deliver to cancer cells. Once EGF on the functionalised SWNT binds to the EGF receptors, the whole DDS will be endocytosed into the cancer cell. Anticancer drugs have been found to be released inside cells and subsequently terminate cancer cells.\textsuperscript{208}
Table 1.6. List of cancer target receptors and their corresponding biomarkers and anticancer drugs

<table>
<thead>
<tr>
<th>Target receptors</th>
<th>Biomarker</th>
<th>Anticancer drugs</th>
<th>Types of CNTs</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGF Receptor</td>
<td>Epidermal growth factor (EGF)</td>
<td>Cl__Cl _H_N_Cl _NH_3</td>
<td>SWNTs</td>
<td>209</td>
</tr>
<tr>
<td>Tyrosine Kinase-7</td>
<td>Sgc8c Aptamer</td>
<td></td>
<td>SWNTs</td>
<td>210</td>
</tr>
<tr>
<td>Endothelial Cells</td>
<td>asparagine–glycine–arginine (NGR)</td>
<td></td>
<td>SWNTs</td>
<td>211</td>
</tr>
<tr>
<td>Folate Receptor</td>
<td>Folate</td>
<td></td>
<td>MWNTs</td>
<td>212</td>
</tr>
<tr>
<td>Integrin αvβ3</td>
<td>Arg-Gly-Asp (RGD)</td>
<td>Doxorubicin</td>
<td>SWNTs</td>
<td>213</td>
</tr>
<tr>
<td>Folate Receptor</td>
<td>Folate</td>
<td>Doxorubicin</td>
<td>SWNTs</td>
<td>214</td>
</tr>
<tr>
<td>Folate Receptor</td>
<td>Folate</td>
<td>Platinum (IV) anticancer complex</td>
<td>SWNTs</td>
<td>215</td>
</tr>
</tbody>
</table>
The research work into the cellular biocompatibility of SWNTs has started almost immediately, once the SWNTs were first functionalised, in study of their potential applying to the biomedical field. Lacerda et al. have shown that SWNT based drug delivery system (DDS) can easily cross the blood brain barriers (BBB) without any external transporter device.\textsuperscript{216} The SWNTs DDS commonly enter cells rapidly in the first hour of internalisation and they distribute in the cytoplasm. There are generally two pathways for the cellular uptake of SWNTs: (a) directly insertion or passive diffusion through the cell membrane. And (b) indirect incorporation \textit{via} endocytosis. The choice of the uptake route depends on the size of the SWNTs, in general, individual SWNTs can enter cell membrane directly, whereas bundles of SWNTs have been shown to enter cells by endocytosis.\textsuperscript{147} For \textit{in vivo} experiments, apart from the conventional oral and injection administration routes, there are also another two ways developed, namely transdermal and ophthalmic drug delivery. The former is designed based on the tiny needle shape of SWNTs and super drug load capacity. Im et al. assembled an electro-sensitive transdermal DDS, which combine carbon nanotubes with polyethylene oxide and pentaerythritol triacrylate polymer and use electrospinning method to control drug release.\textsuperscript{217} As for the SWNTs-based DDS, these have been targeting for ophthalmologic applications designed for several eye disease. Ophthalmic drugs commonly have a short residence time (<5 min) and only 1–5\% of applied therapy may penetrate the cornea and applied to the intraocular tissues. However, the CNTs enhanced ocular DDS has significantly improved the bioavailability of few ophthalmic medications, which have the potential to overcome the limitation of traditional ophthalmic DDS. CNTs could also be used for ocular targeting of different therapeutic agents to the different ocular sites.\textsuperscript{94, 148}

\subsection*{1.4.3. Applications of SWNTs in cellular imaging}

The unique physical properties of SWNTs and their facile functionalisation, make these extremely popular in the biomedical imaging field. SWNTs can be applied in bio-imaging areas in two different ways: (a) SWNTs can be directly used as a contrast reagent. (b) SWNTs materials are used as synthetic scaffolds to carry imaging probes.
to the site of interest. The former one is applicable to imaging techniques, including fluorescence imaging and Raman and photoacoustic (PA) imaging. Semi-conducting SWNTs have a very narrow band gap, which allows SWNTs to emit fluorescence in the near-infrared regions of the spectrum, between 700-1400 nm. Similarly, SWNTs contain a strong resonance Raman scattering with extremely large scattering cross-section. This makes SWNTs promising Raman imaging probes in biological sensing and imaging techniques. Photoacoustic (PA) imaging is a recently developed imaging technique, where a non-ionizing laser pulses are delivered into biological tissues. Some of laser energy will be absorbed and converted into heat, resulting in ultrasonic emissions. This ultrasonic emission are collected by ultrasonic transducers and then analysed to produce images. The latter application of SWNTs in bio-imaging areas include magnetic resonance imaging (MRI), positron emission tomography (PET) and optical imaging. These techniques mainly depend on the contrast agents as probes attached onto the surface of SWNTs.

Figure 1.39 (a) represents schematically a covalent method of assembling DDS probes, which is similar to the assembling method showed in Figure 1.38. As such, SWNTs are oxidised and carboxyl groups are introduced and, subsequently, both EGF and fluorophores are loaded at the defect site and at the end of SWNTs. Hence, the complex can selectively target cancer cells and is traceable in vitro. Instead of the covalently insert of functional groups, a non-covalent wrapping method is normally adopted to coat SWNTs with a water soluble polymer, such as polyethylene glycol (PEG) or β-D-glucan (Figure 1.39 b.). Compared with covalent functionalisation, which allows the use of only a limited number of defect site and ends, glucans have many binding sites available on their surface. Hence, the loading capacity of fluorescent sensors onto glucan-functionalised SWNTs is likely to be improved. In addition, SWNTs have a strong quenching effect to many fluorophores, hence, the direct attachment of fluorescent molecules will cause the loss of fluorescence emission. Furthermore, pristine SWNTs have been shown to be toxic to cells. Therefore, coating SWNTs with biocompatible polymers has been of interest for biological experiments in using these materials.
Figure 1.39. Schematic representations of fluorescent sensor/ SWNTs complex used in cancer imaging (a) Covalent method; (b) Non-covalent wrapping methods with polysaccharides.

1.4.4. Photothermal therapies mediated by the use of SWNTs

Photothermal therapy is a physical-chemical therapy for cancer treatment, which uses optical radiation in the near-infrared wavelength range (700 - 2000 nm). When a laser is focussed on a tissue, photons are absorbed by inter and intra cellular areas and the energy of photons are converted into heat. As a result, the tissue temperature increases, leading to cell and tissue death. NIR light ranged between 700 and 1000 nm and tissue transparency is ideal for optical imaging and phototherapies. Both MWNTs and SWNTs can exhibit strong optical absorption in the near-Infrared (NIR) regions.\textsuperscript{226} Once a SWNT is exposed to the NIR light, this generates heat by light absorption and induce thermal destruction of cancer cells containing significant concentrations of SWNTs (Figure 1.40).\textsuperscript{227}
Carbon nanotube-associated thermal therapy can be combined with time-resolved infrared imaging techniques, and thus enable photothermal therapy to be more selective and traceable for targeting individual cancer cells or their small clusters labelled with carbon nanotubes. This approach can be utilised for the early stage of cancer treatment and it is very efficient for the treatment of small tumours, the edges of tumours and micro-metastases. In recent research, PEG-functionalised SWNTs as described above are normally injected into the tumour mass site. They are found as accumulated in the nearby muscle and skin tissues after the tumours are destroyed and then slowly translocate into the liver and spleen, from which nanotubes are gradually excreted. This research shows that carbon nanotube enhanced photothermal therapy can potentially become a non-invasive and harmless way of treating cancer.

### 1.4.5. Photodynamic therapy (PDT) using SWNTs

Photodynamic therapy (PDT) is a developing technique currently used for malignant tumours and microbial parasites treatment. It is a very similar technique to PTT, and both techniques kill tumour cells from inside. The probes used and they are nontoxic in the absence of light, then become highly toxic under illumination (Figure 1.41). However, they are having fundamental differences from each other, in that cancer cells are killed by heat radiation in PTT, whereas in PDT cells are eliminated via a rising level of ROS generated in situation.
A typical PDT device consists of three components: a photosensitiser, a light source, and tissue oxygen. The photosensitiser is the core part of the system and, it can generate reactive oxygen species from tissue oxygen under illumination. Porphyrins are considered as good photosensitisers due to their low cytotoxicity in the dark, high absorption coefficient in the red spectral region and high quantum yields for singlet oxygen production. Furthermore, in the recent work, quantum dots have been functionalised onto porphyrin framework. These were used as a resonant compound, able to harvest light and transfer energy to porphyrin in order to further enhance the ROS production. Finally, a DDS consisting of SWNTs, a porphyrin and quantum dots has been assembled and data has shown that DDS had a higher capability to generate ROS than the SWNTs alone. It also made SWNTs-enhanced porphyrin type DDSs good candidates as photosensitisers for PDT.

![Diagram of PDT process](image)

**Figure 1.** Application of SWNTs in photothermal therapy in cancer site

### 1.4.6. SWNTs-enhanced gene therapy

Gene therapy is an alternative method to traditional chemotherapy in cancer treatment. The basic principle of gene therapy is replacing the damaged or missing genes with healthy genes. However, the major challenge for this technique is the gene delivery itself. In the past, researchers have used non-pathogenic viruses as vectors, which occasionally led to a significant immune response. Hence, an ideal vector design is
important for this therapy. Many reports have shown that carboxyl-functionalised SWNTs can act as a vector for gene-encoding nucleic acids and plasmid DNA that enhance the gene therapeutic capacity in comparison with DNA alone. At the same time, some reports showed that siRNA can combine with SWNTs for efficient gene delivery. The siRNA (known as short interfering RNA), is only 20-25 nucleotides long and known to be complementary to the mutated gene. Into the cytoplasm, it can bind to the mutated gene and disable its action the cytoplasm. This process is called gene silencing. From the presented works it is clear that direct injection of siRNA is not an effective approach, whereas the siRNA and SWNT complex can be easily taken up by cancer cells and then revealed by immune recognising cells to induce an immune response for the particular gene, in order to kill cells with damaged genes. Furthermore, SWNTs can also be functionalised by single-stranded DNA and subsequently coated with DNA probes. Thus, DNA probes are protected from the enzymatic cleavage and interference from nucleic acid binding proteins in the circulation. After being taken up by cells, most comprising of SWNT modified with DNA could target a specific mRNA inside living cells, with increased self-delivery potential and intracellular bio-stability when compared with free DNA probes. Hence, this new conjugate provides great potential for applications in the field of genetic engineering.
1.5. Specific objectives of this project

The overall aim is to create a new biosensor devise based on functionalised single walled carbon nanotubes for applications toward cancer cell imaging and therapy.

In the first instance, new types of biotin boronic acid-based fluorescent molecules having the ability to act as fluorescence sensors will be designed and synthesised. Afterward, the titration between different types of fluorescent sensors and β-D-glucan will be carried out and compared with own sensors, in order to determine the binding constant in this boronic acid-glucan interacts prior to the incorporation into the SWNT. Then individual fluorescent sensors will be delivered to different cells to perform MTT assays to study the cytotoxicity effect and also obtain cell images through confocal microscopy techniques, including fluorescence lifetime.

For the SWNTs scaffold material part, SWNTs (CVD-made) is available from Thomas Swan were purified by a steam purification method. Then resulting SWNTs will be functionalised by β-D-glucan through non-covalent methods. After characterisation by TEM, SEM and Raman spectroscopy, these glucan-wrapped SWNTs will be delivered to HeLa cells to investigate any effect on the cytotoxicity. On the other hand, purified SWNTs and opened will be used to encapsulate small ions, such as Zr⁴⁺, Na⁺ and Cu²⁺ as models for unconventional PET imaging agents (⁶⁴Cu, ⁸⁹Zr) and their carriers (NaOAc). After wrapping with β-D-glucan, the composites will be characterised by TEM and Raman spectroscopy. Eventually, these two types of functionalised SWNTs will be incorporated with fluorescent sensors and rendered usable towards in cancer imaging in vivo.
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Chapter 2. Synthesis and characterisation of fluorescent sensors

2.1. Overview

This project builds on some initial work carried out in the Pascu and James group. Each group have previously used biotargeting molecules, in the Pascu group this was with the aim of cancer targeting, whereas in the James group a boronic acid was used to sense sugar molecules. The main purpose of this project was to find a way of combining both methods, to optimise the process and finally to develop a dual-function fluorescence sensor, which can both target cancer and interact with sugar molecules. Biocompatibility and sensitivity for the respective target were the next concerns. Fluorescein isothiocyanate (FITC) is the first fluorophore chosen for the synthesis, due to its relatively strong fluorescence and weak cytotoxicity. Biotin can target sodium-dependent multi vitamin transporters (SMVT) which are generally overexpressed on cancer cells. Boronic acid was used to interact with a β-D-glucan functionalised nanoparticle. Finally xylene di-amine was used as a spacer to separate the other three groups. All groups assembled together could create a fluorescence device for cancer targeting and imaging in different cancer cell lines.

There were four main steps involved in the synthesis of the biotin boronic acid fluorescent sensors for incorporation onto the surface of β-D-glucan functionalised SWNTs. Initially, the synthesis involved the use of a commercially available biotin, xylene di-amine, 4-formyl boronic acid and fluorescein isothiocyanate (FITC) (Aldrich), and each step was followed by thin layer chromatography after completion. Furthermore, purification was achieved by column chromatography on a silica column using MeOH: DCM 2:98. The major product collected was characterised by $^1$H NMR spectroscopy and ESI mass spectrometry. After synthesis of the sensor was confirmed, the biocompatibility of the resulting compound was examined by MTT assays, the biodistribution of the fluorescent sensor was tested in confocal fluorescence microscopy.
in several different cell lines, such as cervical cancer cells (HeLa)\(^5\) and epithelial fibroblast cells (FEK-4), breast cancer cells (MCF-7)\(^6\) and prostate cancer cells (PC-3)\(^7\).

### 2.2. Optimisation of synthetic route.

The protocol of synthesis has been modified several times during the period of project, it gradually changed from the initial scheme, which used 1, 6-hexanediamine as linker and synthesis in a fairly unpleasant solvent, like pyridine, to an alternative plan, by replacing the linker 1, 6-hexanediamine with p-Xylylenediamine. Then several different solvent systems were followed. Eventually, different reaction route were tried to optimise the efficiency of the reaction.

**Method A:**

As it can be seen in Scheme 2.1, N, N-Dicyclohexylcarbodiimide (DCC) was used to enhance the electrophilicity of carboxylate, the carboxyl group on biotin is deprotected and act as a nucleophile, attacking the central carbon in DCC. This allowed DCC to attach temporarily onto biotin and form a highly electrophilic intermediate (Biotin-NHS intermediate), which is unstable and attacked by NHS. Biotin-NHS and dicyclohexylurea were formed as products.\(^8\)
From the $^1$H NMR spectroscopy results (recoded at 300 MHz, in d$_6$-DMSO at room temperature) there are two peaks 4.0 and 4.2 ppm. These are characteristic resonances of the alkyl protons (NHC$_3$H) of the ureido moiety within the biotin ring. A characteristic peak was also found at 2.8 ppm which integrates 3.8 with respect to the ureido CH resonances calibrated to 1.0. This resonance was assigned to the protons from the NHS group. In addition, mass spectrometry displays a main peak at m/z 342.2089, which corresponds to the mass proposed for compound 1 plus one proton, \([C_{14}H_{20}N_3O_5S]^+\). Therefore, the spectroscopic data confirm the formation of compound 1 as the desired biotin-NHS.
Scheme 2.2 shows the synthetic pathway of compound 2; the reaction was initiated with compound 1 from step 1 on reaction with 1, 6-hexanediamine. Due to the instability of intermediate (Biotin-diamine intermediate) formed and the NHS group was a good leaving group, therefore, NHS groups would be cleaved from the intermediate. As a result, biotin-hexanediamine was formed.

There are several problems that needed to be solved in order to improve the yield and the purity of the product emerging from this step in further experiments. First of all, a large excess of amine was added to prevent compound 1 from reacting with each end of the hexanediamine. Next, a pressure equalised dropping funnel was used to control of dropping rate of compound 1. Compound 1 needed to be added slowly to ensure that compound 1 only attacked at one end of the hexanediamine, but this slow addition rate appeared to limit the whole reaction rate. Finally, pyridine / water (50% /
50%) mixture was found to be the most suitable solvent to dissolve both compound 1 and hexanediamine in this experiment. However, pyridine was a rather toxic chemical especially in the higher quantities required to dissolve the biotin NHS and control the pH of the reaction. Therefore, a DMF / TEA mixture was found to replace pyridine in further experiments, to reduce the toxicity risks. At the same time, in order to avoid biotin- NHS being attacked at both ends of the amine, a tert-butyloxycarbonyl (tBoc) protecting group was introduced to protect one end of the hexanediamine, prior to biotin-ester formation. The Boc protecting group could subsequently be easily removed by treatment with TFA: CH₂Cl₂ (1: 1). However, this method increased the number of steps and experimental costs overall but seemed to lead to a purer product that was more easily separated from the starting materials.

As described in the experimental part, it was clear that both ¹H NMR spectroscopy and MS spectra showed positive results. As such in ¹H NMR the diagnostic peaks were at 1.83–1.34 ppm and mass spectrometry showed a peak at m/z = 341.2040.

Scheme 2.3 represented the reaction between compound 2, pinacol and 1, 4-formyl phenylboronic acid to produce compound 3. In Figure 2.3 a schematic mechanism showing how pinacol could react with boronic acids via a condensation process, resulted in the formation of a ring structure, in such that the boronic acid is protected by pinacol.

Then Scheme 2.3 b, shows how the primary amine reacted with the aldehyde group on phenylboronic acid via a reductive amination. The amine first reacted with a proton to form a hemiaminal species, which reversibly lost one molecule of water via alkylimino-de-oxo-bisubstitution, to form an imine. The equilibrium between aldehyde and imine could be shifted toward imine formation by removal of the formed water through physical means, herein molecular sieves (3Å) were added to the mixture to absorb H₂O and drive the thermodynamic equilibrium towards imine formation.
The $^1$H NMR spectroscopy (300 MHz, d$_6$-DMSO) and ESI$^+$ mass spectrometry results obtained were not as conclusive for compound 3, indicating that a possible mixture of products remains present. $^1$H NMR spectroscopy showed the occurrence of some large, sharp resonances at 1.10-1.80 ppm and several other weaker resonances (at 2.0 - 3.0 ppm range) inhibit the spectrum baseline. Consequently, NMR spectra suggested that compound 3 was impure with presence of remaining starting materials and solvent. As for mass spectra, there are several other un-assignable peaks which could be assignable to the starting materials. Hence, it was necessary to further purify the resulting product at least two or three times. In this project column chromatography on silica was used to separate product from impurities by using a mixture of MeOH:
DCM 2:98% as the mobile phase. The resulting white powder resulting from the purification and subsequently removal of all solvents was used directly in Step 4 as described below.

**Scheme 2.4.** Schematic representation of the synthesis of Fluorescein isothiocyanate linked biotin-boronic acid fluorescent sensor and proposed mechanism

Scheme 2.4 shows the reaction between compound 3 and Fluorescein isothiocyanate (FITC). The isothiocyanate group of FITC was reacted with the NH group of compound 3 under mildly alkaline conditions to form a thiourea derivative, which was compound 4.

In conclusion, synthesis of the FITC boronic acid fluorescent sensor was well established here and well defined conditions were put in place up to Step 3, according to size, lipophilicity and complex separation issues occurring in the synthesis of compound 4. These made compound 4 difficult to characterise and, the formation of compound 4 was confirmed by the mass spectrometry. However, due to the low yield, neither $^1$H NMR nor $^{13}$C NMR were sufficiently concentrated to characterise compound 4.\textsuperscript{11}
Compound 3 had been modified by replacing the 1, 6-hexanediamine group with p-Xylylenediamine to produce compound 7. This should make the structure more rigid and alter the solubility properties of the compound. Hence, it would be easier to separate the final product from the starting materials by recrystallization or column chromatography.

**Method B:**
Once the condition of the reaction and solvent system was confirmed, the rigid linker was introduced and the new compound was synthesised as described below and in experimental section. However, the difficulty to synthesise the fluorescent sensor varies dependents on the routes of assembling the necessary building blocks, as described below.

![Scheme 2.5](image)

**Scheme 2.5.** Schematic representation for the synthesis of Fluorescein linked biotin-boronic acid fluorescent sensor with a p-Xylylenediamine linker (route 1).
Figure 2.1. NMR spectrum of compound 5, (a) $^1$H NMR spectrum (b) $^1$H COSY spectrum and (c) correlation of N-H to the alkyl protons and N-H to $\alpha$ protons of biotin ring (red), in $d_6$-DMSO.
As can be seen in the Scheme 2.5, the initial route was the same as the route used to assemble compound 4. Both routes started with biotin-NHS and the desired linker, subsequently boronic acid and pinacol were introduced. The fluorescent group was introduced at the final stage, to produce the target compound 5. The formation of compound 5 is characterised in Figure 2.1 (a), the characterisation peaks appeared at 4.3 and 4.1 ppm, which referred to two protons from biotin structures, as well as a typical peak at 1.39 ppm, which represents the boc protection group from the xylidencyanediamine group. The presence of the biotin apparent in Figure 2.1 (b) and (c), which indicated the correlation between N-H and α protons of biotin ring. 1H NMR spectrum of compound 7 is represented in Figure 2.2, Characteristic resonances of the α protons of the biotin appeared at 4.30 and 4.10 remained in the same region of the spectrum for compound 5 in Figure 2.1. However, the resonance of a boc group at 1.39 ppm was not observed,
which indicated that the boc protection group was successfully removed. There were more resonance peaks added in the aromatic region between 6.0 and 8.0 ppm, which integrates 16.8 with respect to the ureido CH resonances calibrated to 1.0. This resonance reveals the attachment of the boronic acid group and fluorophore group. The peaks appeared at 4.70 ppm suggest the presence of the alkyl proton in the boronic acid. Finally, there was no resonance detected at 1.27 ppm, which indicated that the pinacol protection group was removed during the synthesis.

Despite the simplicity of this method, one problem remained, which was that Biotin-NHS was difficult to dissolve in common solvents. Therefore, all reactions were carried out in DMF. As a result, the evaporation of DMF was time consuming and this made this route rather inefficient. More importantly, due to lack of solubility of the reaction compounds, a significant amount of the starting material remained as an impurity, and was difficult to remove this from the product. Hence, in order to improve the efficiency of the synthesis, a different route was attempted, as displayed in Scheme 2.6.

**Scheme 2.6.** Synthesis of Fluorescein isothiocyanate linked biotin-boronic acid fluorescent sensor with a p-Xylylendiamine linker (route 2)
Route two started with a reaction between boc protected xylylenediamine and fluorescein isothiocyanate. Subsequently, biotin was introduced to form compound 9. Route two did not proceed further due to the strong electronegativity of the sulphur group, which drew the lone pair electron on the xylylenediamine toward itself, preventing a further reductive amination. The advantages of this approach is that xylylenediamine and fluorescein isothiocyanate are soluble in DMF, which improve the rate and efficiency of the reaction. However, due to the strong interaction of –NH–CS–, additional boronic acid could not be functionalised onto compound 9 via a reductive amination. Finally, compound 9 was used as a comparison to compound 7, in order to investigate the difference between a compound with a boronic acid group and the other without a boronic acid group in both chemical and biological experiments.

Figure 2.3. $^1$H NMR spectrum of compound 8 in d$_6$-DMSO
Compound 8 formed in Scheme 2.6 was characterised by the result of $^1$H NMR spectrum showed in Figure 2.3. There were many peaks appeared in low field region between 6.5 and 8.5 ppm, indicated the aromatic protons from the boc protected xylylenediamine and FITC group, the characteristic resonances at 1.39 ppm revealed the presence of the boc protection group. Finally, the m/z result found at 624.1861 (ESI-MS) in negative mode also in support of the formation of compound 8.

![Figure 2.4. $^1$H NMR spectrum of compound 9 in deuterated methanol](image)

Figure 2.4 shows the $^1$H NMR spectrum of compound 9. Characteristic resonances of the $\alpha$ protons of the biotin ring at 4.30 and 4.10 remained in the same region of the spectrum as showed in Figure 2.1 for compound 5. However, the resonances of the boc group at 1.39 ppm were not observed. In the low field region of the spectrum, a new peak was detected and confirmed at 10.70 ppm by using 2D COSY techniques (Appendix D.5). This was assigned to the carboxyl group of the FITC moiety.
Furthermore, there were more resonance present in the aromatic region between 6.0 and 8.0 ppm. Finally, the formation of compound 9 was also confirmed by mass spectrometry.
As can be seen in Scheme 2.7, Route three was established to replace Route two in order to synthesise compound 7. The reaction began with boronic acid reacting with
pinacol to form a pinacol-protected boronic acid (compound 10). Compound 10 was then reacted with boc protected xylylenediamine to form compound 11. Subsequently, a FITC group was introduced and generated compound 12. Finally, the t-boc group was removed by TFA and the biotin-NHS was added to the system to form compound 7.

The $^1$H NMR spectrum in Figure 2.5 represents the characterisation of compound 10 synthesised in Scheme 2.7. It is apparent that there were two characteristic resonances detected at the low field region at 10.05 ppm and 7.95 to 7.85 ppm respectively, which integrates 3.63 with respect to the aldehyde proton calibrated to 1.0. This resonance was assigned to the protons from the aromatic group. Subsequently, pinacol protons were found at 1.35 ppm as a single peak, which integrates 11.9 with respect to the aldehyde proton resonances calibrated to 1.0. There remained some starting material and the yield calculated from integration was 81.9%.

This route was an efficient pathway to produce compound 7. The main advantage of this route was that the first two steps could be processed in common solvents, which made the reaction efficient, and the products were easier to collect and analyse. However, there were still some drawbacks, for instance, introduction of boronic acid significantly increased the difficulty of purification via column chromatography, affecting the final yield of the product. At the same time, an early import of FITC group would make the NMR assignment extremely challenging. Finally, early activation of FITC consumes the life of the fluorescence, if compound 12 was not protected properly, the quality of the final compound would be influenced remarkably. Therefore, the FITC group was considered to be added in the last step, this lead to the establishment of route four, which is given in Scheme 2.8.

Route four was the final method designed to assemble this boronic acid-based fluorescent sensor. This route was very similar to route three, apart from the fact that it involved the synthesis of the linker before introducing the fluorescent groups. Although it had the disadvantage in aspects of purification caused by earlier addition of the boronic acid group and the fact that only a small yield of compound 7 could be collected from column chromatography, the amount was enough for both chemical and biological experiments. Therefore, this route would be suitable for small laboratory scale synthesis of the boronic acid based fluorescent sensor for optical imaging experiments.
Scheme 2.8. Synthesis of Fluorescein isothiocyanate linked biotin-boronic acid fluorescent sensor with a p-Xylylenediamine linker (route 4)

2.3. Kinetic stability tests for fluorescent sensors

All experiment involved in this section were with the purpose of testing the stability of the fluorescent sensors under aqueous environments. There were two conditions tested for: (1) time dependency, in which samples were examined at regular time intervals and (2) solvent dependency, in which samples were dissolved in different solvent and examined, by fluorescence spectroscopy.\textsuperscript{13, 14} Although both UV-Vis and fluorescence spectroscopy techniques can be used to study kinetic stability of fluorescent molecules, fluorescence spectroscopy can provide extra information in quenching and photobleaching, hence, fluorescence spectroscopy was chosen to examine the stability.
Figure 2.6. Fluorescent stability study of compound 7 in a period of time in methanol: water mixture (1:40)

Figure 2.6 displays the stability test results of compound 7 in a methanol: water mixture (1:40). The fluorescence of solution was measured every 30 minutes in 4.5 hours. Although there was little fluctuation in the intensity, the variation of intensity was in the range of ± 10 A.U., in addition the peak of all measurement stayed constant at 525 nm. All signs indicated that compound 7 was a stable fluorescent sensor and its emission was not affected over the time period.
This part of the experiment was specifically designed for the fluorescent sensors that were used for biological applications. Samples would be used for applications in living cells cultured in serum media or serum-free media, therefore the variations in the fluorescence emission need to be recorded first under similar conditions but in the absence of cells. Figure 2.2 shows that, there was a significant growth in interaction from 270 A.U. to 420 A.U. and 630 A.U. in the fluorescence intensity for serum media and serum free media in samples recorded under same conditions. It indicated that the fluorescence of compound 7 was enhanced in the culture media and it was favoured in the biological applications. Simultaneously, there was a slight red shift from 525 nm to 550 nm in the emission wavelength for both media, this phenomena indicated compound 7 may bind to some biomolecules present in the media and altered its emission wavelength.\(^{15}\)
Figure 2.8. Fluorescence emission stability assay of compound 7@ glucan@ SWNTs in a period of time in water

Figure 2.8 showed fluorescent stability of compound 7@ glucan@ SWNTs (synthesised as described in experimental section) over 4.5 hours. It was obvious that compound 7@ glucan@ SWNTs was not stable in water, the initial intensity was 250 A.U., with it ending at 400 A.U. The increased rate was fast at the beginning, however the increase gradually reduced until it was insignificant towards the end. In addition, all peaks appeared at 525 nm, hence, no conformational changes occurred during the experiment. This might be due to SWNTs aggregating and precipitating out of the system, thus less fluorophores were quenched, which resulted in an increment in the fluorescence.
2.4. Qualitative avidin binding tests for the fluorescence sensors

In this project biotin had been chosen as a general biomarker for all as made fluorescent sensors due to the strong binding affinity of biotin avidin. Therefore, an avidin binding test was necessary to investigate whether the binding between biotin and avidin would affect the fluorescence of fluorescent sensors.\(^{16}\)

![Fluorescence spectra of compounds](image)

**Figure 2.9.** Fluorescence spectra of compounds in methanol and water mixture (1:100) before and after addition of avidin (1:1)

As described in Figure 2.9, compound 7 alone had a relatively intensive fluorescence peak at 425 A.U., once avidin was added, there was a significant drop in the fluorescence intensity to 110 A.U.. Thus activation of the biotin-avidin complex would remarkably quench the fluorescence intensity of compound 7. More interestingly, compound 9 which lacks a boronic acid group possessed a reduced intensity peak at 250 A.U. and it behaved similarly to compound 7 in that once it was bound to avidin, there was a dramatic decrease in fluorescence emission intensity from 250 A.U. to 60 A.U.
As a result, it could be summarised that a compound accompanied with a boronic acid would have a stronger fluorescence emission intensity and binding to avidin would quench the fluorescence regardless of boronic acid existence.

### 2.5. Direct interaction between fluorescence sensor and purified SWNTs

Most of the fluorescent sensors in this project were functionalised on to \( \beta \)-D-glucan wrapped SWNTs. Experiments were designed to explore whether or not the fluorescent sensors can functionalise directly onto the SWNTs in the absence of glucans.\(^{17-19}\)

![Fluorescence emission measurement](image.png)

**Figure 2.10.** Fluorescence emission measurement of compound 7, compound 7 @ SWNTs and compound 7 @ glucan @ SWNTs

The resultant spectra (Figure 2.10) showed that a direct contact of SWNTs would immediately quench the compound 7 and it was also obtained that this quenching
dose not recover during time. This result demonstrated that covalent functionalisation of SWNTs would not be ideal. The quenching effect might be due to a strong surface energy of SWNTs, hence, this effect would be reduced via increasing the distance between compound 7 with pristine SWNT strands. β-D-glucan wrapping was not only blocking the direct contact of compound 7 from SWNTs, but also increased the distance between compound 7 and SWNTs. Hence, it would significantly reduce the fluorescence quenching effects. Although there was still a remarkable drop in the fluorescence intensity from 840 A.U. to 240 A.U., there was a clear fluorescence emission detected at 525 nm, compared with compound 7 @ SWNTs.

2.6. Fluorescence titration of designed sensors

A fluorescence titration was performed to examine the binding constant of fluorescent sensors to β-D-glucan. Theoretically, once the fluorescent sensor binds onto β-D-glucan, there would be a change in the fluorescence intensity via either quenching or enhancing of fluorescence. All peaks of the intensity at increasing concentration of β-D-glucan were collected and plotted in Figure 2.11 in order to work out the binding constant. The titration began with testing the fluorescence of solvent, β-D-glucan and β-D-glucan @SWNTs, there was no significant fluorescence emission from the conditions mentioned above.
Figure 2.11. Fluorescent titration of compound 7 and compound 13 and estimates of binding constant to β-D-glucans. Note: an $r^2$ of 0.74 denotes a low level of reliability for this fitting regarding compound 13: this could be due to the interaction between Bodipy moiety and the glucan, and generation of multiple binding equilibria in solution.
It was clearly showed in Figure 2.11 that the fluorescence emission of both compound 7 and 13 were quenched by β-D-glucan. The binding constant for compound 7 and 13 were 6.5 \times 10^4 \text{ M}^{-1} and 5.6 \times 10^5 \text{ M}^{-1} respectively. A greater binding constant refers to a more stable binding, because compound 13 had two boron groups in its structure and this extra boron group might facilitate to make the binding between compound 13 and glucan more stable. Figure 2.6 also shows that compound 13 binds less reliably than 7 suggesting that multi binding events may take place. The binding constant is determined by the following equation:

\[
I = \frac{I_0 + I_f K_D^{-1} [C]}{1 + K_D^{-1} [C]}
\]

The equation is modified and used to fit all results from the fluorescence titration:

\[
y = \frac{(1+P1*P2*x)}{(1+P1*x)}
\]

Where \(y\) is \(I/I_0\), P1 stands for binding constant \(K_D^{-1}\), P2 refers to \(I_0/I_f\), \(x\) is the concentration of compound.

2.7. Quantum yields of fluorescence sensors

The quantum yield of compound 7 and compound 7@glucan @SWNTs has been calculated using the equation mentioned in the introduction,\(^{20,21}\)

\[
\phi = \frac{\text{the number of photons emitted}}{\text{the number of photons absorbed}} \rightarrow \phi = Q_R \times \frac{D_S}{D_R} \times \frac{A_R}{A_S} \times \frac{I_R}{I_S} \times \left(\frac{n_S}{n_R}\right)^2
\]

Fluorescein was chosen as a reference and 0.4 \times 10^{-6} \text{ mol of compound 7} was dissolved in methanol, the data collected are given, in Table 2.1. The reference quantum yield of fluorescein (\(Q_R\)) is 0.91, hence, the results of compound 7 and compound 7@glucan @SWNTs were calculated as 0.62 and 0.46 respectively, which indicated there was a decrease in the quantum yield after functionalisation of fluorescein onto the biotin-xylylene-boronic acid linker and the quantum yield was reduced further after compound
Chapter 2. Synthesis and characterisation of fluorescent sensors

7 functionalised onto the glucan wrapped SWNTs. These values are still promising for applications in cellular imaging assays.

Table 2.1. Quantum yield calculation of compound 7 and compound 7@glucan@SWNTs

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<th>A</th>
<th>I</th>
<th>n</th>
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<td>Fluorescein</td>
<td>76900</td>
<td>0.0622</td>
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<td>Compound 7</td>
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<td>Compound 7@glucan@SWNTs</td>
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<td>0.0666</td>
<td>583.66</td>
<td>1.33</td>
</tr>
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2.8. Summary of chapter two

Several synthetic pathways to make a dual function fluorescent sensor were established and tested. The route that assembled biotin-xylylene-boronic linker first and then attached with different fluorophores, was found to be the most efficient and flexible method. The products were purified with column chromatography and then characterised by mass spectrometry, NMR spectroscopy and HPLC. These studies were followed by UV-Vis spectroscopy (Appendix G) and fluorescence spectroscopy investigations, to calculate the quantum yield (QY). The results showed that there was a decrease in the QY compared with the reference fluorophore, which indicated a quenching effect was induced to the fluorophore after the attachment to the linker group and boronic acid. A further decrease in the QY occurred when the fluorescence sensor was bound to the glucan @SWNTs, which demonstrated that anchoring onto the glucan would also quench the fluorophore. A fluorescent titration confirms the results of QY, with the concentration of glucan increasing in the solvent there was a significant drop in the intensity of the fluorescence. The stability test of the fluorescent sensor represented that it was stable in the solvent systems tested, binding to SWNTs would make fluorescence of devices quenched. The fluorescence of the compounds tends to increase over time in a given solvent. The fluorescence of fluorescent sensor was
enhanced in the biological solvent, such as serum free medium and serum medium without precipitation occurring. This indicated the fluorescent sensor is usable in the cellular experiment. The avidin binding test confirmed that biotin is a good biomarker and binding to the target receptor will result in quenching of the fluorescence.

2.9. Reference for chapter two


Chapter 3. Purification and characterisation of SWNTs

3.1. General information

The results discussed in this section include the purification, modification and characterisation of the SWNTs scaffold. These involve the Raman spectroscopy, TEM, SEM microscopy and EDX analysis of SWNTs, in order to investigate their quality. Biotin and boronic acid-based fluorescent tags, containing one of the following fluorophores: fluorescein isothiocyanate (FITC), coumarin, rhodamine and bodipy, were subsequently synthesised and analysed via mass spectrometry, $^1$H NMR, UV-vis and fluorescence spectroscopies in order to shed light into the structure, purity, absorption, quantum yield and emission intensity of the fluorescent systems synthesised. A series of anticancer reagents in different cancer cell lines were studied to establish a robust protocol for MTT assays, then this assay method was applied to examine the cytotoxicity of SWNTs and different fluorescent sensors in different cancer cell lines. Eventually, confocal microscopy or epi-fluorescence microscopy imaging was performed for the different fluorescent sensors in cells.

3.2. Purification methods for SWNTs

SWNTs were made by different methods, hence the products yielded normally contained different impurities. More interestingly, the purification methods needed depend on the method of synthesis for the as-made SWNTs. SWNTs were purified by modifying the method originally used in Pascu’s group and which was widely used within the Oxford Nanotube Group since 2004. TEM, SEM, and EDX analysis were carried out at the University of Bath between 2011 and 2012. 

As it can be seen in Figure 3.1 the raw SWNTs, (Elicarb variety, as-made which were provided by Thomas Swan Ltd and which are related to the batch in use at the Oxford Nanotube Group) produced by a CVD method, present three main impurities: amorphous carbon, metal catalyst and multi-shell carbon nanoparticles. The presence of metallic impurities, such as Fe, was confirmed by EDX analysis.
Two different methods to purify the raw SWNTs were used, one of which is steam purification,\textsuperscript{1} the other one is a solvent-based oxidation method,\textsuperscript{2,3} both of which were modified based on literature protocols. The fundamental principle of purification is to remove a maximum amount of impurities, whilst causing minimum damage to the SWNT structure. Because multi-shell carbon nanoparticles have a similar structure to SWNTs, they resist high temperature and hence cannot be removed by the gas oxidation method. Whereas, in the solvent oxidation method, multi-shell carbon nanoparticles are “cracked” via a microwave pre-treatment, which makes carbon nanoparticles more vulnerable to oxidative treatment.\textsuperscript{4} Therefore, the solvent oxidation method was chosen to purify these batches of SWNTs.

\textbf{Figure 3.1.} TEM Micrograph of raw SWNTs with varieties of impurities
Complete characterisation of these products was subsequently carried out via TEM, EDX and Raman spectroscopy. A TEM micrograph showing typical as-made SWNTs available from Thomas Swan is given in Figure 3.1.

![TEM micrograph showing typical as-made SWNTs](image)

**Figure 3.1.** TEM micrograph showing typical as-made SWNTs available from Thomas Swan (CVD made, Elicarb® and steam-purified by S. Pascu and her collaborators with the Oxford Nanotube Group).

As it can be seen in Figure 3.2a, the SWNTs can be purified via solvent oxidation method. For comparison in Figure 3.2b, shows SWNTs purified by the steam method, which were provided by Dr Pascu and originated, for consistency reasons, from a large scale batch purified for Thomas Swann using a method devised within the Oxford Nanotube Group (Professor Malcolm Green). It is apparent that although purification via solvent oxidation removes most of amorphous carbon from the SWNTs (Figure 3.2a), there is a thick layer formed by stacking of SWNTs, and also some impurities, such as metal catalysts and contaminants like silica, remain in the layers, which indicates that the oxidation method cannot completely eliminate all the metal
catalysts and due to the multistep methodology, it was highly contaminated. Therefore, the quality of the SWNTs in the Figure 3.2a was lower than that in Figure 3.2b. Though there was still some amorphous carbon left in the industrially supplied sample Figure 3.2b, most carbon nanotubes were clean and the structure of SWNTs was intact, and therefore ready for use in the majority of the experiments which require relatively large scale of materials. In this work, most of the experiments were carried out using the batches of SWNTs with TEM micrographs show in Figure 3.2b.

![Figure 3.3. EDX analysis of purified SWNTs via solvent oxidation method](image)

Result represented in Figure 3.3 showed the EDX analysis obtained from purified SWNTs. It clearly reveals that the target area of SWNT is very clean. Although there are signals from copper, silicon and oxygen, they usually came from copper grid and glass (SiO₂) used in the procedure. However, with this results it is not possible to confirm the 100% purity of the whole batch of SWNTs. Because the laser beam was focused onto an extremely tiny spot (nm size), hence, this spectrum only refers to a small area of the sample. Thus in order to prove that the bulk of SWNTs was purified, multiple site of SWNTs have been examined, and the results encouraged us to proceed into their sample.
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Figure 3.4. SEM micrographs and EDX analysis of purified SWNTs via solvent oxidation method. These micrographs were recorded on samples purified for this programme, jointly with Zhiyuan Hu, and the technical support was that of Dr John Mitchels (MAS Bath)
SEM microscopy was done after TEM to analyse the surface morphology of purified SWNTs. As it can be seen clearly from Figure 3.4, there are many SWNT bundles in between supporting particle aggregates. An EDX analysis was subsequently carried out. The results clearly show that no significant amount of metal catalysts remained. Although there was still aluminium and gold elements detected, they were from substrate and coating elements used respectively. Hence, it can be concluded that only carbon is detected in the actual SWNT sample. However, similar to TEM, information from one site is not representative for the whole SWNTs batch, therefore six sites were analysed shown in Figure 3.5 in order to understand the purity of SWNTs overall. The first two sites have been examined as a spot and then the rest sites have been investigated via an EDX spectra. As displayed in Figure 3.5b there are signals from Br and Rb from site one, which are contaminations at the site presumably from the instrument. Finally, there were oxygen peaks found in four sites, which means there were certainly functionality still exist, which carried oxygen (O₂), due to the presence of absorbed the O₂, H₂O and CO₂ from air at these sites. Therefore, results conclude that metal catalysts are removed reliably from SWNTs, but SWNTs may be partially oxidised during the oxidative treatment due to the prolonged oxidation process.
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3.3. Functionalisation methods of SWNTs

In general, two methods were applied to functionalise SWNTs: non-covalent coating of SWNTs with β-D-glucan, and filling of SWNTs with metal ions. The functionalised SWNTs were characterised by TEM, SEM microscopies and Raman spectroscopy. Purified SWNTs were provided by S. Pascu with the Oxford Nanotube Group and functionalised by modifying the method originally used by Dr Pascu as a member of the Oxford Nanotube Group (2005-2007) and which was repeated and adapted by Dr. Zhiyuan Hu. However, there were concerns about the purity of the material and therefore an adapted method was devised, which shows reliability for biomedical investigations. Here, TEM, SEM, and EDX analysis were carried out (at the University of Bath between 2011 and 2012) and Raman spectroscopy were record at University of Bath between 2013 and 2015 and the Research Complex at Harwell under the training provided by Prof Tony Parker.

Figure 3.5. a.) SEM micrograph of purified SWNTs via a solvent oxidation method with b.) Six corresponding EDX spectra
Scheme 3.1 represents a non-covalent functionalisation of SWNTs with β-D-glucan. Subsequently fluorophores are loaded on the surface of β-D-glucan. A fluorescence modified with a boronic acid may bind to diol on glucan coated SWNTs via an interaction showed as follow:

After filtering and washing with water, the final product was prepared for TEM microscopy and Raman spectroscopy.
Chapter 3. Purification and characterisation of SWNTs

Micrographs exhibited in figure 3.6 presents SWNTs wrapped with β-D-glucan and fluorophore. There are two types of SWNTs revealed under TEM, one is a curved SWNT bundle (Figure 3.6 a) and the other one is a straight SWNT bundle (Figure 3.6 b). Both types of SWNT bundles are covered by a polymer layer and there are several black spots on the layer. These are likely glucan particles layer present on SWNTs bundle shown, and also there is more aggregated glucan accumulated at the base of the SWNTs.

A series of Raman experiments have been carried out to examine the interactions between SWNTs and glucan and fluorophores (for this programme, by this author, at the University of Bath, 2015). The experiments were carried out under different laser conditions with several different types of SWNTs in order to compare them. Experiments started using a laser with an operation wavelength of 532 nm. In the obtained spectra (Figure 3.7) peaks corresponding to SWNTs are observed for both samples, but no useful information can be obtained at their wavelength. A significant rise in the baseline of the Raman spectrum was observed for the sample SWNTs...
contains both glucan and fluorophores, which indicates a luminescence phenomenon occurring from glucan and fluorophore functionalised SWNTs, which prevents a reliable Raman spectroscopy experiment to be carried out.

![Raman spectra of pristine SWNTs and SWNTs functionalised with glucan & fluorophores in using laser at 532 nm wavelength](image)

**Figure 3.7**. Raman spectra of pristine SWNTs and SWNTs functionalised with glucan & fluorophores in using laser at 532 nm wavelength

The results represented in Figure 3.8 show the opposite outcome, using a 785 nm wavelength laser, a wavelength at which SWNTs started to illuminate. There was a significant increase in the intensity between 1600 to 3500 cm\(^{-1}\) Raman shift. Whereas in SWNTs with glucan and fluorophore, SWNTs seemed to be protected by glucan layer, hence there was no luminescence emitted from SWNTs and the Raman spectrum baseline remained steady. Data from 785 nm were believed to be more informative than data from 532 nm due to an improvement in the resolution. Therefore, I\(_D\)/I\(_G\) ratio of SWNTs was 18.18 % and I\(_D\)/I\(_G\) ratio of SWNTs with glucan and fluorophore was
7.69%, hence, there was a remarkable increase in the quality of SWNTs after non-covalently functionalised with glucan.

**Figure 3.8.** Raman spectra of SWNTs and SWNTs functionalised with glucan & fluorophores in laser at 785 nm wavelength

**Figure 3.9.** Raman spectra of SWNTs and SWNTs functionalised with glucan and fluorophores in laser at 958 nm wavelength
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By contrast, the Raman spectra data from a laser at 958 nm wavelength, which is at a visible light region, was carried out (figure 3.9). It is obvious that the baseline of glucan coated SWNTs raised again in this spectra, it was probably caused by fluorophores anchored onto the glucan, which absorbed and reflected visible light. However, due to a low laser energy import, the resolution of spectra was too low to be analysed.

Scheme 3.2. Schematic presentation of SWNTs filling with metal ions and capping with fullerenes.

Scheme 3.2 shows the method be used to fill SWNTs’ internal cavity with metal ions. Purified and opened SWNTs after the steam purification protocol, available from Oxford Nanotube Group, were dispersed and stirred in a zirconium acetate solution. Then C_{70} fullerenes are dispersed and stirred in the solution. After filtering and rinsing with excess of water and toluene, a zirconium filled SWNT capped with C_{70} was prepared for a TEM investigates and Raman spectroscopy.\textsuperscript{7,8} (Zr^{4+} filled SWNTs were obtained by modifying the method developed by Dr. Sofia Pascu at Memorial Sloan Kettering 2011 and repeated initially by Zhiyuan Hu. However, both of those available samples showed extremely low filling yields and this project set out to investigate the reasons why this was the case. This project reports the adapted and improved synthetic method (see Experimental section). TEM, SEM, and EDX analysis were carried out by this author at the University of Bath between 2011 and 2012. Raman spectroscopy was carried out at Research Complex at Harwell in 2013 on the samples provided by the
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author by Dr. Boyang Mao under the supervision of Dr Sofia Pascu and Prof Tony Parker. The interpretation and processing of the raw data belongs entirely to this author.

Figure 3.10 exhibits TEM micrographs of a SWNT and a bundle of SWNTs filled with Zr$^{4+}$ ions, the result images clearly showed the surface of either SWNT or SWNT bundle is clean, but the resolution of TEM is not high enough to reveal the detail of the internally trapped metal ions. Hence, an EDX analysis focused on SWNT strand region shows in Figure 3.10 a, was recorded, and the result is showed as follows, in Figure 3.11.

![TEM micrographs](image)

**Figure 3.10.** TEM micrographs of zirconium filled a. SWNT and b. bundle of SWNTs
Chapter 3. Purification and characterisation of SWNTs

Figure 3.11. EDX of zirconium-filled SWNTs

The EDX analysis certainly contains zirconium peaks. However, it also shows the overlapping peak of Mo and other elements from impurities in the microscope, copper and nickel from sample holder and preparation. Sulphur elements might come from a sample contamination or it was an artificial overlaid peak of Mo.

Figure 3.12. SEM images of (a) bundle of SWNTs at 1K magnification. (b) A bundle of SWNTs at 33K magnification, filled with zirconium acetate

Figure 3.12 represents the result of SEM micrographs at x1000 and x33000 magnifications resolutions. As can be observed, at lower magnification, SWNTs bundles appear simulatively with carbon supporting materials. At the higher
magnification, it is shown that the surface of individual SWNT bundles is clean, and there appear to be no amorphous carbon or nanoparticles attached to these. However, there are no further details available as revealed by this method due to the limitation of the SEM equipment available. When a high voltage was applied to SWNTs in order to improve the quality of image, there was an electronic charge on the surface of SWNTs and as a result, a highly bright micrograph is obtained without improving the resolution.

Figure 3.13. (a) SEM micrograph of zirconium acetate filled SWNTs, and (b) Five corresponding EDX spectra
Figure 3.13b shows EDX spectra of zirconium acetate-filled SWNTs, there are many peaks from different elements in the EDX spectra. In spectra 1, 2, 4 and 5, the majority of elements are C and O from SWNTs, gold (Au) from gold coating, Pt (from many catalyst) and F from instrument contamination. There are also force peaks from Fe detected due to a contamination from tweezers during the sample preparation. There are no zirconium signals in these explored four sites. This may be caused by the covering of SWNT bundles with a thick carbon layer. Site 3 may have SWNTs presented at the spot, but the peak of aluminium element is dominant, which hides the peaks from other elements. Hence, there is only one peak observable in spectrum 3.

Figure 3.14. Raman spectra of zirconium and copper filled SWNTs compared with purified SWNTs. This data was recorded at the Research Complex at Harwell under the supervision of Prof Tony Parker, by the Pascu Group of original samples by this author.
For comparison, a copper acetate filling experiment was carried out, as it can be seen in Figure 3.14, SWNTs are filled with either zirconium acetate or copper acetate respectively. The results clearly show that all characterisation peaks are remained the same at 250 cm\(^{-1}\), 1250 cm\(^{-1}\) and 1600 cm\(^{-1}\). This means the structure of SWNTs are intact and stable, the calculated I\(_D\)/I\(_G\) ratio of SWNTs filled with zirconium and copper are 19.97 % and 18.15 % respectively, which is higher than the I\(_D\)/I\(_G\) ratio of SWNTs functionalised with \(\beta\)-D-glucan alone (7.69 %). However, this ratio is close to the ratio of purified SWNTs, which is 14.52 %. Hence, it proves that filling of SWNTs with ions will neither damage the structure of SWNTs nor improve its structure. Furthermore, The RBM of filled SWNTs are different from purified SWNTs, which indicate a change in the diameter of SWNTs and refers to SWNTs were filled with Zr\(^{4+}\) or Zr\(^{4+}\) and Na\(^+\). The RBM is not changed during two different filling process, which indicates that filling SWNTs with either zirconium or copper ions will not cause a significant change in the diameter of SWNTs.

Figure 3. 15. Raman spectra of zirconium and zirconium & sodium filled SWNTs compared with purified SWNTs. This data was recorded at the Research Complex at Harwell under the supervision of Prof Tony Parker, by the Pascu Group of original samples by this author.
As it is represented in Figure 3.15, a zirconium filled SWNT is compared with a SWNT carrying both zirconium acetate and sodium acetate in a 1:1 mixture. It is obvious that characterisation peaks stay at the same position, hence the structure of SWNT is unchanged. However, the $I_D/I_G$ ratio of SWNTs increase to 20.93%, which refers to an increase in the defect rate when SWNTs are filled with two metal ions. In addition, the RBM has reduced to half of its original value, which indicate that the diameter of SWNT changed.

Figure 3.16. Raman spectra of zirconium ions and simultaneously present $C_{70}$ fullerene and zirconium acetate and sodium acetate and $C_{70}$ fullerene in filled SWNTs and compared with purified SWNTs. This data was recorded at the Research Complex at Harwell under the supervision of Prof Tony Parker, by the Pascu Group of original samples by this author.

$C_{70}$ fullerene, which has a similar size (11.2 Å)\textsuperscript{9} to the diameter of SWNT, is used in this experiment to seal the open end of SWNTs in order to block metal ions
inside SWNTs. Similar to the case of the other two Raman spectra recorded, there is no shift in the characterisation peaks. The $I_D/I_G$ ratio of [Zr$^{4+}$ + C$_{70}$] and [Zr$^{4+}$ +Na$^+$ + C$_{70}$] filled SWNTs are 19.06 % and 18.76 % respectively; although a small improvement from 19.97 % and 20.93 % was observed, it appeared to reduce the defect ratio slightly and makes SWNT higher quality. In addition, the RBM of filled SWNTs are overlaid after introduction of C$_{70}$ fullerene, hence there was no modification of the diameter of SWNTs.

### 3.4. Summary of chapter three

Results from TEM, EDX, SEM and Raman indicates that the solvent oxidation method appeared to as-made SWNTs (either as-made or CVD available) successfully removes all of the metal catalysts and most of the amorphous carbon from SWNTs. However, the efficiency and quality of the products was still not comparable to the commercially available purified SWNTs from Thomas Swann Company and provided by S. Pascu from Oxford Nanotube Groups. The SWNTs purified via steam-based gas phase oxidation methods have been shown to be good enough to be used for research purposes through TEM, Raman spectra and MTT assays. However, because the resolution of TEM available so far was rather low, higher resolution TEM imaging of metal salt filled SWNTs is still needed to clearly demonstrate the expected filling and estimate the filling yield. β-D-glucan and fluorophore coated SWNTs were successfully characterised by TEM imaging and Raman spectra, with further investigations of the SWNTs hybrids carried out in Chapter 4 and 5 to explore the potential of this biocompatible device in the biomedical research areas.
3.5. **Reference for chapter three**

Chapter 4. Investigations into the cellular cytotoxicity

4.1. Overview

As described in the introduction, the majority of the organic fluorophores are aromatic molecules which can react with oxygen to produce reactive oxygen species (ROS), which can in term damage structures of the living cell. The same principle applies to the SWNTs, impure or not well functionalised SWNTs can elevate oxidative stress in the cell due to their high surface energy.\(^1,2\) Therefore a preliminary study of cytotoxicity prior to their application in biological experiments is necessary, in order to determine the safe concentration to avoid causing damage to cells, and this was devised and will be described hereby.

Figure 4.1. Schematic representation of mechanism of MTT assay
Chapter 4. Investigations into the cellular cytotoxicity

The MTT assay is a quantitative colorimetric assay and it is the major assay used for cytotoxicity test in this project. In this assay, a yellow tetrazolium salt MTT (3- [4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) is reduced by living cells to formazan crystals, which can be dissolved in DMSO. This formazan solution can absorb energy at 570 nm which can be measured by a spectrometer. The mechanism is shown in Figure 4.1; NADP (nicotinamide adenine dinucleotide phosphate) is reduced to NADPH (nicotinamide adenine dinucleotide phosphate reduced form) in cellular mitochondria of living cells. Then an electron of NADPH is transferred to 1-methoxy PMS, which is an electron carrier on the cell membrane and subsequently 1-methoxy PMS is reduced to the 1-methoxy PMS reduced form by NADPH in the cytoplasm. Finally, tetrazolium is reduced by the 1-methoxy PMS reduced form in the medium.

4.2. MTT assays of raw, as-made SWNTs

The result of MTT assays were collected and plotted into a dose response curve and an EC$_{50}$ value was calculated. EC$_{50}$ stands for the half maximum effective concentration, which is the concentration of a sample solution that induces a response halfway between the baseline and maximum after a period of time. In this case EC$_{50}$ values represent the toxicity of the sample solution. Therefore, the higher the EC$_{50}$, the higher the concentration required to cause 50% cell toxicity. Compounds with an EC$_{50}$ value greater than 100 mg/L (0.1 mg/mL) can be considered nontoxic.\textsuperscript{5}
Figure 4.2. Dose response curves and calculated EC$_{50}$ of un-purified SWNTs in HeLa cells (a) for 24 hours, EC$_{50}$ = 1.78x10$^{-8}$ ± 1.03 x 10$^{-9}$ mg/mL (b) for 48 hours. EC$_{50}$ = 1.14x10$^{-8}$ ± 5.14 x 10$^{-9}$ mg/mL.
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The dose response curves of HeLa cells treated with SWNT-OX were determined, and the results are given in Figure 4.2 (a) and (b). Both MTT assays used pristine commercial available SWNTs prior to purifications in HeLa cells over two different time periods (24 hours and 48 hours respectively). Data shows a significant level of cell death which occurred in both conditions. The EC$_{50}$ value for 24 hours was 1.78×10$^{-8}$ mg/mL and for 48 hours it was 1.14×10$^{-8}$ mg/mL. In both cases, samples were found to be roughly 1×10$^{7}$ fold more toxic than the desired non-toxic criteria for any material. Exposure of cells to un-purified SWNTS for 24 hours gave a 35.96% lower EC$_{50}$ value than that compared to 48 hours EC$_{50}$. SWNTs become more toxic to cells or cells become more vulnerable to SWNTs over a longer time of exposure, i.e. more cells were killed by the same quality of SWNTs on time. The results prove that raw SWNTs were toxic in cells and that toxicity increases with increased exposure time. Therefore, purification and functionalisation is critically important for SWNTs’ toxicity.

(a)
Bar graph plots were also drawn with the data from the MTT assays. It shows that the viability of HeLa cells dropped slowly from 100 % to 47 % as the concentration of SWNTs increased from $5 \times 10^{-10} \text{ mg/mL}$ to $5 \times 10^{-7} \text{ mg/mL}$. Similar results were obtained from cells treated with SWNTs for 48 hours. As can be seen in Figure 4.3 b, the viability of HeLa cells decrease gradually from 100 % to 47.23 %, when the concentration of the SWNTs increased from $5 \times 10^{-10} \text{ mg/mL}$ to $5 \times 10^{-7} \text{ mg/mL}$. In conclusion, unpurified SWNTs were toxic to HeLa cells, but did not cause 100 % cell death. HeLa cells adapted to SWNTs during prolonged incubation as there were a similar percentage of cells that survived in the same concentration of SWNTs after 48 hours treatment, compared with 24 hours treatment.

**Figure 4.3.** Viability of HeLa cells after treatment after (a) 24 hours (b) 48 hours, where SWNT-OX is the pristine SWNTs commercially available from Thomas Swan Ltd, used in Oxford Nanotube Group for purification and functionalisation
4.3. MTT assays of purified SWNTs

Two different batches of purified SWNTs, one is from Thomas Swan Elicarb and the other one is from the Oxford Nanotube Group, were examined by MTT assays. In order to find other whether different purification method would affect the cytotoxicity of purified SWNTs.

Figure 4.4. Dose response curve determines EC$_{50}$ of purified SWNTs in HeLa cells (a) from Thomas Swan Elicarb, purified by solvent method, EC$_{50} = 3.38 \times 10^{-7} \pm 1.51 \times 10^{-7}$ mg/mL (b) Steam purified and CVD-made SWNTs, available from Oxford Nanotube Group, EC$_{50} = 1.17 \times 10^{-7} \pm 5.12 \times 10^{-8}$ mg/mL.
As can be seen in Figure 4.4, two batches of purified SWNTs were tested for cell toxicity by MTT assay. One was purchased from Thomas Swan Ltd (Elicarb), and the other one was also from Thomas Swan but steam purified by S. Pascu with the Oxford Nanotube Group. The dose response curve clearly showed that purified SWNTs whether by solution methods, or by steam purification still caused cytotoxicity and led to cell death. However, compared with EC$_{50}$ from Figure 4.2, the toxicity had roughly decreased by 10 fold. The EC$_{50}$ of Elicarb© solvent purified (CVD-made) SWNTs was $3.38 \times 10^{-7} \pm 1.51 \times 10^{-7}$ mg/mL and Oxford steam purified (CVD-made) SWNTs was $1.17 \times 10^{-7} \pm 5.12 \times 10^{-8}$ mg/mL. The EC$_{50}$ of Elicarb SWNTs was therefore higher than that of the steam-purified SWNTs so Elicarb SWNTs were ca. 50 % less toxic than the steam-purified SWNTs available from Oxford Nanotube Group.
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Figure 4.5. Viability of HeLa cells after 48 hours treatment of CVD-made purified SWNTs from (a) Elicarb (b) steam-purified and provided by the Oxford Nanotube Group.

The viability of HeLa cells was reduced at a slower rate and at a higher concentration for both purified SWNTs (whether solution purification, or steam-purification), compared with raw SWNTs after 48 hour treatment. Both batches of SWNTs led to a high survival rate at concentrations of $1 \times 10^{-8}$ mg/mL and $1 \times 10^{-7}$ mg/mL, then the viability of HeLa cells treated with steam-purified SWNTs suddenly dropped from 94.36% to 49.94% at the concentration of $2.5 \times 10^{-7}$ mg/mL, whilst the viability of HeLa cells treated with Elicarb© SWNTs (solution purified) was gradually reduced from 93.65% to 87.23%. Subsequently, both SWNTs batches induced drops in the viability of HeLa cells tested to levels between 50.47% and 55.04%.

4.4. MTT assays of β-D-glucan functionalised SWNTs

Figure 4.6 represents the MTT assays using β-D-glucan wrapped SWNTs in HeLa cells over 48 hours. From the plot, EC$_{50}$ for sugar wrapped SWNTs was estimated to be
5.03×10^{-6} \text{ mg/mL}. This is 100 fold greater than the result described in Figure 4.2 and 10 times greater than that shown in Figure 4.4.

**Figure 4.6.** The dose response curve of HeLa cell line treated β-D-glucan functionalised SWNTs over 48 hours, EC_{50} = 5.03×10^{-6} \pm 1.12 \times 10^{-7} \text{ mg/mL}

**Figure 4.7.** The viability of HeLa cells after 48 hours treatment of β-D-glucan SWNTs
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The viability graph (Figure 4.7) shows that the survivability of HeLa cells was stable at around 100%, after treatment with SWNTs at concentrations ranging between $1 \times 10^{-9}$ mg/mL and $1 \times 10^{-6}$ mg/mL. Then the viability reduced steadily from 98.27% to 69.69% when $5 \times 10^{-6} \pm 1.12 \times 10^{-7}$ mg/mL of β-D-glucan functionalised SWNTs was applied to HeLa cells. Therefore, it can be concluded that β-D-glucan functionalisation of SWNTs significantly reduced the cytotoxicity of SWNTs and rendered these more biocompatible as a vector for medical applications or life sciences assays. This data also proved that β-D-glucan was an ideal material to functionalise SWNTs, it not only provide a scaffold for boronic acid to anchor, but also isolate SWNTs from the environment to avoid causing damage to living organisms, as a consequence, more volume of sugar wrapped SWNTs can apply to the cells, which would significantly enhance the result of the confocal microscopy.

Figure 4.8. The dose response curve of PC-3 cells treated with compound 7 @glucan @ SWNTs over 48 hours

Figure 4.8 displays the dose response curve of PC-3 cells treated with compound 7 @glucan @ SWNTs composition. Only very few of the cells died in the experiment,
thus the EC$_{50}$ value could not calculated with precisely from the graph given in the Figure 4.8. However, more than 80% of cells were survived during the experiment, which indicated the composition is largely non-toxic. As it can be seen in the viability data in Figure 4.9, cells died at an extremely slow rate under the conditions used, from 100% at 1×10$^{-9}$ mg/mL to 84% at 5×10$^{-6}$ mg/mL. There was a significant improvement in the survivability compared with 50% for purified SWNTs at 5×10$^{-6}$ mg/mL in Figure 4.5, but there was also a clear reduction in the survival rate compared with 98% at 5×10$^{-6}$ mg/mL in Figure 4.7. This might have been caused by the inclusion of compound 7 within the β-D-glucan layer which wrapped the SWNTs.

Figure 4.9. The viability of HeLa cells after 48 hours treatment of compound 7 @ SWNTs@β-D-glucan

4.5. MTT assays of boronic acid-based fluorophore

After MTT assays were carried out for a series of different types of SWNTs. MTT assays were also performed on HeLa cells treated with the boronic acid-based fluorescent compounds synthesised and some related molecules as benchmarks. As
shown in Figure 4.8, the viability of HeLa cells was calculated as $EC_{50} = 5.32 \times 10^{-4} \pm 1.03 \times 10^{-5}$ M (0.53 mg/mL). This value was higher than the lowest toxic standard, which was 0.1 mg/mL. As a result, compound 7 was considered non-toxic in this cell line and therefore safe to use to the biological experiments and assays in a concentration range between $10^{-9}$ and $10^{-4}$ M.

**Figure 4.10.** The dose response curve of compound 7 was raised for an assay in HeLa cells carried out over 48 hours, $EC_{50} = 5.32 \times 10^{-4} \pm 1.03 \times 10^{-5}$ M.
A viability diagram (Figure 4.11) was plotted to examine the survivability of HeLa cells after 48 hours treatment of compound 7 at different concentration. The viability of compound 7 was similar to that observed for the glucan functionalised SWNTs. Both samples cause little toxicity at lower concentrations followed by a gradual drop to their highest concentration. This viability plot further shows that compound 7 was a biocompatible compound for cells and it is safe for a biological application into the concentration ranges used hereby ($10^{-9} - 10^{-4}$ M).

The cytotoxicity of some fluorescent sensors designed and provided by collaborators were also tested in MTT assays to try and estimate whether the ranges defined hereby were with available ranges and consistent with that of a large library of probes. These fluorescent sensors were structurally similar to the fluorescent sensor synthesised in this project so could contribute to a deeper understanding of the relationship between the cytotoxicity and the structure of the compound through the comparison amongst the compounds including in the library. The compounds of the library studied are given at the beginning of this project (Appendix C).
Figure 4.12. The dose response curve of HeLa cell lines after their treatment with compound \textbf{24} over 48 hours, $\text{EC}_{50} = 4.08 \times 10^{-6}$ M.

The result in figure 4.12 shows, $4.08 \times 10^{-6}$ M (2.2 $\times 10^{-3}$ mg/mL) of compound \textbf{24} caused half of the cells to die during 48 hours. This effective concentration was below 0.1 mg/mL, thus this compound was theoretically cytotoxic. The toxicity of compound \textbf{24} was higher than toxicity of compound \textbf{7} by comparing Figure 4.11 and 4.13. There was more than 90% cell survival at 1$\times$10$^{-6}$ M concentration when cells were treated with compound \textbf{7} whereas, only 78% of cells remained when treated with compound \textbf{24}. These data suggests that inclusion of a diamine linker and of a biotin group in the structure can make compounds more biocompatible.
Chapter 4. Investigations into the cellular cytotoxicity

Figure 4.13. The viability of HeLa cells estimated after 48 hours for treatment with compound 24

This viability diagram (Figure 4.13) can be used to compare results between compound 7 and compound 24. The general trend showed that compound 24 was generally nontoxic over the concentration range used because even at the maximum concentration of 5×10^{-6} M, the cell survival is still 58.43 %. However, the initial drop in viability caused by compound 24 appeared at the concentration of 2.5×10^{-7} M. Then the survival rate of cells then reduces slowly. Compared with data in Figure 4.11, there was only one sharper decrease at the concentration of 2.5×10^{-4} M. These results prove the toxicity of compound 24 was higher than the toxicity of compound 7. Therefore, compound 7 is still the most biocompatible in the series and suitable to be used in biological experiments.
Compound 13 is a derivative of compound 7, in which a fluorescein group is replaced by a boron-dipyrrromethane (bodipy) group. As a result, the cytotoxicity is examined to analyse the difference introduced by this replacement. The EC$_{50}$ value calculated from Figure 4.14 was $5.20 \times 10^{-5} \pm 6.74 \times 10^{-6}$ M, which is smaller than the EC$_{50}$ value of compound 7 ($5.32 \times 10^{-4} \pm 1.03 \times 10^{-5}$ M) from Figure 4.11. By replacing the fluorescein with a bodipy group the cytotoxicity is increased 10 fold. It is also evident that changing the fluorophore in the fluorescent sensor will alters the cytotoxicity significantly of the fluorescent sensor.

As can be seen in Figure 4.15, the viability of cells was above 95% at the concentration of $1 \times 10^{-9}$ M and $5 \times 10^{-7}$ M, which indicated compound was non-toxic in this concentration range. Then from $1 \times 10^{-6}$ M to $5 \times 10^{-5}$ M the survival rate of cells dropped slowly from 80% to 70%, indicating only a weak toxicity to cells. Finally, from $1 \times 10^{-4}$ M, a strong toxicity was introduced by compound 13 that reduced the viability of cells down to 14%.
Chapter 4. Investigations into the cellular cytotoxicity

Figure 4.15. Viability of PC-3 cells after 48 hours treatment of compound 13

Figure 4.16. Dose response curve of PC-3 cells treated with compound 15 over 48 hours, $EC_{50} = 1.41 \times 10^4 \pm 1.42 \times 10^5$ M.
Particularly, compound 15 is another boronic acid derivative related to compound 7 and compound 13, where a coumarin group (instead of bodipy group or a fluorescein) is attached onto the biotin-diamine-boronic acid linker. As mentioned previously, introducing a different fluorophore could change the toxicity of the fluorescent sensor. Results displayed in Figure 4.16 shows compound 15 causes a different cytotoxic effect than compound 7 and compound 13. The EC$_{50}$ of compound 15 was 1.41×10$^{-4}$ ± 1.42 × 10$^{-5}$ M, which was less toxic than compound 13 (5.20×10$^{-5}$ ± 6.74 × 10$^{-6}$ M), but more toxic than compound 7 (5.32×10$^{-4}$ ± 1.03 × 10$^{-5}$ M).

The viability plot (Figure 4.17) also proved compound 15 was toxic but not less so than compound 13. The viability of cells drops slowly down to 61% from 1×10$^{-9}$ M to 1×10$^{-4}$ M, followed by a sudden decline to 20% at 2.5 ×10$^{-4}$ M. This trend indicates compound 15 has a more stable cytotoxicity profile than compound 13 and therefore has better potential for biological applications.

![Figure 4.17](image.png)

**Figure 4.17.** Viability of PC-3 cells after 48 hours treatment of compound 15
4.6. **Summary of chapter four**

Two sets of MTT assays were established successfully to investigate samples in either molar concentration or mass concentration. All the fluorescent molecules and nanomaterials synthesised hereby were tested for cytotoxicity prior their application in confocal imaging. In general, SWNTs were found to be rather toxic, but after purification and functionalisation of biocompatible materials, a significant drop in their cytotoxicity was observed. Therefore, a great potential in the biomedical research can be forecast based on these evolution. As for the fluorescent sensors applied in this project, the cytotoxicity of the fluorescent sensors mainly originates from the fluorophore tags. Thus the cytotoxicity of fluorescent sensors varied from one system to another. From MTT assays of the fluorescent sensors, it was obvious that majority of these sensors seemed non-toxic at low concentrations until it reached the threshold at high concentration. The most common concentration used in confocal imaging in this project was 100 µM over 15 minute observation. Most of the fluorescent molecules in the assays studied could easily maintain more than 50% of cell survival at that concentration for over 48 hours observation. Therefore, most of the fluorescent specimens are appeared to be non-toxic in the applications in cancer cells, or can be tolerated in the cell experiments over 48 h observations.

4.7. **Reference to chapter four**


Chapter 5. In vitro fluorescence imaging of fluorescence sensors and functionalised SWNTs

5.1. Overview

Following on from purification and functionalisation protocols for SWNTs-based materials, as well as from the characterisation of organic fluorophores equipped with boronic acids, and synthesis & characterisation of fluorescent sensors, these two parts have been combined as described in Figure 5 – 1 to form a drug delivery device (DDS) which was then tested in biological experiments.

As seen in Figure 5.1, purified SWNT samples were functionalised with β-D-glucan in a biocompatible environment such as DMSO and water mixtures. (SWNTs were functionalised by modified the method used in S.I.P. group during 2008-2011). Then fluorescent sensors were loaded on the surface of glucan wrapped SWNTs via boronic acid-diol functionalisation using interactions involving the hydroxyl groups on the glucan backbones. Subsequently, these fluorescent sensor and SWNTs devices were filtered, rinsed and delivered to cancer cells and imaged.
The biological experiments in Chapter 5 include laser scanning confocal and epifluorescence imaging techniques to examine the morphology and fluorescent distribution of the fluorescent sensor in cells. This was followed by a fluorescence-lifetime imaging microscopy (FLIM) using two photon imaging to analyse the lifetime change of fluorescent sensors in cells. A variety of different conditions were investigated including: (a) different fluorescent organic dyes in cells, (b) different fluorescent tags anchored upon SWNTs in cells, (c) the same fluorescent systems tested under different conditions, such as, concentrations, incubation times and temperatures.

5.2. **Cell imaging of biotin-boronic acid based fluorescent sensors (compounds 4 and 7)**

Compound 7 was incubated for an hour at 100 µM, 1% DMSO (Figure 5.2). The figure clearly shows that the compound passed the cell membrane and distributes within HeLa cells. From the DIC image Figure 5.2 (c), the structure of the cells appears to be visually intact in terms of morphology, although some bubbles appeared due to a prolonged exposure to a laser. This may be led to the ROS generated from photodynamic effect accumulated over time and thus changing the homeostasis of the cells. According to MTT assays, more than 90% of cells survived at 100 µM for 48 hours with compound 7 alone. Under the laser condition (λ_{ex} = 488 nm for one photon, λ_{ex} = 910 nm for two photon), this cellular disruption occurred within minutes. The HeLa cell control experiment showed in appendix A.1 also proved that HeLa cells were slightly disrupted under the laser conditions, even in the absence of the compound. As a result, it can be concluded that compound 7 has a strong membrane penetrability and a lower power laser needed to be tried to test whether it could reduce the damage.
Figure 5.2. Confocal microscopy images of HeLa cells treated with 100 µM of compound 7 in 1% DMSO, incubated at 37 °C for 1 hour. The sample was excited at 488 nm wavelength and long-pass filtered at 515 nm. Image (a) was merged image of images (b) and (c). Image (b) was green fluorescence channel. Image (c) represented for image took from DIC. Scale bar: 20 µm.

Figure 5.3 shows an experiment designed to determine the co-localisation of compound 7. Hoechst was applied to CHO cells to enable distinction of the nuclei and to determine whether compound 7 underwent nuclear uptake. The green fluorescence observed upon excitation with 488 nm (representing the compound 7 nuclear stain) could be overlaid with an image of the blue fluorescence with excitation at 405 nm (representing Hoechst). In the overlay, where nuclear uptake of compound 7 had occurred, a cyan colour can be observed. Similar to HeLa cells, compound 7 showed good membrane penetrability into CHO cells, nuclei of CHO cells were successfully stained by Hoechst. A clear colocalisation between the green and blue stains was observable in Figure 5.3 (a). Therefore, this shows that compound 7 not only enters cells and travels to the cytoplasm, but also could access the nucleus of the CHO cell. There was another observation is found from Figure 5.3 (b), which was that most nuclei remain intact under the confocal microscopy. This may suggest that both compound 7 and Hoechst dyes leave cells undamaged. As it is shown in using images from in Figure 5.3 (d), most of the cells appear undamaged in terms of morphology. Figure 5.3 (d) also showed that were some less well-defined cells had appeared, in this situation, the problem was not only raised by the cytotoxicity of compound 7 under a prolonged incubation, but also involved an overgrowth issue, which could further have elevated the oxidative stress effects and break the homeostasis of the cells.
Figure 5.3. Confocal microscopy images of Chinese hamster ovary (CHO) cells treated with 100 μM of compound 7 in 1% DMSO, incubated at 37 °C for 1 hour. Followed by a 15 minute treatment with Hoechst. The sample was excited at 488 nm wavelength. Image (a) was merged image of images (b), (c) and (d). Image (b) was captured at > 450 nm wavelength. Image (c) was green fluorescence channel. Image (d) represented for image took from DIC. Scale bar: 20 μm.

Figure 5.4 represents the confocal microscopy images of HeLa cells treated with compound 4. As seen in Figure 5.4 (a) and (b), compound 4 could pass through the cell membrane, but the distribution of fluorescence was similar to that of compound 7. The fluorescence emission is observed as spread across the cytoplasm and there was no
prove that compounds across the cytoplasm into cell nucleus. Although the morphology of cells altered slightly (the surface of the cells became less well defined), the integrity of the nuclei were unchanged (Figure 5.4 c). Therefore, cells appeared to remain alive with the duration of the observation. Perhaps the poorer definition observed could be explained accumulation of compound 4 on the surface of the cell membrane. This phenomenon could also be observed in Figure 5.4 (a) and (b), there was a significant amount of compound 4 aggregated on the surface of cells due to the solubility problem, this aggregation could interrupt the structure of the cell membrane and cause damages to the morphology. A diluted concentration might help to improve the quality of the image. In conclusion, a replacement of the diamine linker in the fluorophore used would not affect the permeability of the fluorescent sensor, but this change of linker seemed to reduce the membrane damage caused by compound 7. Further LDH assays could investigate the details of the membrane damage caused by both compound 4 and 7.

Figure 5.4. Confocal microscopy images of HeLa cells treated with 100 µM of compound 4 in 1% DMSO, incubated at 37 °C for 15 minutes. The sample was excited at 488 nm wavelength and long-pass filtered at 515 nm. Image (a) was merged image of images (b) and (c). Image (b) was green fluorescence channel. Image (c) represented for image took from DIC. Scale bar: 20 µm

Figure 5.5 (b) and 5.6 (b) represents the HeLa cells imaged after treating with compounds 7 and compound 4 respectively. Furthermore, after incubation with these compounds, a Lysotracker (red) dye was applied to both dishes of cells. Lysosome red tracker is a red dye that stains lysosomes in cells and emits a red fluorescent signal at 590 nm wavelength. As seen in the results, both cells showed a well distributed of
Lysotracker in the cytoplasm in Figure 5.5 (c) and 5.6 (c). HeLa cells with compound 7 had undamaged morphology and intact nuclei, which indicate compound 7 and lysotracker did not cause any disruptions towards cells. Whilst in Figure 5.6, the aggregation of compound 4 appeared again on the cell membrane and that the fluorescence of the compounds and lysotracker gathered in corresponding areas of the cytoplasm, these altogether indicate distribution of fluorescent samples and morphology of cells. The results clearly showed that compound 4, 7 and lysotracker were absorbed by cells, in Figure 5.5, compound 7 emitted a green signal (Figure 5.5 b), and lysotracker gave a red fluorescence (Figure 5.5 c), thus a merged image of (b) and (c) exhibited in yellow, which indicate compound 7 stained lysosome as well. Compound 4 behaved in a similar manner to compound 7, as shown in Figure 5.6, whereby it distributed throughout the cytoplasm, including inside lysosomes.
Chapter 5. In vitro fluorescence imaging of fluorescence sensors and functionalised SWNTs

Figure 5.5. Confocal microscopy images of HeLa cells treated with 100 µM of compound 7 in 1% DMSO, incubated at 37 ºC for 1 hour. Followed by a 15 minute treatment with Lysotracker Red. The sample was excited at 488 nm. Image (a) merged image of images (b), (c) and (d). Image (b) long-pass filtered at 515 nm, Image (c) long-pass filtered at 605 nm. Image (d) represented for image took from DIC. Scale bar: 20 µm.
5.3. Cellular imaging of biotin-boronic acid based fluorescent sensors attached onto β-D-glucan and β-D-glucan functionalised SWNTs

This section describes the series of confocal microscopy cell images captured using compound 7 after its attachment to β-D-glucan or β-D-glucan @ SWNTs nanomaterials. Together with HeLa and PC-3, additional cell line (CHO, Chinese Hamster Ovarian) was used allowing comparisons of the compounds in more than one accessible cell line.
Figure 5.7. Confocal microscopy images of HeLa cells treated with 100 µM of compound 7 functionalised with 1 mg/mL β-D-glucan in 1% DMSO, incubated at 37 ºC for 15 minutes. The sample was excited at 488 nm and long-pass filtered at 515 nm. Image (a) was the merged image of images (b) and (c). Image (b) green fluorescence channel. Image (c) represented for image took from DIC. Scale bar: 20 µm.

Figure 5.7 represents the confocal images captured from cells incubated with compound 7 @β-D-glucan. The result clearly shows that compound 7 @glucan could pass though the cell membrane and enter the nuclei, moreover the shape of the cell membrane and the nuclear membrane were still intact, i.e. the structure of cells remains unchanged. In addition, there was significant fluorescence observed on the surface of the cells, which might be due to the solvent system change from DMSO to serum free medium environment, resulting in aggregation of β-D-glucan to form a cluster too large to endocytose. As a conclusion compound 7 @glucan had good cell permeability and possesses a reasonably low cytotoxicity to HeLa cells.

Figure 5.8. Confocal microscopy images of CHO cells treated with 100 µM of compound 7 functionalised with 1 mg/mL β-D-glucan in 1% DMSO, incubated at 37 ºC for 15 minutes. The sample was excited at 488 nm and long-pass filtered at 515 nm. Image (a) was the merged image of images (b) and (c). Image (b) green fluorescence channel. Image (c) represented for image took from DIC. Scale bar: 20 µm.

Figure 5.8. Confocal microscopy images of CHO cells treated with 100 µM of compound 7 functionalised with 1 mg/mL β-D-glucan in 1% DMSO, incubated at 37 ºC for 15 minutes. The sample was excited at 488 nm and long-pass filtered at 515 nm. Image (a) was the merged image of images (b) and (c). Image (b) green fluorescence channel. Image (c) represented for image took from DIC. Scale bar: 20 µm.

As seen in Figure 5.8, HeLa cells were replaced by CHO cells in these experiments. It has been observed that compound 7 @glucan does enter CHO cells, however, most of the compounds remains attached to the cell membrane and only a small amount of the compound migrated into the cytoplasm. This might be due to the type of cells in Figure 5.8 being different from that in Figure 5.7, therefore, the uptake
rate of compounds and glucan could be distinct. The shape of cells was well defined that indicated cells remained healthy and undamaged. Thereby, compound 7 @glucan showed a major difference depending on the types of cell line (HeLa cell line VS the CHO cell line).

Figure 5.9. Confocal microscopy images of CHO cells treated with 50µL of 20 mM compound 7 functionalised with 1 mg/mL β-D-glucan wrapped SWNTs (1:1) in water, incubated at 37 ºC for 15 minutes. The sample was excited at 488 nm and long-pass filtered at 515 nm. Image (a) was the merged image of images (b) and (c). Image (b) green fluorescence channel. Image (c) represented for image took from DIC. Scale bar: 20 µm.

Figure 5.9 displays confocal images of CHO cells treated with compound 7 @glucan @ SWNTs. Compounds were dispersed in water, thus stock samples could be applied to more than 1% of the total volume on the cell plate. As a result of increase in the concentration of the sample, there was significantly greater uptake, this therefore enhanced the fluorescent intensity and the cytotoxicity inside cells. Thereby, compound 7@glucan @SWNTs distributed all over the cell, it was believed to have entered the nucleus. Subsequently, cells become unstable and cells morphology altered slightly, which indicate that cells could not tolerate this amount of compounds. The aggregation of compound nearby cells could also demonstrate there were excess samples in the environment. In conclusion, the compound 7 @ glucan @SWNTs could facilitate compound 7 into the cytoplasm. Dispersed 1 mg/mL compound 7 @ glucan @SWNTs in water was a good way to reduce the cytotoxicity of compound 7 @ glucan @SWNTs, but excess of samples could still cause harmful effect to cells.
Figure 5.10. Confocal microscopy images of CHO cells treated with 100 µM of compound 9 functionalised with 1 mg/mL β-D-glucan wrapped SWNTs in water, incubated at 37 ºC for 15 minutes. The sample was excited at 488 nm and long-pass filtered at 515 nm. Image (a) was the merged image of images (b) and (c). Image (b) green fluorescence channel. Image (c) represented for image took from DIC. Scale bar: 20 µm.

Compound 9 was a compound found through the alternative synthetic route from the compound 7 synthesis, which was formed at the end of Route two in the system shown in scheme 2.6. The compound 9 was considered as a precursor of compound 7. The newly synthesised compound 9 and used as a comparison for compound 7. In this experiment, compound 9 @ glucan @SWNTs was incubated with CHO cells at 37 ºC for 15 minutes. The results showed that compound 9 @ glucan @SWNTs could not pass through the cell membrane and did not enter the cells, the intensity of the fluorescence in Figure 5.10 (b) indicated that most of compound 9 @ glucan @SWNTs were rinsed away. The remaining compounds, accumulated on the CHO cell membrane and there was only a slight sign for the aggregation of compound 9 @ glucan @SWNTs on the cell surface. As a result, the observed cellular damage introduced by compound 9 was not as evident as that showed in in Figure 5.9.

In conclusion, introduction of boronic acid group does not only affect the effectiveness of this types of fluorophores functionalised onto the glucan wrapped SWNTs, but also influences greatly the permeability of compounds through the cell membrane. Notably, fluorescein alone is shown not to be taken up by cells in the absence of extensive functionalisation.¹
5.4. **Cell imaging of biotin-boronic acid based fluorescent sensors under different environmental conditions**

Aliquots of compound 7 and compound 7 @glucan were added to cells, and incubated at different temperatures and over different period of times to investigate how these conditions might affect the compounds’ uptake and to assess any damages in the morphology of cells.

![Confocal microscopy images](image)

**Figure 5.11.** Confocal microscopy images of CHO cells treated with 100 µM of compound 7 in 1% DMSO, incubated at 37 ºC for 15 minutes. The sample was excited at 488 nm and long-pass filtered at 515 nm. Image (a) was the merged image of images (b) and (c). Image (b) green fluorescence channel. Image (c) represented for image took from DIC. Scale bar: 20 µm

This series of imaging experiments started with CHO cells treated with compound 7 at 37 ºC for 15 minutes (Figure 5.11). The results clearly show that most compounds accumulated on the surface of cell membrane in Figure 5.11 (a). This might due to a low concentration of compound or due to the fact that there was not enough incubation time, considering the number of cells visible in the picture below. However, the cells’ morphology was undamaged and the shape of cells was well defined.
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Figure 5.12. Confocal microscopy images of HeLa cells treated with 100 µM of compound 7 in 1% DMSO, incubated at 37 ºC for 1 hour. The sample was excited at 488 nm and long-pass filtered at 515 nm. Image (a) was the merged image of images (b) and (c). Image (b) green fluorescence channel. Image (c) represented for image took from DIC. Scale bar: 20 µm.

In Figure 5.12, HeLa cells were treated with compound 7 for one hour at 37 ºC, in order to examine whether a prolonged incubation of compound 7 would affect the state of the cells. As displayed in Figure 5.12 (b), compound 7 successfully entered the cell membrane, and in some of the cells, compound 7 also appeared to stain the nuclei. However, the morphology of cells became unstable when compared to Figure 5.11 (c). The cell membrane became indistinct and some cells detached from dishes. All these results indicated that prolonged incubation of compound 7 would increase the intensity of fluorescence in HeLa cells and simultaneously increase the toxicity of compound 7 towards HeLa cells.

Subsequently, the temperature of the incubation condition dropped from 37 ºC to 4 ºC. Regarding the uptake method of compound 7 in cells, there could be three different pathways to uptake compound 7; including (a) diffusion (b) active transfer and (c) endocytosis. However, the active transfer and endocytosis are known to be inhibited by the low temperature, and diffusion could occur at a slow rate. Then, compound 7 was loaded again (with new cells) to test whether there were any difference caused by temperature. The result displayed in Figure 5.13 (b) clearly showed that fluorescent signal was detected, but unlike data showed in Figure 5.13 (b), the compound 7 did not distributed across cells, and most of the fluorescent signals were emitted nearby the cell membrane instead. This phenomena indicated that a decrease in
temperature can lead to a decrease in the rate of uptake. As for the morphology of cells, although the shape of cells was intact, the surface of the cell membrane became ill-defined during the observation. Thus this demonstrated some problems had occurred due to accumulation on the cell membrane which affected the quality of the image captured or disrupted the structure of the cell membrane. From these experiments it is possible to conclude that compound 7 has either entered the cell membrane or has accumulated on top of it. Due to the slightly less distinct nature of the cell membrane it may be more likely that the compound is on top of the cell membrane rather than in it. A cell membrane stain would determine which hypothesis is correct in further experiments.

![Image of HeLa cells treated with 100 µM of compound 7 in 1% DMSO, incubated at 4 ºC for 15 minutes. The sample was excited at 488 nm and long-pass filtered at 515 nm. Image (a) was the merged image of images (b) and (c). Image (b) green fluorescence channel. Image (c) represented for image took from DIC. Scale bar: 20 µm.](image)

**Figure 5.13.** Confocal microscopy images of HeLa cells treated with 100 µM of compound 7 in 1% DMSO, incubated at 4 ºC for 15 minutes. The sample was excited at 488 nm and long-pass filtered at 515 nm. Image (a) was the merged image of images (b) and (c). Image (b) green fluorescence channel. Image (c) represented for image took from DIC. Scale bar: 20 µm.

Similarly to the case shown in Figure 5.13, a prolonged incubation experiment was repeated. In Figure 5.14 HeLa cells were treated with compound 7 at 4 ºC for 60 minutes. After 60 minutes, compound 7 remained on or in the cell membrane. However, the distribution of fluorescence and morphology of the cells seems didn’t change compared with cells in figure 5.13 (b). it indicated that a low temperature would not cause damage to the cells in a short period of time and slow the absorption of compound 7.
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**Figure 5.14.** Confocal microscopy images of HeLa cells treated with 100 µM of compound 7 in 1% DMSO, incubated at 4 ºC for 60 minutes. The sample was excited at 488 nm and long-pass filtered at 515 nm. Image (a) was the merged image of images (b) and (c). Image (b) green fluorescence channel. Image (c) represented for image took from DIC. Scale bar: 20 µm.

**Figure 5.15.** Confocal microscopy images of HeLa cells treated with 20µL of 10 mM compound 7 functionalised with 1 mg/mL β-D-glucan in 1% DMSO, incubated at 37 ºC for 15 minutes. The sample was excited at 488 nm and long-pass filtered at 515 nm. Image (a) was the merged image of images (b) and (c). Image (b) green fluorescence channel. Image (c) represented for image took from DIC. Scale bar: 20 µm.

The following four imaging experiments described in (Figure 5.15-5.18) represent HeLa cells treated with compound 7 @glucan, but instead of 10 µL, 20 µL of the compound was applied to cells in these experiments. (Figure 5.8)

The results in Figure 5.15 clearly showed compound 7 @ glucan could pass through the membrane and also access to nuclei. Obviously, there were many intensive fluorescence signals appeared in cells. These were believed to be aggregated compound 7 @ glucan attached inside the cell. Simultaneously, most of cells retained their shape,
this indicated that compound 7 @ glucan has limited toxicity to this cell line at 37 °C within 15 minutes observation.

Figure 5.16. Confocal microscopy images of HeLa cells treated with 20µL of 10 mM compound 7 functionalised with 1 mg/mL β-D-glucan in 1% DMSO, incubated at 37 °C for 60 minutes. The sample was excited at 488 nm and long-pass filtered at 515 nm. Image (a) was the merged image of images (b) and (c). Image (b) green fluorescence channel. Image (c) represented for image took from DIC. Scale bar: 20 µm.

Figure 5.16 showed the results of a prolonged incubation of compound 7 @ glucan for 60 minutes at 37 °C. There was a significant change in the morphology of the cells, most of cells became unstable and began to die. The fluorescence of the cells in 5.16 (b) indicated that the structure of cell nuclei was also altered, likely leading to an interruption of the nuclei’s normal function and resulting in a moderate damage of cells’ morphology.
Figure 5.17. Confocal microscopy images of HeLa cells treated with 20µL of 10 mM compound 7 functionalised with 1 mg/mL β-D-glucan in 1% DMSO, incubated at 4 ºC for 15 minutes. The sample was excited at 488 nm and long-pass filtered at 515 nm. Image (a) was the merged image of images (b) and (c). Image (b) green fluorescence channel. Image (c) represented for image took from DIC. Scale bar: 20 µm.

The results in Figure 5.17 demonstrated experiment which incubated cells with 2% of compound 7 @glucan at 4 ºC, the hypothesis is there were less uptake of compositions at low temperature. In fact, there were less absorption of compound 7 @glucan observed in Figure 5.17 compared with the outcome as shown in Figure 5.16, although compound 7 @ glucan still enter cells, with some appearing to enter the nucleoli. The overall morphology of cells, however looked better than that in Figure 5.16 (c). Although there were cells in a poor morphological state, there were no cell structures collapsed in the picture. Therefore, a reduced temperature method of incubation not only decreased the uptake rate of compounds, but also decreased the cytotoxicity effect of compounds. As a result of the slow uptake, compound 7 @ glucan aggregated outside cells and then accumulated on the cell membrane or diffused into cells, thereby there were more fluorescence signals on the cell membrane or emitted out of cells.

Figure 5.18. Confocal microscopy images of HeLa cells treated with 20µL of 10 mM compound 7 functionalised with 1 mg/mL β-D-glucan in 1% DMSO, incubated at 4 ºC for 60 minutes. The sample was excited at 488 nm and long-pass filtered at 515 nm. Image (a) was the merged image of images (b) and (c). Image (b) green fluorescence channel. Image (c) represented for image took from DIC. Scale bar: 20 µm.
In Figure 5.18, HeLa cells were incubated with compound 7 @ glucan for 60 minutes, the intensity of fluorescence increased as the increment of incubation time, because more of the compound available on the plate was absorbed into cells cytoplasm or remained attached onto the cell membrane. However, it was surprising that even a prolonged incubation of cells with these compounds did not cause many cells to die. As it was described in Figure 5.16. This result further suggested that the low temperature incubation techniques would significantly reduce the cytotoxic effect to cells, even over prolonged periods of incubation.

5.5. Fluorescence-lifetime imaging microscopy (FLIM) data of boronic acid-based fluorescent sensors

Two-photon fluorescence lifetime imaging (FLIM) is an imaging technique which combined with laser confocal microscopy, has the ability to build on the information resulting from a highly sensitive single-photon counting lifetime measurements to examine the fluorescence intensity, distribution and lifetime of small molecules in cells. The fluorescence lifetime is an invaluable datum obtained from this technique, which is characteristic to an individual fluorescence molecule. The stability of the excited state and therefore the fluorescent lifetime, may be affected by interactions with intracellular ions or oxygen, conformational changes caused by binding to other molecules or pH difference in the environment during the experiment. As a result, FLIM is also an ideal tool to study the aggregation, viscosity and distance between molecules.

FLIM was used in this project to investigate the lifetime of various fluorophores and its interactions with other molecules or ions. Experiments began with testing the lifetime of compounds in solvent (Time Correlated Single-Photon Counting, TCSPC),\textsuperscript{7} followed by an \textit{in vitro} FLIM investigation.\textsuperscript{8} These experiments were performed at the Rutherford Appleton Laboratory (RAL) in central Laser Facilities (CLF) and assisted by Prof S. W. Botchway, Dr P Burgos, Mr A. McKenzie and Mr A. Henman.\textsuperscript{9}
Table 5. 1. Two-photon Time Correlated Single Photon Counting data of fluorescein, compound 4 and compound 7 at 10 mM concentration in DMSO, the laser for both fluorescein and compound 4 were excited at 810 nm, and for compound 7 was excited at 910 nm, laser power was in between 4.0 – 7.0 mW.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Fluorescein</th>
<th>Compound 4</th>
<th>Compound 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent system</td>
<td>DMSO</td>
<td>DMSO</td>
<td>DMSO</td>
</tr>
<tr>
<td>$\chi^2$</td>
<td>1.11</td>
<td>1.17</td>
<td>1.13</td>
</tr>
<tr>
<td>$\tau_1$ (ns)</td>
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<td>1.3</td>
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<td>$\tau_1$ %</td>
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<tr>
<td>$\tau_2$ (ns)</td>
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<tr>
<td>$\tau_2$ %</td>
<td>-</td>
<td>40.9</td>
<td>18.3</td>
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</table>

A series of compounds, including fluorescein, compound 4 and compound 7, were tested by TCSPC and the results are displayed in Figure 5.19. Each photon detected and collected in TCSPC would be plotted to calculate the lifetime of fluorescence. The results obtained from TCSPC consists of three parts, a chi square, lifetime and lifetime percentage, if more than one component was modelled. As seen in Figure 5.19, fluorescein only had one lifetime, which was 3.9 ns, which represents the duration within which this molecule existed in an excited state. Whereas, both compound 4 and 7 contained two components, which meant there were more than one component that was fluorescent and the fluorescence lifetimes were individually different.
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- Fluorescein
- Fluorescein fit curve:
- Compound 4
- Compound 4 fit curve:
- Compound 7
- Compound 7 fit curve:

Figure 5.19. TCSPC decay curves for commercially available fluorescein, compound 4 and compound 7 at 10 mM concentration in DMSO, the laser for both fluorescein and compound 4 were excited at 810 nm, and for compound 7 was excited at 910 nm, laser power was in between 4.0 – 7.0 mW.

This variation between components could be due to several reasons, for instance, existence of isomers, conformation change in the solvent, or pH-dependent protonated and un-protonated analogues of a molecule. The percentage of lifetime given represents the portion of lifetime individual results contributed to the overall lifetime ($\tau_m$). As for the chi squared, there are three different range of $\chi^2$ values in the results, the best $\chi^2$ value is 1.0, which indicates an optimal fitting. Then when $\chi^2$ value is in between 1.0 and 1.3 demonstrates incomplete single exponential fit, whilst $\chi^2$ value is greater than 1.5, indicates there was significant noise within the TCSPC setup or more than on component decay profile. Therefore, all results in Table 5.1 are reliable. Due to the structural similarities, compound 4 and 7 would carry a similar lifetime to fluorescein, thus the lifetimes of compound 4 and 7 were $\tau_2 = 3.2$ ns (40.9%) and $\tau_2 = 4.3$ ns (18.3%) respectively. Because of the high purity of the sample a single component was expected, however it can be speculated that a second conformation of the compound was present.
due to interactions with the solvent (e.g. polarity effects, protonation/deprotonation etc.). Therefore, $\tau_1 = 1.3$ ns (59.1%) and $\tau_1 = 0.9$ ns (81.7%) for compound 4 and compound 7 respectively.

Table 5.2. Two-photon Time Correlated Single Photon Counting data of compound 7 at 10 mM in DMSO, compound 7 @glucan at 1mg/mL in DMSO, compound 7 @glucan @SWNTs at 1mg/mL in water and compound 9 @glucan @SWNTs at 1mg/mL in water. $\lambda_{ex} = 910$nm, laser power was in between 2.5 – 8.7 mW.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Compound 7</th>
<th>Compound 7 @ β-D-glucan</th>
<th>Compound 7@β-D-glucan @SWNTs</th>
<th>Compound 9@β-D-glucan @SWNTs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent</td>
<td>DMSO</td>
<td>DMSO</td>
<td>Water</td>
<td>Water</td>
</tr>
<tr>
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<tr>
<td>$\tau_2$ (%)</td>
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<td>43.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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- Compound 7
- Compound 7 fit curve
- Compound 7 @β-D-glucan
- Compound 7 @β-D-glucan fit curve:

**Figure 5.20.** TCSPC decay curves for compound 7 at 10 mM in DMSO, compound 7 @glucan at 1mg/mL in DMSO, compound 7 @glucan @SWNTs at 1mg/mL in water and compound 9 @glucan @SWNTs at 1mg/mL in water. λ<sub>ex</sub> = 910nm, laser power was in between 2.5 – 8.7 mW.

In Figure 5.20, 10 mM compound 7, 10 mM compound 7 @ β-D-glucan in 1% DMSO and 10 mM compound 7 @ β-D-glucan @ SWNTs (1:1) in water were tested by TCSPC. Two components were found to be in both compound 7 (τ<sub>1</sub> = 0.9 ns, τ<sub>2</sub> = 4.3 ns) and compound 7 @ glucan (τ<sub>1</sub> = 1.0 ns, τ<sub>2</sub> = 2.8 ns), τ<sub>1</sub> of compound 7 and compound 7@glucan were slight differ in DMSO, it is not only because of interactions with the solvent, but also compound 7 bind to glucan lead to a conformational change. Compound 7 has a 4.3 ns lifetime and inducing from glucan therefore slightly accelerates the lifetime of compound 7 to τ<sub>2</sub> = 2.8 ns. It indicated the lifetime of excited state of compound 7 @ glucan is less than compound alone in DMSO, hence the fluorescence of compound 7 @glucan returned to the ground state faster. Because compound 7 @glucan @SWNTs were dispersed in water, the polarity of solvent
increased in the water could resulted in one component model was favoured for compound 7 @glucan @SWNTs and compound 9 @glucan @SWNTs.

**Figure 5.21.** FLIM images of cells treated with 100 µM of compound 4 or 7 in 1% DMSO, cells were incubated at 37 ºC where (a) two photon fluorescence intensity diagram of compound 4 in HeLa cells, (b) the fluorescence lifetime map of $\tau_m$ of compound 4 in HeLa cells (c) two photon fluorescence intensity diagram of compound 7 in HeLa cells, (d) the fluorescence lifetime map of $\tau_m$ of compound 7 in HeLa cells, (e) two photon fluorescence intensity diagram of compound 7 in CHO cells and (f) the fluorescence lifetime map of $\tau_m$ of compound 7 in CHO cells.
Figure 5.21 represents the two photon FLIM mapping and intensities, which allow comparisons between compounds 4 and 7, in HeLa and CHO cells. Each experiment was modelled to fit two components, the fluorescence observed from the fluorescence lifetime map was represented by the fluorescence lifetime intensity map and its fluorescence lifetime map, whereby the colour indicates the lifetime (red signifies a shorter and blue is a longer lifetime), which are used to calculate the mean \( \tau_m \).

From the Figure 5.21 (a) and (c), it can be observed that compound 4 enters the cells as well as appearing to accumulate on the cell surface. In comparison, however, images of compound 7 in cells are much brighter, indicating either significantly better uptake of compound 7, or quenching of compound 4. In Figure 5.21 (c) there was no obvious sign of compound 7 precipitating on the surface of the cells, however, compound 7 also disturbed the normal function of cells, thus cells looked less healthy than cells in Figure 5.21 (a). Subsequently, a comparison could be made between Figure 5.21 (c) and (e), which indicated that compound 7 could enter both HeLa and CHO cell lines.

The fluorescence lifetime map was used to display the fluorescence distribution across the whole diagram’s field of view. This fluorescence map explored the overall fluorescence lifetime \( \tau_m \), individual lifetime \( \tau_1 \) and \( \tau_2 \) and their portion of contribution to the overall fluorescence. The fluorescence lifetime map summarised each individual fluorescence detected from the cells and their environment, thus to fully analyse of each individual spot on the maps was not appropriate.

Table 5.3 and figure 5.22 together exhibited the corresponding fluorescence lifetimes obtained from the cell FLIM data. First of all, a \( \chi^2 \) value of approximately 1 indicated these data were reliable results. Then the lifetime of compound 4 in HeLa was found to be \( \tau_2 = 3.4 \text{ ns} \), 51.9\%, FWHM was 1.2 ns. The full width at half maximum (FWHM) was calculated from the lifetime distribution curve (Figure 5.23) and it is a distance between points on the curve at which half its maximum value was reached. The results of FWHM were used to assess the error. The smaller the FWHM, the sharper the peak is, hence, the more reliable the data is. Compared with compound 4 in solvent, which was \( \tau_2 = 3.2 \text{ ns} \), 40.9\% in Table 5.1. Because the value of 3.4 ± 1.2 ns is covered
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in the range of 2.2 ns – 4.6 ns (error range), it undoubtedly showed that there was no significant change in the fluorescence lifetime once compound 4 entered cells. Whereas, \( \tau_1 = 1.3 \) ns was dropped remarkably to \( 0.6 \pm 0.6 \) ns, due to compound 4 interacted with the complicated cellular environment in cells. By contrast, compound 7 changed significantly in lifetime when it was applied in HeLa cells. \( \tau_2 \) reduced from 4.3 ns to 3.1 \( \pm 0.5 \) ns, FWHM of compound 7 in HeLa was 0.5 ns. However, the lifetime of compound 7 changed slightly to 3.8 \( \pm 1.8 \) ns once it was applied to CHO cell lines. It again indicated there was no considerable change in fluorescence lifetime when compound 7 was absorbed by CHO cells.

Table 5.3. FLIM data of compound 4 and compound 7 at 100 \( \mu \)M in DMSO in different cell lines. The laser for compound 4 was excited at 810 nm, and for compound 7 was excited at 910 nm, laser power was in between 4.3 – 7.0 mW.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Compound 4</th>
<th>Compound 7</th>
<th>Compound 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell line</td>
<td>HeLa</td>
<td>HeLa</td>
<td>CHO</td>
</tr>
<tr>
<td>( \chi^2 )</td>
<td>1.10</td>
<td>1.03</td>
<td>1.02</td>
</tr>
<tr>
<td>( \tau_1 ) (ns)</td>
<td>0.6</td>
<td>0.9</td>
<td>0.8</td>
</tr>
<tr>
<td>( \tau_1 ) %</td>
<td>48.1</td>
<td>45.0</td>
<td>24.9</td>
</tr>
<tr>
<td>FWHM (ns)</td>
<td>0.6</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>( \tau_2 ) (ns)</td>
<td>3.4</td>
<td>3.1</td>
<td>3.8</td>
</tr>
<tr>
<td>( \tau_2 ) %</td>
<td>51.9</td>
<td>55.0</td>
<td>75.1</td>
</tr>
<tr>
<td>FWHM (ns)</td>
<td>1.2</td>
<td>0.5</td>
<td>1.8</td>
</tr>
</tbody>
</table>
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Figure 5.22. FLIM decay curves for compound 4 and 7 in HeLa and CHO cell line in individual point inside cells

Figure 5.23. Fluorescence lifetime distribution curve of compound 4 and 7 in HeLa and CHO cells
Figure 5.23 shows three fluorescence lifetime distribution curves, these were the overall fluorescence summarised from Figure 5.21 (b), (d) and (f). From the plot, it can be easily observed that the fluorescence lifetime distribution of compound 4 was in the range between 0 and 4.0 ns, compound 7 in HeLa was within 1.0 to 3.0 ns and compound 7 in CHO was in between 1.25 ns and 4.75 ns. The distribution of lifetime in this curve was based on the corresponding components, i.e. the proportion of individual lifetime. For example, 75.1 % of the overall lifetime (\( \tau_m \)) in compound 7 in CHO was 3.8 ns, which draws the blue lifetime distribution curve towards 3.5 ns. The other reason to plot the lifetime distribution curve was it could be used to estimate the FWHM in order to access the error of \( \tau_m \).
Figure 5.24. FLIM images of cells treated with 10 µL of 10 mM compound 7 with 1 mg/mL β-D-glucan in 1% DMSO, 20 mM compound 7 with 1 mg/mL glucan and 1 mg/mL of SWNTs in water or 20 mM compound 9 with 1 mg/mL glucan and 1 mg/mL of SWNTs in water, cells were incubated at 37 ºC where (a) two photon fluorescence intensity diagram of compound 7 @glucan in CHO cells, (b) the fluorescence lifetime map of $\tau_m$ of compound 7 @glucan in CHO cells (c) two photon fluorescence intensity diagram of compound 7 @glucan @SWNTs in CHO cells, (d) the fluorescence lifetime map of $\tau_m$ of compound 7 @glucan @SWNTs in CHO cells, (e) two photon fluorescence intensity diagram of compound 9 @glucan @SWNTs in HeLa cells and (f) the fluorescence lifetime map of $\tau_m$ of compound 9 @glucan @SWNTs in HeLa cells.
Another three samples were tested by FLIM, namely, Compound 7 @ glucan, Compound 7 @ glucan @SWNTs and Compound 9 @ glucan @SWNTs. From the microscopy results displayed in Figure 5.24, each compound show different behaviour different. In Figure 5.24 (a), CHO cells were treated with Compound 7 @ glucan, compared the result exhibited in 5.24 (c), similarly, the fluorescence lifetime mainly distributed in the cytoplasm from the fluorescence lifetime map, however, the brightness of the colour was significantly reduced in 5.24 (c), which indicated a longer lifetime, i.e. quench effect induced by SWNTs. Lifetimes were calculated from each pixels of the fluorescence image. As seen in Figure 5.24 (d), Compound 7 @ glucan @SWNTs enters cells and fluorescence lifetimes were mostly calculated from cytoplasm, but it seemed compound 7 suffered a quenching effect from SWNTs, hence, the lifetime of fluorescence was remarkably decreased. At the same time, due to the aggregation problems caused by SWNTs, there were an obvious accumulation of composition on the cell membrane and it was highly fluorescent (Figure 5.24 d), the fluorescence lifetime discovered in cells was close to the lifetime of Compound 7 @ glucan @SWNTs in solvent. It indicated the fluorescence lifetime of Compound 7 @ glucan @SWNTs did not change remarkably in the cellular environment. The results displayed in Figures 5.24 (e) and (f) showed a totally different result compared with previous compounds and compositions. Compound 9 was carried by glucan @SWNTs and entered HeLa cells, but unlike other samples, which gave an evenly distributed fluorescence lifetime in the cytoplasm, it spread randomly in cells instead. There were two hypothesis made, one was cells died during the experiment, so compositions located unregularly. The other one was the amount of compositions carried by glucan @SWNTs was not enough to distribute across the whole cytoplasm, hence, the pattern of distribution presented in the fluorescence mapping looked like compositions aggregated in specific regions of the cell.
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Table 5.4. Two-photon Time Correlated Single Photon Counting data of compound 7 at 100 µM in DMSO, compound 7@glucan@SWNTs at 10 µg/mL in water and compound 9@glucan@SWNTs at 10 µg/mL in water in different cell lines. $\lambda_{ex} = 910$ nm, laser power was in between 2.5 – 8.7 mW.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Compound 7 @ glucan</th>
<th>Compound 7@glucan@SWNTs</th>
<th>Compound 9@glucan@SWNTs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell line</td>
<td>CHO</td>
<td>CHO</td>
<td>HeLa</td>
</tr>
<tr>
<td>$\chi^2$</td>
<td>1.01</td>
<td>1.10</td>
<td>1.25</td>
</tr>
<tr>
<td>$\tau_1$ (ns)</td>
<td>1.8</td>
<td>0.9</td>
<td>0.8</td>
</tr>
<tr>
<td>$\tau_1$ %</td>
<td>39.4</td>
<td>39.3</td>
<td>80.1</td>
</tr>
<tr>
<td>FWHM (ns)</td>
<td>0.5</td>
<td>0.5</td>
<td>1.1</td>
</tr>
<tr>
<td>$\tau_2$ (ns)</td>
<td>4.0</td>
<td>3.3</td>
<td>3.3</td>
</tr>
<tr>
<td>$\tau_2$ %</td>
<td>60.6</td>
<td>60.7</td>
<td>19.9</td>
</tr>
<tr>
<td>FWHM (ns)</td>
<td>1.8</td>
<td>1.8</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Figure 5.25. FLIM decay curves for compound 7 at 100 µM in DMSO, compound 7@glucan@SWNTs at 10 µg/mL in water and compound 9@glucan@SWNTs at 10 µg/mL in water in HeLa and CHO cell line. $\lambda_{ex} = 910$ nm, laser power was in between 2.5 – 8.7 mW.
Figure 5.25 and Table 5.4 displayed the corresponding lifetime and lifetime related information to Figure 5.25. The first and most important parameter observed from the table was the $\chi^2$ value. Each value was approximately 1, therefore all data listed on this table are considered statistically reliable. Then, the lifetime from these experiment were compared. There was an increase from 2.8 ns to 4.0 ±1.8 ns in the $\tau_2$ lifetime of compound 7 @ glucan when it had been moved from DMOS solvent system into CHO cells (Table 5.2). This expected results mainly due to ions, biomolecules (sugar, proteins etc.) existed in the cells, which could bind to compound 7 @ glucan and caused a conformational change of the structure, which prolonged the fluorescence lifetime and result in an increase of the fluorescence lifetime. Likewise, the lifetime of compound 7 in CHO cells was 3.8 ± 1.8 ns (Table 5.3), which was not much different from 4.0 ± 1.8 ns. Thus it was indicated that introduction of glucan did not introduce a dramatic changes in the fluorescence lifetime in the same cell line and shift of solvent system would cause a considerable change in the fluorescence lifetime. Both Compound 7 @ glucan @SWNTs and Compound 9 @ glucan @SWNTs had similar fluorescence lifetimes in water and cells, however, the lifetime proportion in cells varied. Both samples have two components in cell FLIM. Compound 7 @ glucan @SWNTs’ lifetime changed from 3.6 ns (100%) in water to $\tau_1$=0.9 ± 0.5 ns (39.4%, FWHM 0.5 ns), $\tau_2$=3.3 ± 1.6 ns (60.7%, FWHM 1.6 ns) in CHO cells, whereas the lifetime of compound 9 @ glucan @SWNTs decreased sharply from 3.5 ns(100%) in water to $\tau_1$=0.8 ± 1.1 ns (80.1%, FWHM 1.1 ns), $\tau_2$=3.3 ± 1.4 ns (19.9%, FWHM 1.4 ns) in HeLa cells.

Figure 5.26 represented the fluorescence lifetime distribution of compound 7 @ glucan in CHO, compound 7 @ glucan @SWNTs in CHO and compound 9 @ glucan @SWNTs in HeLa cells. The range of lifetime for three samples were distinct from each other, compound 7 @ glucan had the most wide range from 0 to 5 ns and also had two peaks at 1.8 ns and 3.3 ns respectively, it might be due to binding to a protein in cells to give a second fluorescence lifetime peak. Compound 7 @ glucan @SWNTs stayed in the range of 1 to 4 ns and peaked at 2.5 ns. Whilst Compound 9 @ glucan @SWNTs ranged from 0 to 2.6 ns and had a rough peak at 0.7 ns. Compound 7 @ glucan displayed the most fluorescent signals, compound 7 @ glucan @SWNTs ranked
second, Compound 9 @ glucan @SWNTs had the least fluorescence signals, indicating that SWNTs functionalised with compound 7 @ glucan are better suited for imaging than with compound 9 @ glucan.

![Fluorescence lifetime distribution curve of compound 7 and 9 composition in different cell lines. $\lambda_{ex} = 910$ nm, laser power was in between 2.5 – 8.7 mW.]

5.6. FLIM data of boronic acid-based fluorescent sensor under various conditions

Figure 5.27 to 5.32 show the fluorescence lifetime images corresponded to all confocal images listed in chapter 5.4, which studied how fluorescence differentiated in varies conditions. Figure 5.27 focus on the lifetime changes of compound 7 in distinct circumstances. Whereas 5.31 mainly explored the lifetime alteration of compound 7 @ glucan.

Images exhibited in Figure 5.27 showed that after incubated cells with 100 µM of compound 7 at 37 ºC for 60 minute, fluorescence lifetime could be clearly observed.
from the cell cytoplasm. Images labelled Figure 5.27 (e) and (f) similarly supported the results represented in Figure 5.13, lifetime of compound 7 was revealed from the cell membrane, diffusion and moving speed inside cells were remarkably inhibited at 4 ºC in 15 minutes, which lead to a brighter colour (shorter lifetime) from the cell membrane and darker colour (longer lifetime) was detected from inside of cells.

**Figure 5.27.** FLIM images of HeLa cells treated with 100 µM compound 7 in 1% DMSO, cells were incubated at 37 ºC or 4 ºC for 15 minutes or 60 minutes. where (a) two photon fluorescence intensity diagram of compound 7 in HeLa cells (37 ºC, 60 minutes incubation), (b) the fluorescence lifetime map of $\tau_m$ of compound 7 in HeLa cells (37 ºC, 60 minutes incubation), (c) two photon fluorescence intensity diagram of compound 7 in HeLa cells (4 ºC, 15 minutes incubation) and (d) the fluorescence lifetime map of $\tau_m$ of compound 7 in HeLa cells (4 ºC, 15 minutes incubation), (e) two photon fluorescence intensity diagram of compound 7 in HeLa cells (4 ºC, 60 minutes incubation) and (f) the fluorescence lifetime map of $\tau_m$ of compound 7 in HeLa cells (4 ºC, 60 minutes incubation).
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Because of the cold environment, some precipitations were detected in the image. The result found in 60 minutes (Figure 5.27 f) had no much different from 15 min (Figure 5.27d) incubation at low temperature, fluorescence lifetime remained concentrated at the cell membrane, but there was an increased amount of fluorescence detected inside cytoplasm, which indicated although the active absorption of compounds were inhibited, there was compound 7 across cell membrane and entered cells via diffusion during time period.

Table 5.5. Two-photon Time Correlated Single Photon Counting data of HeLa cells treated with compound 7 at 37 ºC or 4 ºC for 15 minutes or 60 minutes at 100 µM in DMSO. λ_ex = 910 nm, laser power was 1.8 – 7.0 mW.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Compound 7</th>
<th>Compound 7</th>
<th>Compound 7</th>
<th>Compound 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell line</td>
<td>HeLa</td>
<td>HeLa</td>
<td>HeLa</td>
<td>HeLa</td>
</tr>
<tr>
<td>Temperature ( ºC)</td>
<td>37</td>
<td>37</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Incubation time (min)</td>
<td>15</td>
<td>60</td>
<td>15</td>
<td>60</td>
</tr>
<tr>
<td>( \chi^2 )</td>
<td>0.96</td>
<td>1.14</td>
<td>1.05</td>
<td>1.00</td>
</tr>
<tr>
<td>( \tau_1 ) (ns)</td>
<td>0.9</td>
<td>0.9</td>
<td>1.1</td>
<td>1.3</td>
</tr>
<tr>
<td>( \tau_1 ) %</td>
<td>72.2</td>
<td>28.6</td>
<td>36.6</td>
<td>15.5</td>
</tr>
<tr>
<td>FWHM (ns)</td>
<td>0.6</td>
<td>1.2</td>
<td>1.5</td>
<td>0.39</td>
</tr>
<tr>
<td>( \tau_2 ) (ns)</td>
<td>3.8</td>
<td>3.4</td>
<td>3.6</td>
<td>3.3</td>
</tr>
<tr>
<td>( \tau_2 ) %</td>
<td>27.8</td>
<td>71.4</td>
<td>63.4</td>
<td>84.5</td>
</tr>
<tr>
<td>FWHM (ns)</td>
<td>1.2</td>
<td>0.8</td>
<td>1.7</td>
<td>1.4</td>
</tr>
</tbody>
</table>
Table 5.5 and Figure 5.28 listed all the information of fluorescence lifetime corresponding to Figure 5.27, all data showed in the FLIM decay curve carried an acceptable low $\chi^2$ value, which indicated these data were reliable and usable in the analysis. Compound 7 cultured at all four conditions carried two components, the lifetime of compound 7 in cells cultured at 37 ºC for 15 minutes was $\tau_1 = 0.9 \pm 0.6$ ns (72.2%, FWHM 0.6 ns), $\tau_2 = 3.8 \pm 1.2$ ns (27.8%, FWHM 1.2 ns), compared with the lifetime of compounds in cells cultured at same condition in 60 minutes, which was $\tau_1 = 0.9$ ns $\pm 1.2$ (28.6%, FWHM 1.2 ns), $\tau_2 = 3.4 \pm 0.8$ ns (71.4%, FWHM 0.8 ns). It clearly showed a considerable change in the percentage of components, at 15 minutes $\tau_1$ was dominant, whereas with the increase of incubation time, more compound 7 absorbed by cells, hence the percentage of $\tau_2$ was raised. Similar situation occurred at 4 ºC, the percentage of $\tau_2$ increase from 63.4% to 84.5%.
In conclusion, temperature or incubation time change would influence not only the ratio of two lifetime components in cells, but also it had little effect on the fluorescence lifetime of compounds in cells, which remained within the nanoseconds range as expected.

**Figure 5.29.** Fluorescence lifetime distribution curve of HeLa cells treated with compound 7 in different conditions. $\lambda_{\text{ex}} = 910$ nm, laser power was $1.8 - 7.0$ mW.

Figure 5.29 represented the fluorescence distribution of each condition, from the plot, it was clearly exhibited that most conditions peaked around about $3 - 4$ ns apart from cells treated with compound 7 at $37^\circ$C for 15 minutes, as was discussed previously, due to smaller component represented a larger proportion of the fluorescence lifetime, the overall fluorescence was distributed mainly in the range of $0.6$ ns to $3$ ns and peaked at $1.7$ ns. There were two conditions had two peaks in its system, which happened in cells treated with compound 7 at $37^\circ$C for 60 minutes and compound 7 at $4^\circ$C for 60 minutes. Because all experiments apply the same batches of compound 7, hence, the possible reason of this unexpected fluorescence distribution might be due to the
prolonged incubation of compounds, leads to two components with different lifetime accumulated and stabilised during the period of incubation.

Figure 5.30. FLIM images of HeLa cells treated with 20 µL of 20 mM compound 7 with 1mg/mL β-D-glucan in 1% DMSO, cells were incubated at 37 ºC or 4 ºC for 15 minutes or 60
minutes, where (a) two photon fluorescence intensity diagram of compound 7 @glucan in HeLa cells (37 ºC, 15 minutes incubation), (b) the fluorescence lifetime map of $\tau_m$ of compound 7 @glucan in HeLa cells (37 ºC, 15 minutes incubation) (c) two photon fluorescence intensity diagram of compound 7 @glucan in HeLa cells (37 ºC, 60 minutes incubation), (d) the fluorescence lifetime map of $\tau_m$ of compound 7 @glucan in HeLa cells (37 ºC, 60 minutes incubation), (e) two photon fluorescence intensity diagram of compound 7 @glucan in HeLa cells (4 ºC, 15 minutes incubation) and (f) the fluorescence lifetime map of $\tau_m$ of compound 7 @glucan in HeLa cells (4 ºC, 15 minutes incubation), (g) two photon fluorescence intensity diagram of compound 7 @glucan in HeLa cells (4 ºC, 60 minutes incubation) and (h) the fluorescence lifetime map of $\tau_m$ of compound 7 @glucan in HeLa cells (4 ºC, 60 minutes incubation).

Figure 5.30 shows FLIM images of HeLa cells treated with 2% of compound 7 @glucan in 2% DMSO, incubated at 37 ºC for 15 minutes, 37 ºC for 60 minutes, 4 ºC for 15 minutes and 4 ºC for 60 minutes respectively. It can be observed from all pictures that samples entered cells in all conditions. There were precipitations accumulated on top of cells and in 5.30 g & h, it looked like there was a layer of fluorescent substances covered the cells. These problem were mainly caused by the excess amount of glucan applied to cells, which carried fluorescent compounds and was difficult to wash away. At 37 ºC, compound 7 with glucan appeared to enter cells and mainly stayed in cytoplasm and the cells’ morphology was unchanged. At 37 ºC 60 minutes incubation, the image captured was again out of focus, Hence, it became difficult to extract useful information, but it could still be seen that there was fluorescence signal detected from cells, although it was difficult to tell whether it was due to the fluorophore located in the cell cytoplasm or attached to the cell membrane, and there was precipitation of fluorescence accumulated on cells. As for the images at 4 ºC, there were an interesting phenomenon, it was discovered that with the assistance of the glucan, compound 7 was no longer accumulated on the cell membrane, but entered the cells and even seemed to enter the nuclei. After a 60 minutes period of incubation, compound 7 @glucan appeared to accumulate inside the cell membrane, which resulted in an intensive fluorescence out of cells and made the washing of the cells extremely difficult.

As seen in Figure 5.31, all the TCSPC decay curves for compound 7 @ glucan in different conditions carried a similar fitting curve, although the fluorescence counts for cells treated with compound 7 @glucan at 4 ºC for 15 minutes were significantly higher than cells cultured in other conditions. They would yield a similar result of the lifetime.
The results represented in Table 5.6 showed that the all the lifetimes calculated from Figure 5.31 were similar to each other and in the range of 3 ns, it also did not differ much from Table 5.5, which indicated there was no significant effect in lifetime when functionalised compound 7 onto glucan. However, combined compound 7 and glucan did affect proportions between components one and two, which keep the percentage of both components at around about 50%; there was a slight drop of lifetime in all components two and the change of the overall fluorescence lifetime $\tau_m$ distribution was clearly showed in Figure 5.32.

**Table 5.6.** Two-photon Time Correlated Single Photon Counting data of HeLa cells treated with compound 7 at 37 °C or 4 °C for 15 minutes or 60 minutes. $\lambda_{ex} = 910$ nm, laser power was 2.5 mW.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Compound 7 @glucan</th>
<th>Compound 7 @glucan</th>
<th>Compound 7 @glucan</th>
<th>Compound 7 @glucan</th>
</tr>
</thead>
<tbody>
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<td>Cell line</td>
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<td>HeLa</td>
<td>HeLa</td>
<td>HeLa</td>
</tr>
<tr>
<td>Temperature (°C)</td>
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<td>4</td>
</tr>
<tr>
<td>Incubation time (min)</td>
<td>15</td>
<td>60</td>
<td>15</td>
<td>60</td>
</tr>
<tr>
<td>$\chi^2$</td>
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<td>1.05</td>
<td>0.99</td>
</tr>
<tr>
<td>$\tau_1$ (ns)</td>
<td>1.0</td>
<td>0.5</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>$\tau_1$ %</td>
<td>50</td>
<td>59.8</td>
<td>56.2</td>
<td>34.5</td>
</tr>
<tr>
<td>FWHM (ns)</td>
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<td>0.6</td>
<td>1.7</td>
<td>1.4</td>
</tr>
<tr>
<td>$\tau_2$ (ns)</td>
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<td>3.1</td>
<td>3.0</td>
<td>3.4</td>
</tr>
<tr>
<td>$\tau_2$ %</td>
<td>50</td>
<td>40.2</td>
<td>43.8</td>
<td>65.5</td>
</tr>
<tr>
<td>FWHM (ns)</td>
<td>1.8</td>
<td>1.6</td>
<td>0.3</td>
<td>1.3</td>
</tr>
</tbody>
</table>
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Figure 5.31. FLIM decay curves for compound 7 @ glucan in HeLa in different incubation conditions. \( \lambda_{\text{ex}} = 910 \text{ nm} \), laser power was 2.5 mW.

Figure 5.32. Fluorescence lifetime distribution curve of HeLa cells treated with compound 7 @ glucan in different conditions. \( \lambda_{\text{ex}} = 910 \text{ nm} \), laser power was 2.5 mW.
As was mentioned above, use of functionalised compound 7 onto glucan dropped the percentages of the second component in the system, this resulted into a shift of the overall fluorescence lifetime distribution towards the shorter lifetimes. For instance, cells treated with compound 7 at 4 °C for 60 minutes had a lifetime distributed in a range of 0 to 4 ns, peaked at 3.2 ns, when combined compound 7 and glucan and then incubated at the same condition, the range of the composition became 0.4 – 4 ns and which peaked at 2.8 ns. It can be conclude that by functionalisation of compound 7 with glucan, this would not affect the fluorescence lifetime of compound 7. However, after the introduction of β-D-glucan, the morphology of cell and uptake route of composition appeared significantly changed, and as a result, the overall fluorescence lifetime in cells was changed due to the significant changes in the environment of the fluorophore.

5.7. Cell imaging and FLIM data of compounds from collaborating projects under various conditions

There were many fluorescent sensors in this general family, available from the research groups of Pascu and James and which were studied to explore the different use of
boronic acid and fluorophores in biological applications. For example, compound 24 (designed and synthesised by Sabrina Wang, in Tony James’ group, University of Bath) was a compound simply made up of boronic acid and FITC, without a linker and a biotin. This compound was used as a comparison for compound 7 to see whether there was a difference without a diamine linker and a targeting group. Compound 25 (designed by Prof Tony James in University of Bath) and 26 (synthesised by Dr. Stephen Flower, in University of Bath) were prototypes of compounds 15 and 13 respectively. Compound 27 (designed and synthesised in Tony James’ group University of Bath) was another boronic acid-based compound, which was designed to have altered fluorescence properties upon binding metal ions.11 Whereas for compound 7, the boronic acid was developed to attach onto glucan wrapped SWNTs, i.e. it was attached onto SWNTs indirectly. Through investigations of these novel boronic acid-based fluorescent sensors, additional boronic acid-based fluorescent sensor derivatives would be designed, developed and applied in research areas, and the methods devised hereby were generalised.

**Figure 5.33.** Confocal microscopy images of HeLa cells treated with 100 µM of compound 24 in 1% DMSO, incubated at 37 ºC for 15 minutes. The sample was excited at 488 nm and long-pass filtered at 515 nm. Image (a) was the merged image of images (b) and (c). Image (b) green fluorescence channel. Image (c) represented for image took from DIC. Scale bar: 20 µm.

Figure 5.33 represents HeLa cells treated with compound 24, which had a similar structure to compound 7 and 9. Thus it was a good candidate to test how a structural difference might affect function. Although the results displayed in Figure 19 (b) clearly showed the compound 24 penetrated the cell membrane and distributed
mainly in cytoplasm, unlike compound 7 in Figure 5.2, little of compound 24 could breach the cell nuclei. Nuclei appear to be stained by compound 24. The morphology of the cells was unchanged, which showed a similar level of cytotoxicity of compound 7 showed in Figure 5.2 (c) and confirmed the result of MTT assays exhibited in Figure 4.9 and 4.11, thus indicating that the nuclear uptake was not as a result of damage.

**Figure 5.34.** Confocal microscopy images of HeLa cells treated with 20µL of 10 mM compound 25, incubated at 4 ºC for 15 minutes. The sample was excited at 405 nm wavelength and emitted at 450 nm long pass. Image (a) was the merged image of images (b) and (c). Image (b) green fluorescence channel. Image (c) represented for image took from DIC. Scale bar: 20 µm.

Compound 25 possessed a coumarin group, which absorbs light at 405 nm and emitted at 450 nm. This enables compound 25 to emit a blue fluorescence signal. The result in Figure 5.34 (b) clearly revealed there was a significant fluorescence signal appearing in the cell cytoplasm at 4 ºC, at which temperature it should have a reduced uptake rate of the compounds as well as uptake exclusively due to passive methods. The distribution of fluorescence demonstrated that the compound does not entered the nuclei. The morphology of cells was damaged as expected, due to the strong laser energy had been introduced onto cells, photodynamic effect occurred, which elevated the level of oxidative stress and formed a lot of “bubbles” in the cells. The surface of cells also become unstable; this indicated that there was a certain level of damage introduced on the membrane, to disturbing the homeostasis of the cell membrane.
Chapter 5. In vitro fluorescence imaging of fluorescence sensors and functionalised SWNTs

Figure 5.35. Confocal microscopy images of HeLa cells treated with 100 µM of compound 25 functionalised with 1 mg/mL β-D-glucan in 1% DMSO, incubated at 4 ºC for 15 minutes. The sample was excited at 405 nm wavelength and emitted at 450 nm long pass. Image (a) was the merged image of images (b) and (c). Image (b) was taken at >450 nm wavelength. Image (c) represented for image took from DIC. Scale bar: 20 µm.

In Figure 5.35, β-D-glucan was applied in order to improve the survivability of cells. Similar to Figure 5.34, compound 25 @ glucan easily entered cells without breaching the cell nuclei. There was an increment in the fluorescence in Figure 5.35 (b), compared with Figure 5.34 (b). This phenomenon indicated that binding to glucan enhanced the fluorescence of compound 25 and facilitated the entrance of the compound 7. However, the damage caused by fluorescence to cells increased as well, because the morphology of cells in figure 5.35 (c) is significantly worse than that in figure 5.34 (c). As a result, the outcome displayed in Figure 5.35 (c) did not show any improvement in the cell survivability.

Figure 5.36. Confocal microscopy images of HeLa cells treated with 100 µM of compound 26 in 1% DMSO, incubated at 37 ºC for 15 minutes. The sample was excited at 543 nm wavelength
and emitted at 605 nm long pass. Image (a) was the merged image of images (b) and (c). Image (b) was taken at > 605 nm wavelength. Image (c) represented for image took from DIC. Scale bar: 20 µm.

Rhodamine was another fluorophore chosen for study, which was excited at 543 nm and emitted at 605 nm, therefore, it emitted a red fluorescence. The reason to choose rhodamine was it absorbed and emitted light at a high wavelength, hence, it need low energy laser to activate the fluorescence. Therefore, a boronic acid attached rhodamine (compound 26) was delivered into HeLa cells. However, there were severe problems inspected in Figure 5.36 (c), in which most cells were moderately damaged, the shape of cells were irregular and cell nuclei were slightly affected. Fluorescent image 5.36 (b) indicated that most fluorescence were held in the cytoplasm and seemed concentrated in ER and Golgi, a further colocalisation study is require to confirm, but the size and frame of nuclei were unusual compared with nuclei displayed in Figure 5.36 (b). This result illustrated that compound 26 itself was toxic to cells.

![Image](image_url)

**Figure 5.37.** Confocal microscopy images of HeLa cells treated with 100 µM of compound 26 in 1% DMSO, incubated at 4 ºC for 15 minutes. The sample was excited at 543 nm wavelength and emitted at 605 nm long pass. Image (a) was the merged image of images (b) and (c). Image (b) was taken at > 605 nm wavelength. Image (c) represented for image took from DIC. Scale bar: 20 µm.

This experiment was designed to show that the reduced uptake of compound 26 would reduce the cytotoxicity by decreasing the uptake. As experiment was repeated at 4 ºC, there were less compound 26 absorbed into cells, consequently, there fewer fluorescence signals were detected from 5.37 (b) compared that was detected in 5.36.
(b). as a result, the shape for both cell membrane and nuclei looked more intact than that in Figure 5.36 b and c. In order to further investigate the cytotoxicity of the compound 26, MTT assays would need to be performed in the future research.

**Figure 5.38.** Confocal microscopy images of HeLa cells treated with 10µL of 5 mM compound 27 in 1% DMSO, incubated at 37 ºC for 15 minutes. The sample was excited at 408 nm wavelength and long-pass filtered at 515 nm. Image (a) was the merged image of images (b) and (c). Image (b) green fluorescence channel. Image (c) represented for image took from DIC. Scale bar: 20 µm.

The results in Figure 5.38 represent those resulting from the study of a different type of fluorescent sensor. The boronic acid group of compound 27 was used as a detector rather than an anchor as in compound 7. Compound 27 could bind to copper ions directly to form a complex, hence, it was designed to sense the level of copper concentration in the living organism. HeLa cells treated with compound 27 (Figure 5.38) were compared with HeLa cells pre-treated with copper then treated with compound 27 (Figure 5.39), in order to examine whether elevated the copper level would cause any effect on the fluorescence of compound 27.11

In the situation of compound 27 only, there was a strong fluorescence appearing in the cell cytoplasm, the hollow cavity surrounded by the fluorescence represented cell nuclei. The shape of nuclei in 5.38 (b) combined with the structure of cell membrane revealed in Figure 5.38 (c) altogether demonstrated that compound 27 could pass through cell membranes freely but would not penetrate into cell nuclei and it possessed limited cytotoxicity that would not cause a visible disruption of cells.
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Figure 5.39. Confocal microscopy images of HeLa cells were pre-treated with 10 µL of 50 mM CuCl$_2$ in 1% DMSO (37 ºC, 60 minutes incubation), subsequently 10 µL of 5 mM compound 27 was added incubated at 37 ºC for 15 minutes. The sample was excited at 405 nm wavelength and long-pass filtered at 515 nm. Image (a) was the merged image of images (b) and (c). Image (b) green fluorescence channel. Image (c) represented for image took from DIC. Scale bar: 20 µm.

When cells were pre-treated with copper ions, as seen in Figure 5.39 b, there was a significant decrease in the strength of fluorescence in side cells when there were 5 eq. of copper ions were added. Consequently, compound 27 could still enter the cell membrane freely without breaching the nuclei, but caused much less fluorescence at the same concentration and same temperature. It showed that the copper ions present in excess would remarkably influence the function of compound 27, which made compound 27 a potential sensor for copper. On the other hand, results from the Figure 5.39 concluded that both pre-treatment of copper and the formation of compound 27 – copper complexes would not damage cells. This phenomenon demonstrated that compound 27 and its copper complexes were biocompatible, which was one of the premise condition to design a sensor or detector for living organisms.

Figure 5.40 and Table 5.7 together revealed information regarding the fluorescence lifetime of compound 24 and compound 24 @glucan respectively. Both samples contained a second lifetime, which were fitted on multi-functional exponential with two components and the $\chi^2$ were 1.15 and 1.2 respectively. These indicated a reasonable fitting parameters in data analysis. The lifetime of compound 24 had a lifetime of 3.6 ns and the lifetime dropped to 3.0 ns when compound 24 was functionalised onto glucan. This lifetime alteration was due to interaction between compound 24 and glucan. The fitting in Figure 5.40 also shows that for the values this lifetime, as seen in the diagram
and their gradients of both fitting were visually different, the gradient of compound 24 was slightly higher than that of compound 24@glucan. This indicated that the lifetime of compound 24 was slightly longer than compound 24@glucan.

### Table 5.7

<table>
<thead>
<tr>
<th>Compound</th>
<th>Compound 24</th>
<th>Compound 24 @glucan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent system</td>
<td>DMSO</td>
<td>DMSO</td>
</tr>
<tr>
<td>$\chi^2$</td>
<td>1.15</td>
<td>1.2</td>
</tr>
<tr>
<td>$\tau_1$ (ns)</td>
<td>1.4</td>
<td>1.1</td>
</tr>
<tr>
<td>$\tau_1$ %</td>
<td>60.5</td>
<td>55.8</td>
</tr>
<tr>
<td>$\tau_2$ (ns)</td>
<td>3.6</td>
<td>3.0</td>
</tr>
<tr>
<td>$\tau_2$ %</td>
<td>39.5</td>
<td>44.2</td>
</tr>
</tbody>
</table>

Figure 5.40. TCSPC decay curves for compound 24 and compound 24@β-D-glucan in 1% DMSO. $\lambda_{ex} = 910$ nm. Laser power was in between 2.0 and 8.8 mW.
Figure 5.40 and Table 5.7 has all the information about fluorescence lifetime of compound 24 and compound 24 @glucan. Both samples contained two components, the chi square were 1.15 and 1.2 respectively, which were a reasonably acceptable value for data analysis. The lifetime of compound 24 had a lifetime of 3.6 ns and the lifetime dropped to 3.0 ns when compound 24 was functionalised onto glucan. This lifetime alteration was due to a conformational change of compound 24 when bound to glucan. This tendency in Figure 5.40 also shows this lifetime, as seen in the diagram, the gradients of both tendency were visually different, the gradient of compound 24 was significantly higher than that of compound 24 @glucan, which indicated the lifetime of compound 24 was longer than compound 24 @glucan.

Table 5.8. Two-photon Time Correlated Single Photon Counting data of compound 25 and compound 25 @glucan in 1% DMSO. $\lambda_{ex} = 810$ nm, laser power was 8.0 mW

<table>
<thead>
<tr>
<th>Compound</th>
<th>Compound 25</th>
<th>Compound 25 @glucan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent system</td>
<td>DMSO</td>
<td>DMSO</td>
</tr>
<tr>
<td>$\chi^2$</td>
<td>1.47</td>
<td>1.43</td>
</tr>
<tr>
<td>$\tau_1$ (ns)</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>$\tau_1$ (%)</td>
<td>41.4</td>
<td>41.3</td>
</tr>
<tr>
<td>$\tau_2$ (ns)</td>
<td>1.3</td>
<td>0.9</td>
</tr>
<tr>
<td>$\tau_2$ (%)</td>
<td>40.8</td>
<td>38.2</td>
</tr>
<tr>
<td>$\tau_3$ (ns)</td>
<td>4.2</td>
<td>3.1</td>
</tr>
<tr>
<td>$\tau_3$ (%)</td>
<td>17.8</td>
<td>20.8</td>
</tr>
</tbody>
</table>

Table 5.8 and Figure 5.41 were set of data represented all the solvent FLIM information of compound 25 and its composite with the glucan. The results showed that both samples carried an unusually large $\chi^2$ compared with that of compound 24, which might be due to the noise from the background or the interaction of compound with cellular environment. As such, fitting of the data on two exponential components was needed. There were also three components exposed in the sample solution, $\tau_1 = 0.4$ ns
(55.6%), $\tau_2 = 2.6$ ns (44.4%) and there was a slight decrease in the lifetime from 4.2 ns to 3.1 ns in $\tau_2$. When compound 25 was bound onto glucan, this feature of TCSPC indicated that binding to glucan would not cause a significant change in lifetime for compound 25.

![Figure 5.41. TCSPC decay curves for compound 25 at 10 mM and compound 25 @ β-D-glucan at 1 mg/mL in 1% DMSO. $\lambda_{ex} = 810$ nm, laser power was 8.0 mW](image)

**Table 5.9.** Two-photon Time Correlated Single Photon Counting data of compound 26 and compound 26@glucan in 1% DMSO. $\lambda_{ex} = 910$ nm. Laser power was in between 2.0 and 8.8 mW.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Compound 26</th>
<th>Compound 26 @glucan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent system</td>
<td>DMSO</td>
<td>DMSO</td>
</tr>
<tr>
<td>$\chi^2$</td>
<td>1.01</td>
<td>1.35</td>
</tr>
<tr>
<td>$\tau_1$ (ns)</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>$\tau_1$ %</td>
<td>50.6</td>
<td>52</td>
</tr>
</tbody>
</table>
Table 5.9 and Figure 5.42 exhibit a set of data representing all the solvent FLIM information of compound 26 and its composition with glucan. Both samples possessed reasonable chi squared values, which were 1.01 and 1.35 for compound 25 and compound 25@glucan respectively. The fluorescence lifetime showed the opposite results after binding to glucan, compared with compound 24 and 25. The lifetime of compound 26 was slightly increased from 2.5 ns to 2.7 ns, instead of decreasing as was observed with compounds 24 and 25. This result was calculated from the plot in Figure 5.42, there were more fluorescence signals detected in the FLIM of compound 26 @glucan, the gradients of compound 26 and compound 26 @glucan were different and the gradient of compound 26 was slightly smaller than compound 26 @glucan, thus the lifetime of compound 26 was slightly shorter.

<table>
<thead>
<tr>
<th>( \tau_2 ) (ns)</th>
<th>2.5</th>
<th>2.7</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \tau_2 % )</td>
<td>49.4</td>
<td>48.0</td>
</tr>
</tbody>
</table>

Figure 5.42. TCSPC decay curves for compound 26 and compound 26 @ D-glucan in 1% DMSO. \( \lambda_{ex} = 910 \) nm. Laser power was in between 2.0 and 8.8 mW.
Figure 5.43. FLIM images of HeLa cells treated with 10 µL of 10 mM compound 24 and 10 mM compound 25 @ 1 mg/mL glucan in 1% DMSO, cells were incubated at 37 ºC for 15 minutes. where (a) two photon fluorescence intensity diagram of compound 24 in HeLa cells (37 ºC, 15 minutes incubation), (b) the fluorescence lifetime map of $\tau_m$ of compound 24 in HeLa cells (37 ºC, 15 minutes incubation) (c) two photon fluorescence intensity diagram of compound 25 @glucan in HeLa cells (37 ºC, 15 minutes incubation), (d) the fluorescence lifetime map of $\tau_m$ of compound 25 @glucan in HeLa cells (37 ºC, 15 minutes incubation).

Figure 5.43 showes FLIM images of cells cultured with compound 24 and compound 25 @glucan. The results displayed in Figure 5.43 (b) was compared with Figure 5.33 (b), fluorescence distribution was similar, compound 24 entered cells and spread in the cytoplasm. During the imaging there was notable presence of precipitations of the compound on the membrane of cells, and the morphology of cells remain unchanged in 5.43 (b), this appeared to be healthier than the morphology observed in 5.33 (b). In Figure 5.43 (d), compound 25 assisted by glucan easily entered HeLa cells, but, unlike the fluorescence lifetime distribution exhibited in Figure 5.35 (b), there were fluorescence signals discovered in some of the cell nuclei too. However,
it seemed that the morphology of cells were severely interrupted and there was significant increase in the intensity of fluorescence in majority of the cells. All indicated that cell damage occurred and made the compound 25 @glucan with more likely to access to cell nuclei.

### Table 5.10

<table>
<thead>
<tr>
<th>Compound</th>
<th>Compound 24</th>
<th>Compound 25 @glucan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell line</td>
<td>HeLa</td>
<td>HeLa</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>Incubation time (min)</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>$\chi^2$</td>
<td>1.02</td>
<td>1.01</td>
</tr>
<tr>
<td>$\tau_1$ (ns)</td>
<td>0.9</td>
<td>0.8</td>
</tr>
<tr>
<td>$\tau_1$ %</td>
<td>30.5</td>
<td>39.5</td>
</tr>
<tr>
<td>FWHM (ns)</td>
<td>1.2</td>
<td>0.5</td>
</tr>
<tr>
<td>$\tau_2$ (ns)</td>
<td>3.5</td>
<td>2.7</td>
</tr>
<tr>
<td>$\tau_2$ %</td>
<td>69.5</td>
<td>60.5</td>
</tr>
<tr>
<td>FWHM (ns)</td>
<td>1.0</td>
<td>0.6</td>
</tr>
</tbody>
</table>

The results obtained in Table 5.10 were used to access the fluorescence lifetimes differences between compound 24 and compound 25 @glucan. There was no significant contrast between each other. As seen in Table 5.10 and 5.7 together, compound 24 showed little change in its fluorescence lifetime from 3.6 ns to 3.5± 1.0 ns, but it altered significantly in its proportion: the percentage of component two was raised from 39.5% to 69.5%. Hence, the fluorescence of the second lifetime component became more significant, and the overall lifetime of fluorescence shifted towards 3.5 ns. Similar changes were observed for to compound 25 @glucan: its fluorescence
lifetime reduced from 3.0 ns to $2.7 \pm 0.6$ ns, with a percentage change from 20.8% in the solvent to 60.5% in cells for this component.
Figure 5.44. FLIM images of HeLa or PC-3 cells treated with 10 µL of 10 mM compound 26 and 10 mM compound 26 @ 1 mg/mL glucan in 1% DMSO, cells were incubated at 37 ºC or 4 ºC for 15 minutes. where (a) two photon fluorescence intensity diagram of compound 26 in HeLa cells (37 ºC, 15 minutes incubation), (b) the fluorescence lifetime map of $\tau_m$ of compound 26 in HeLa cells (37 ºC, 15 minutes incubation) (c) two photon fluorescence intensity diagram of compound 26 @glucan in PC-3 cells (37 ºC, 15 minutes incubation), (d) the fluorescence lifetime map of $\tau_m$ of compound 26 @glucan in PC-3 cells (37 ºC, 15 minutes incubation). (e) two photon fluorescence intensity diagram of compound 26 in HeLa cells (4 ºC, 15 minutes incubation), (f) the fluorescence lifetime map of $\tau_m$ of compound 26 in HeLa cells (4 ºC, 15 minutes incubation).

The results exhibited in Figure 5.44 represented the two photon FLIM data of the rhodamine-tagged fluorescent molecule (compound 26). They are linked to Figure 5.36 and Figure 5.37. As was revealed in Figure 5.44 (a) and (b), the fluorescence lifetime of compound 26 was mainly from cytoplasm. In Figure 5.44 (c) and (d), cells were cultured with compound 26 @glucan at 37 ºC in order to change the uptake route of compound 26. The outcome showed a significant change in the cell morphology, although limited change in fluorescence lifetime was detected from the cytoplasm. The same phenomena was observed in Figure (e) and (f), as the temperature was dropped to inhibit the active uptake of compound 26. Although the outcome was not as good as that observed for figure 5.44 (c) and (d), the fluorescence lifetime mainly comes from the cytoplasm, the colour combines blue and green indicate at least two components existed.

Table 5.11 Two-photon Time Correlated Single Photon Counting data of HeLa or PC-3 cells treated with compound 26 and compound 26 @glucan at 37 ºC or 4 ºC for 15 minutes. $\lambda_{ex} = 910$ nm. Laser power was in between 2.0 and 8.8 mW.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Compound 26</th>
<th>Compound 26 @glucan</th>
<th>Compound 26</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell line</td>
<td>HeLa</td>
<td>PC-3</td>
<td>HeLa</td>
</tr>
<tr>
<td>Temperature ($^\circ$C)</td>
<td>37</td>
<td>37</td>
<td>4</td>
</tr>
<tr>
<td>Incubation time (min)</td>
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<td>15</td>
<td>15</td>
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</table>
Table 5.1: FLIM parameters for compounds 26 and 26@glucan in HeLa and PC-3 cell line.

<table>
<thead>
<tr>
<th></th>
<th>Compound 26 in HeLa</th>
<th>Compound 26@glucan in PC-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\chi^2)</td>
<td>1.08</td>
<td>1.25</td>
</tr>
<tr>
<td>(\tau_1) (ns)</td>
<td>0.4</td>
<td>0.6</td>
</tr>
<tr>
<td>(\tau_1) %</td>
<td>48.8</td>
<td>72.3</td>
</tr>
<tr>
<td>FWHM (ns)</td>
<td>0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>(\tau_2) (ns)</td>
<td>2.4</td>
<td>2.9</td>
</tr>
<tr>
<td>(\tau_2) %</td>
<td>51.2</td>
<td>27.7</td>
</tr>
<tr>
<td>FWHM (ns)</td>
<td>2.6</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Figure 5.45: FLIM decay curves for compound 26 and compound 26@glucan in HeLa and PC-3 cell line. \(\lambda_{ex} = 910\) nm. Laser power was in between 2.0 and 8.8 mW.

Figure 5.45 and Table 5.11 together determined the lifetime of compound 26 and compound 26@glucan. It could be observed from Table 5.11 that compound 26 was a compound with a short lifetime. Whereas there was a slight increase in the lifetime when treated cells with compound 26 at 4°C or compound 26@glucan at 37°C. The lifetime was raised from 2.4 ± 2.6 ns to 2.9 ± 2.7 ns and 2.8 ± 0.9 ns respectively. At the same time, there were a remarkable drop in the proportion of fluorescence, which
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was from 51.2% (compound 26 alone, 37 °C) to 27.7% (compound 26 @glucan, 37 °C) and 17.2% (compound 26 alone, 4 °C) respectively. This might because the route of uptake changed when combined with glucan, lead to a slow absorption, or uptake of compound reduced at 4 °C. The tendency in Figure 5.45 also proved that there were less fluorescence signals counted in compound 26 @glucan and compound 26 at 4 °C condition.

Figure 5.46. Fluorescence lifetime distribution curve of HeLa cells treated with compound 26 and compound 26 @glucan in different conditions. $\lambda_{ex} = 910$ nm. Laser power was in between 2.0 and 8.8 mW.

The overall lifetime distribution of compound 26 in HeLa at 37 °C was mainly distributed in between 0 and 4 ns, it had peaks at 0.8 ns and 1.3 ns respectively. When compound 26 was on β-D-glucan, the range of fluorescence distribution shortened to 0.6 ns to 3.2 ns and peaked at 1.9 ns. For compound 26 at 4 °C as well, due to a reduced uptake rate, the fluorescence photon counts were significantly lower than at 37 °C, and the lifetime distribution ranged in between 0 to 2.6 ns with a broad peak at 1.3 ns.
Table 5.12. Two-photon Time Correlated Single Photon Counting data of compound 27 and compound 27 with CuCl2 in 1% DMSO. \( \lambda_{ex} = 910 \text{ nm}, \) laser power was 0.25 – 0.5 mW.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Compound 27</th>
<th>Compound 27 with CuCl2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent system</td>
<td>DMSO</td>
<td>DMSO</td>
</tr>
<tr>
<td>( \chi^2 )</td>
<td>1.06</td>
<td>1.23</td>
</tr>
<tr>
<td>( \tau_1 ) (ns)</td>
<td>9.0</td>
<td>4.4</td>
</tr>
<tr>
<td>( \tau_1 ) %</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Figure 5.47. TCSPC decay curves for compound 27 and compound 27 with CuCl2 in 1% DMSO. \( \lambda_{ex} = 910 \text{ nm}, \) laser power was 0.25 – 0.5 mw.

Figure 5.47 with table 5.12 fully explained the lifetime of compound 27 and compound 27 with CuCl2 in 1% DMSO. It was showed clearly in both diagram and table that there was a significant difference in fluorescence lifetime between compound 27 and compound 27 with CuCl2 in 1% DMSO.
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27 and compound 27 bound to copper ions. The lifetime dropped from 9.0 ns to 4.4 ns when compound 27 was bound to copper ions. There was only one component found in both conditions and the chi squared values were 1.06 and 1.23 respectively, both in a reasonably acceptable range.

Figure 5.48. FLIM images of HeLa cells treated with 10 µL of 10 mM compound 27 in 1% DMSO, cells were incubated at 37 ºC for 15 minutes. where (a) two photon fluorescence intensity diagram of compound 27 in HeLa cells (37 ºC, 15 minutes incubation), (b) the fluorescence lifetime map of τm of compound 27 in HeLa cells (37 ºC, 15 minutes incubation) (c) two photon fluorescence intensity diagram of compound 27 in HeLa cells, which were pre-treated with 5 eq. of CuCl₂ for 60 min (37 ºC, 15 minutes incubation), (d) the fluorescence
lifetime map of $\tau_m$ of compound 27 in HeLa cells, which were pre-treated with 5 eq. of CuCl$_2$ for 60 min (37 °C, 15 minutes incubation).

FLIM images displayed in Figure 5.48 showed that compound 27 entered HeLa cells without entering the cells’ nuclei, the fluorescence signal mainly emitted from cytoplasm. The morphology of cells did not change significantly, which indicated that compound 27 was non-toxic to HeLa cells. In Figures 5.48 (c) and (d), a similar pattern was observed, compound 27 easily entered HeLa cells that pre-treated with CuCl$_2$, there were no significant fluorescence signals visualised in nuclei. However, there was a considerable drop in the fluorescence intensity. At the same time, there was a slight increase in the size of the nuclei, which might due to a prolonged incubation of copper ions during the period of pre-treatment.

Table 5.13. Two-photon Time Correlated Single Photon Counting data of HeLa cells treated with compound 27 and compound 27 with CuCl$_2$ at 37 °C for 15 minutes. $\lambda_{ex} = 910$ nm, laser power was 0.25 – 0.5 mW.
Figure 5.49. FLIM decay curves for compound 27 and compound 27 with CuCl₂ in HeLa.
λ_ex = 910 nm, laser power was 0.25 – 0.5 mW.

Figure 5.49 and table 5.13 together represented all lifetime information corresponding to Figure 5.48, first of all, the chi square for both compound 27 and compound 27 with copper were 1.05 and 1.07 respectively, they were in the reliable range of analysis. The lifetime of compound 27 in HeLa cells was 12 ± 3.8 ns, which was higher than that in 1% DMSO, hence, the fluorescence cycle of compound 27 was processed slower in cells. Then the fluorescence lifetime was reduced remarkably to 6.8 ± 4.6 ns when band to copper ions. The tendency of compound 27 and compound 27 and copper complexes were similar, but the gradient of compound 27 was slightly more flattened than that of compound 27 –copper complexes, which resulted a longer lifetime for the compounds’ fluorescence emission in this environment.12
Figure 5.50 fluorescence lifetime distribution curve of HeLa cells treated with compound 27 and compound 27 – copper complexes at 37 °C for 15 minutes. \( \lambda_{\text{ex}} = 910 \text{ nm} \), laser power was 0.25 – 0.5 mW.

As seen in Figure 5.50, the lifetime distribution of compound 27 was in 0 to 11 ns, the peak was appeared at 7 ns. Whereas compound 27 with copper had lifetime distributed in between 2 ns and 13 ns, the peak was also appeared at 7 ns. The difference in the fluorescence counts was due to different strength of laser power applied.
5.8. **Summary of chapter five**

This Chapter detail an investigation into a number of fluorophores and imaging within different cell lines and under distinct environmental conditions, using laser scanning confocal microscopy and FLIM, with single photon and two photon excitation.

In confocal microscopy, first of all, compound 4 was shown to have a good penetrability through cell membrane, but not through the nuclear membrane. Compound 7 was found to pass through both membrane, but most of compound 7 was retained in the cytoplasm. A Hoechst dye co-localisation experiment was carried out to confirm the presence of compound 7 in the nucleus. In addition, Lysotracker experiments were also performed to confirm the distribution of compounds in the lysosomes. Secondly, biocompatible SWNTs with fluorescent sensors were examined. The β-D-glucan, attached to a compound 7 as a composite, was structured with two different cell lines. Both cell types showed a reasonable uptake of the composites and there was a reduction in the fluorescence signal from the nucleus. This indicated that there were fewer compounds (or composites), which had the ability to entered the cell nucleus. However, imaging of SWNTs@ glucan@ compound 7 showed a clear penetration of both cell membrane and nuclear membrane, which gave a significant fluorescent signal from cells and slight disruption of cell homeostasis. SWNTs @glucan @compound 9 was also studied as a comparison, because there were no boronic acid present in the structure to bind to the glucan surface. As such most of compound 9 was washed out from cells and imaging showed only a weak fluorescent signal. In the next step, the same concentration of fluorescent compounds were studied at different incubation times and temperatures. It was clear that the increase in the time of incubation would significantly increase the fluorescence signals and toxicity in cells too. Incubated cells at lower temperatures (4 °C) could considerably reduce the uptake rate of fluorescent compounds and therefore inhibited the damage caused by these compounds. Finally, a list of fluorescent sensors with different fluorophores from collaborators were investigated, most of compounds showed a good uptake, sharp fluorescence and reasonable damage to cells, however those fluorophores could be potentially useful in life science imaging experiments, after some modification of their structures. The result of compound 27 revealed potential applications of boronic acid
based fluorescent sensors in ion detection, which could be very useful in the search for cancer diagnostics.

As for the FLIM, the fluorescence lifetime and components distribution varied under various conditions, which referred to lifetime and distribution of components. This could be influenced by the solvent or the cells’ nature, under different environmental conditions. In TCSPC measurement, the fluorescence lifetime appeared from multi-exponential fitting functions using single components or two components, which percentage or the ratio of the two components was commonly closed to 1:1. With cells, most of the fluorescence lifetimes were presented in the two-component form and the multi- exponential fitting components were distributed in a more variable ratio, which may be due to the complicated cellular environment and the presence of the biomolecules affecting the binding and conformation of the compound. As a result, most of the $\chi^2$ values of the FLIM were reasonably acceptable. The FWHM corresponded to the lifetime and binding of molecules, in general, a fluorophore with a high fluorescence lifetime commonly had a larger FWHM. The binding of a small molecule to a biomolecule inside cells would lead to significant conformational changes, resulting in an altered FWHM. Furthermore, temperature and incubation time modification affected the uptake of fluorescent sensors, leading to a change in the distribution of the components and in different FWHM values. Most commonly, it was shown that higher temperatures and longer incubation times yield a broader uptake of compounds or composites and hence produce a wider FWHM. These observations led to a generalised method for studying small molecules, biocompatible polymers and nanomaterials with cells and optical imaging assays.
5.9. **Reference for chapter five**


Chapter 6. Experimental section

6.1. Chemicals and glassware

All solvent and chemicals used in this project were reagent-grade and HPLC-grade respectively unless stated otherwise. These were purchased from Fisher scientific and Sigma Aldrich Company Ltd, and were usually used without further purification, unless specified otherwise. The majority of life science consumables, such as cellular, media, labels and proteins, were obtained from Sigma Aldrich Company Ltd and Invitrogen Company. Carbon dioxide cylinders used in cell culture, was supplied by BOC.

All glassware was bought from Fisher scientific and VWR international and cleaned via the standard base- and acid-bath methods. The base bath was made of 300 g of potassium hydroxide, 10 L of isopropanol and 100 mL of toluene. Glassware was immersed totally in the base bath for 24 hours at room temperature and then rinsed with excess of MilliQ water (MilliQ water is purified via a Milli-Q Reference purification system), whereby the purity of water used was normally characterised in terms of resistivity, which was 18.2 MΩ·cm at 25 °C. The glassware used was subsequently placed in an acid bath, which was made of diluted hydrochloric acid, for 24 hours at room temperature. Afterwards, glassware was rinsed with MilliQ water to remove the acid and was then dried on the rack or at 60°C oven. This complex protocol was needed to ensure that the carbon nano materials used are not contaminated with ions.

6.2. Thin Layer Chromatography (TLC)

TLC was performed using commercially available Merck aluminium backed plates coated with 200 µm layer of silica gel 60 with fluorescent indicator F254 for detection of colourless substances. These plates were visualised by using either ultraviolet light of 254 nm or 365 nm wavelengths, samples which absorb at 254 nm or at 365 nm will be revealed due to a fluorescence quenching.
6.3. Column chromatography

Silica gel column chromatography was carried out by using silica gel (60 Å, 35-70 μm particle size). The volume of silica gel depended on the mass of the sample available; normally 30 g of silica gel was needed per 1 g of sample. Weighted silica was made into a slurry in excess of methanol and then silica precipitated in the column. Then samples were dissolved in a minimum volume of solvent and added carefully to the column to form a thin layer in between the silica gel and sand layer. Finally, sand was added on top of the silica as a buffer layer.

The column was washed with a mixture of two solutions in different proportion in order to separate samples into different layers. Then the washout was collected into different fractions and each fraction would be checked with a TLC test until the desired spot was found. Eventually, the fraction carried the compound was dried using a freeze drier and further spectroscopic characterisation carried out to confirm the result.

6.4. High performance liquid chromatography (HPLC)

HPLC was carried out using a ThermoFisher HPLC systems, samples of 1 mg/mL concentration in DMSO were injected and passed through a Symmetry® C-18 column (4.6 x 260 mm) through a UltiMate 3000 Diode Array Detector with the UV/visible detection measured at \( \lambda_{\text{obs}} = 200 \text{ nm}, 300 \text{ nm}, 400 \text{ nm}, 450 \text{ nm}, 500 \text{ nm}, 600 \text{ nm}, 700 \text{ nm} \) and 800 nm. The flow rate was 0.8 mL/minute and the eluents involved gradient methods of two solvents: solvent A was made of MilliQ water with 0.1% TFA and solvent B as MeCN in 0.1% TFA.

The HPLC system was switched on 60 minutes prior to the initial measurement, the flow was set at 0.2 mL/minute under UV/Vis detection in order to wash all residuals in the column. Samples were injected and examined with the following method: Start, 95% A reverse gradient until 5% A at 7.5 minutes, kept rinsed samples with 5% A :95% B solvent mixture until 15 minutes, reverse gradient until 17.5 minutes 95% A, then hold to 21 minutes.
Chapter 6. Experimental section

The UltiMate 3000 pump was maintained by the solution made of 80% MilliQ water, 15% MeCN and 5% Isopropanol. All solvents used in HPLC measurements were HPLC grade and the water was deionised.

6.5. Mass spectrometry

Data was acquired on a Bruker Daltonics electrospray ionisation – time-of-flight (ESI-TOF) mass spectrometry. Samples were dissolved in HPLC-grade methanol or distilled water, and under some circumstances, in methanol with one drop of DMSO. The concentration of the solution needs to be kept in a range of 1 µg to 10 µg, the formula of samples were identified or confirmed via positive or negative loop injection. The results were analysed using the Bruker Daltonics Data Analysis 4.0 software package.

6.6. Nuclear magnetic resonance spectroscopy (NMR)

$^1$H NMR, $^{13}$C NMR, COSY NMR, (including solvent suppression techniques), HSQC and HMBC spectra were recorded on a Bruker 300 Ultrashield™ ($^1$H: 300 MHz, $^{13}$C: 75.5 MHz) at the room temperature, using in the facilities of the University of Bath. All spectra were recorded in chloroform-$d$, methanol-$d$, dimethyl-$d_6$ sulfoxide or combination of deuterated solvents, for instance, in methanol-$D$ with a drop of dimethyl-$d_6$ sulfoxide, depending on the sample solubility. Chemical shifts ($\delta$) of NMR spectra were expressed in parts per million (ppm) and relative to a reference signal, which is usually tetramethylsilane (TMS), as an internal standard. The multiplicity and general assignments of the spectroscopic data were abbreviated to: singlet (s), doublet (d), triplet(t), quartet (q), doublet of doublet (dd), doublet of doublet of doublet (ddd), doublet of triplet (dt) triplet of triplet (tt), unresolved multiplet (m), apparent (app), broad (br) and aromatic (Ar).
6.7. **Fluorescence spectroscopy**

Fluorescence spectroscopy measurements were performed on a Perkin Elmer Luminescence spectrophotometer LS 55 in the Department of Chemistry at University of Bath, utilising 1.2 mL volume quartz cuvettes, with 10 mm path length. The spectrophotometer was usually turn on for 30 minutes prior to use. All solvents used in fluorescence measurements were HPLC grade and the water used was MilliQ quality. The concentration of samples could be varied between $10^{-5}$ and $10^{-7}$ M dependent on the strength of fluorescence; however, a pre-scan in the range of 200 – 900 nm was usually necessary to determine the maximum excitation and emission wavelength. Then the maximum excitation will be applied to acquire the precise emission peak. Data collected was processed via the FL WinLab v1.60 software package and origin 9.1.

6.8. **UV spectroscopy**

Electronic absorption spectroscopy (UV/vis) was examined by using a Perkin-Elmer Lambda 35 spectrometer and spectra were measured using two 1.00 cm quartz cuvettes. Data was collected and analysed via the UV Winlab software package. The instrument was usually switched on 30 minutes prior measurement for stabilisation. All solvents used in UV Vis measurements were HPLC grade and the water was deionised. All measurements started with calibration, which set solvent as standard in the same solvent system, recalibration was essential for switching to another solvent system.

6.9. **Transmission electron microscopy (TEM)**

High resolution transmission electron microscope (HR TEM) images of functionalised SWNTs were captured with Gatan Dualvision digital camera on a JEOL 1200EXII high resolution microscope (operating voltage of 120 kV) coupled with Energy-dispersive X-ray spectroscopy (EDS) (point resolution, 0.16 nm) in the University of Bath. Samples for HR TEM were dispersed in EtOH or MilliQ water environment and load drop wise onto a copper grid coated with holey carbon film. In circumstances, copper grids could be oxidised by exposure to UV light, as a result, an ozone layer was created.
on the surface of the grid to improve the hydrophilicity. Finally, samples would be left in a desiccated environment overnight prior the observation by TEM.

6.10. Scanning electron microscopy (SEM)

SEM images were taken using JEOL JSM6480LV scanning electron microscope and Oxford INCA X-ray analyser used for mapping, scanning and quantitative analysis. Samples of SEM were dispersed in ethanol and mounted on stubs. A convenient adhesive was a double-stick, electrically-conductive carbon tape. Samples were left in a desiccated environment overnight prior to the observation by SEM.

6.11. Raman spectroscopy

Raman spectroscopy was performed at central laser facilities in the Rutherford Appleton Laboratory. A Renishaw upright microscope was adopted and samples were excited at 830 nm wavelength. The working distance (LWD) of objective was 2 cm and operated at 50× magnification. The laser power pulsed at samples was 220 mW.

6.12. SWNTs Purification

The purification of SWNTs was carried out via a solvent oxidation method, which included the steps described below. The whole purification process was started with a microwave heating to expose the metal catalyst from inside of nanoparticles. Next, an ultrasonication was utilised to disperse aggregated SWNTs, then a hydrochloric acid wash followed by filtration to dissolve and rinsed metal catalyst away. Subsequently, an oxidative treatment using concentrated nitric acid was performed in order to oxidise the amorphous carbon and graphite carbon. Finally, a NaOH / aqueous washing and filtration step were carried out to improve the dispersibility of oxidised amorphous carbon and graphite carbon and wash these impurities away from the system.

In a typical experiment, 20 mg of raw SWNTs (as made by the arc discharge method, supplied by Carbolex, Rice) were weighed and added to 20 mL of H₂O₂ in 50
mL centrifuge tube. The mixture was ultrasonicated in an ultrasonic bath (Ultrawave U-500 30 Hz or Agar scientific S0080 Sonomatic 30 HZ, 80W) for five minutes. This step was repeated four times with one minute intervals to minimise the damage to the tubes. Then, the mixture was separated into four 10 mL microwave vials for the microwave treatment (Biotage initiator 2.0), using the microwave setting at 120 W and 40 °C. This step lasted one minute and was repeated over five cycles per vial. Subsequently all the mixtures were collected into 50 mL centrifuge tube and diluted with excess MilliQ water and filtered through a cyclopore track etched membrane. This was a hydrophilic membrane with 0.2 µm pores with resistance to inorganic corrosive aqueous solutions. The solid left on the membrane was then collected and dispersed into 70 mL of MilliQ water, then heated under reflux with 70 mL 16% HCl at 120 °C whilst stirring at 1000 rpm overnight. After that, the mixture was diluted with excess MilliQ water and neutralized with NaOH and subsequently filtered through a cyclopore track etched membrane (0.2 µm pore from Camlab). The solid collected on the membrane was dispersed into 50 mL of MilliQ water and 30 mL of 9 M HNO₃ were added dropwise. Subsequently, the mixture was heated under reflux with stirring at 1000 rpm for 24 hours. Afterwards, the sample was diluted, filtrated and washed with excess MilliQ H₂O and re-dispersed into 20 mL of MilliQ H₂O. The mixture was transferred into a polytetrafluoroethylene (PTFE) container which is base resistant, then treated with 100 mL of 8M NaOH and heated under reflux and stirred at 1000 rpm for 48 hours under nitrogen condition. The mixture was subsequently diluted with MilliQ water and filtrated though a cyclopore track etched membrane and washed with excess (1L) MilliQ water. The final product was washed with excess MilliQ water through the hydrophilic membrane and 300 mL of toluene through a hydrophobic membrane (polyethylene membrane). Purified SWNTs were dispersed into a MilliQ water or ethanol for characterisation, which was carried out via high resolution TEM / SEM coupled with EDX and Raman spectroscopy.
6.13. General synthesis of compounds@ Glucan@ SWNTs

![Scheme 6.1. Synthetic pathway of Glucan@SWNTs component and R-B (OH)$_2$ (compounds) @Glucan @SWNTs, where R = fluorophore.]

1 mg SWNTs was weighed and dispersed into 1 mL DMSO, the mixture was sonicated for five minutes with one minute interval and the procedure was repeated four times. A stock solution of 1 mL glucan in DMSO (1 mg/mL) was added; the mixture was diluted to 10 mL with MilliQ water and stirred overnight at room temperature. Then the mixture was filtered using a cyclopore track etched membrane and washed with excess of MilliQ water. The solid part on the filter membrane was dispersed in 1 mL of water. Boronic acid-based fluorophores were added into the dispersion and stirred for 48 hours at room temperature. The mixture was filtered and washed with excess of MilliQ water. Finally, the residue on the filter membrane was dispersed into MilliQ water, and then products were freeze dried and re-dispersed into 1 mL of MilliQ water.
6.14. General procedure for the internal functionalisation of SWNTs

In a typical procedure, a sample of ca. 1 mg SWNTs was weighed and dispersed into 1 mL MilliQ water, the mixture was sonicated for five minutes with one minute interval and the procedure was repeated four times. 1 mg copper acetate in 1 mL MilliQ water was added; the mixture was stirred overnight at room temperature for two hours. Then 1 mL glucan (2mg /mL) in MilliQ water was added and stirred for an hour. Finally, 1

Scheme 6. 2. Synthetic pathway of copper filled SWNTs

MilliQ H₂O/toluene
washing

C₇₀
1h, r.t.

DMSO
1h, r.t.

β-D-glucan

2h, r.t.

⁶⁴Cu(OAc)₂
mg C\textsubscript{70} was added and stirred at room temperature for an hour. The mixture was filtered using a cyclopore track etched membrane and washed with excess of MilliQ water. The solid part on the filter membrane was dispersed in 1 mL of ethanol. Then the product was filtered again through polyethylene membrane and rinsed with toluene. The residue remained on the membrane, was collected and dispersed into MilliQ water.

6.15. Specific synthetic details and structures

(a) Synthesis of 2,5-dioxopyrrolidin-1-yl 5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanoate (I)

\begin{center}
\includegraphics[width=0.5\textwidth]{structure.png}
\end{center}

Figure 6. 1. Structure of 2,5-dioxopyrrolidin-1-yl 5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanoate

488 mg (2 mmol) N-Biotin and 230 mg (2 mmol) N-Hydroxysuccinimide (NHS) were added into 20 mL of Dimethylformamide (DMF). The mixture was heated to 100 °C until all the suspensions was dissolved. 412 mg (2 mmol) of N, N-dicyclohexylcarbodiimide (DCC) was subsequently added to the solution then the mixture was stirred under nitrogen at room temperature. A white suspension was formed at the end and then been filtered, the filtrate was evaporated to collect 669 mg of white solid products (98% yield).

\textsuperscript{1}HNMR (300 MHz, CD\textsubscript{3}OD, 298 K) $\delta = 6.42$ (m, 2H, H\textsubscript{a,b}), 4.29 (m, 1H, H\textsubscript{9}), 4.13 (m, 1H, H\textsubscript{8}), 2.83 (d, 2H, H\textsubscript{10}, J= 5.43 Hz), 2.89-2.54 (m, 4H, H\textsubscript{1,2}), 1.87-1.26 (m, 9H, H\textsubscript{3-7}).
Mass Spectrum ESI-MS calculated for C_{14}H_{19}N_{3}O_{5}S [M + H]^{+} 342.1182, found 342.1097. HPLC R_{t} = 5.2 minutes.

IR spectrum: 1711 cm\(^{-1}\), 1645 cm\(^{-1}\), 1552 cm\(^{-1}\), 1061 cm\(^{-1}\), 861 cm\(^{-1}\).

(b) Synthesis of N-(6-aminohexyl)-5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide (2)

Figure 6. Structure of N-(6-aminohexyl)-5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide

500 mg (1.46 mmol) of compound 1 was dissolved into 20 mL of DMF solution and 0.3 mL of TEA added. The mixture was sonicated until all white solids were dissolved. Then 1.7 g (14.6 mol) of Hexamethylenediamine was added, dissolved into 50 mL MilliQ water. Subsequently the mixture was stirred overnight under room temperature. Finally, 100 mL of diethyl ether was added to the mixture and the formation of a white solid was observed. This was separated from the supernatant by filtration and dried in the freeze dryer overnight, 200 mg of white solid of collected (40% yield). The formation of the desired product (Compound 2) was confirmed by mass spectrometry (ESI\(^{+}\)) and \(^{1}\)H NMR spectroscopy.

\(^{1}\)HNMR (300 MHz, CD\(_{3}\)OD, 298 K) \(\delta = 4.52\) (t, 1H, H\(_{13}\), J=3.92), 4.34 (s, 1H, H\(_{12}\)), 3.07 (m, 1H, H\(_{11}\)), 3.10 – 2.85 (m, 2H, H\(_{14}\)), 1.83–1.34 (m, 20H, H\(_{1,2,3,4,5,6,7,8,9,10}\)).

(c) Synthesis of 5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-N-(6-((4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)amino)hexyl)pentanamide (3)

![Chemical Structure](image)

**Figure 6.3.** Structure of 5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-N-(6-((4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)amino)hexyl)pentanamide

200 mg (0.61 mmol) of compound 2 were dissolved in 20 mL of DMF. To this 92 mg (0.61 mmol) of 4-formylphenylboronic acid and 72 mg (0.61 mmol) of pinacol were added, as well as activated molecular sieves (3 Å). The mixture was then stirred for 16 hours under an inert atmosphere (N₂). Next, 0.61 mmol sodium triacetoxyborohydride was added to this, and the mixture was stirred for a further 24 hours. Finally, the solution was neutralized by excess ethyl acetate, filtered and evaporated under reduced pressure to give 58 mg of compound 3 as a white solid (11% yield). The purity was verified by TLC and the identity was confirmed by mass spectrometry (ESI⁺) and ¹H NMR spectroscopy.

**¹H NMR** ((CD₃)₂OS, 300 MHz, 298K) δ = 7.89 (d, 2H, H$_{5,8}$, J=8.32 Hz), 7.54 (d, 2H, H$_{6,7}$, J=8.32 Hz), 4.21 (m, 1H, H$_{22}$), 4.04 (m, 1H, H$_{21}$, J= 6.94 Hz), 3.41 (d, 2H, H$_9$, J= 6.93), 2.75 (dd, 1H, H$_{23}$, J= 5.12 Hz), 2.48 (d, 1H, H$_{23}$, J= 12.28 Hz), 1.79-1.30 (m, 21H, H$_{10-15,16-20}$), 1.24 (s, 12H, H$_1-4$)

ESI-MS (m/z) calc. for C$_{29}$H$_{47}$BN$_4$O$_4$S [M+H]$^+$ 559.3411, found 559.3679. HPLC R$_t$= 6.4 minutes.
(d) Synthesis of tert-butyl (4-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)methyl)benzyl)carbamate (5)

250 mg (0.73 mmol) of biotin-NHS and 173 mg (0.73 mmol) boc protected p-xylylenediamine were dissolved in 10 mL DMF, and then 4 eq. of TEA was subsequently added. The mixture was stirred at room temperature overnight. The product was evaporated and yielded 220 mg of white solid. (83% yield)

1H NMR (500 MHz, (CD$_3$)$_2$OS) δ = 8.25 (t, 1H, H$_a$, J = 5.6 Hz), 7.34 (t, 1H, H$_b$, J= 5.8 Hz), 7.1 (m, 4H, H$_{8-12}$), 6.42 (s, 1H, H$_c$), 6.35 (s, 1H, H$_{20}$), 4.30 (t, 1H, H$_d$, J= 5.3 Hz), 4.21 (d, 2H, H$_{13}$, J = 5.5 Hz), 4.11 (m, 1H, H$_{21}$), 4.06 (d, 2H, H$_{6}$, J = 5.5 Hz), 3.08 (m, 1H, H$_{19}$), 2.82 (dd, 1H, H$_{22}$, J = 12.2 Hz, J = 5.0 Hz), 2.58 (t, 1H, H$_{22}$, J=5.0 Hz), 2.13 (t, 2H, H$_{15}$, J = 7.6 Hz), 1.5 (m, 2H, H$_{16}$), 1.8 – 0.98 (m, 4H, H$_{17,18}$), 1.39 (s, 9H, H$_{1-3}$).

13C NMR ((CD$_3$)$_2$OS, 500MHz, 298 K) δ = 173.3 (C$_5$), 172.4 (C$_{14}$), 163.2 (C$_{23}$), 139.1 (C$_7$), 138.5 (C$_{10}$), 127.5 (C$_{8,12}$), 127 (C$_{9,11}$), 78.2 (C$_4$), 61.5 (C$_{21}$), 61.2 (C$_{20}$), 55.9 (C$_{19}$), 43.6 (C$_6$), 42.2 (C$_{13}$), 39.5 (C$_{22}$), 35.6 (C$_{15}$), 33.7 (C$_{16}$), 28.7 (C$_{1-3}$), 28.5 (C$_{17}$), 22.8 (C$_{18}$).

Mass Spectrum ESI-MS calculated for C$_{31}$H$_{43}$BN$_4$O$_4$S [M + H]$^+$ 485.2193, found 485.2300.
IR spectrum: 3298 cm\(^{-1}\), 1778 cm\(^{-1}\), 1695 cm\(^{-1}\), 1637 cm\(^{-1}\), 1522 cm\(^{-1}\), 1163 cm\(^{-1}\), 871 cm\(^{-1}\).

(e) Synthesis of 5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-N-(4-(((4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)amino)methyl)benzyl) pentanamide (6)

Figure 6.5. Structure of 5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-N-(4-(((4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)amino)methyl)benzyl) pentanamide

281 mg (0.5 mmol) of compound 5 was dissolved in 8 mL TFA and DCM (1:3), the mixture was stirred at room temperature for two hours. The solvent was evaporated and white solid was yielded. 220 mg (0.5 mmol) of de-protected compound 5 was mixed with 110 mg (0.5 mmol) 4-formal-benzoic boronic acid, 79 mg (0.5 mmol) pinacol and 142 mg (0.5 mmol) triacetoxyborohydride were subsequently dissolved into 10 mL DMF, the mixture was stirred overnight at room temperature. The solvent was evaporated to yield 260 mg of white solid. (89% yield)

\(^1\)HNMR (300 MHz, CD\(_3\)OD, 298 K) \(\delta = 7.69\) (d, 2H, H\(^8\), J=7.80 Hz), 7.31 (d, 2H, H\(^9\), J=7.72 Hz, overlapping), 7.27 (d, 2H, H\(^17\), J=8.45 Hz, overlapping) and 7.15 (d, 2H, H\(^16\), J=8.33 Hz, overlapping), 4.40 (m, 1H, H\(^30\)), 4.28 (d, 2H, H\(^14\), J=10.86Hz), 4.20 (m, 1H, H\(^28\)), 3.99 (d, 2H, H\(^13\), J=6.56Hz), 3.92 (bd, 2H, H\(^21\), J=5.37Hz), 3.09 (m, 1H, H\(^27\)), 2.90 (s, 2H, H\(^26\)), 2.77 (s, 2H, H\(^31\)), 2.17 (m, 2H, H\(^23\)), 1.60 (m, 4H, H\(^24\)), 1.25 (s, 12H, H\(^1-4\)).
$^{13}$C NMR ($\text{CD}_32\text{OS}$, 300MHz, 298 K) $\delta = 186.2$ (C$^{22}$), 181.0 (C$^{29}$), 176.4 (C$^{18}$), 166.5 (C$^{10}$), 165.3 (C$^{15}$), 136.6 (C$^{7}$), 131.1 (C$^{8}$), 130.1 (C$^{12}$), 129.8 (C$^{9}$), 129.7 (C$^{11}$), 129.6 (C$^{16}$), 129.5 (C$^{20}$), 124.6 (C$^{17}$), 124.5 (C$^{19}$), 85.6 (C$^{5}$), 76.3 (C$^{6}$), 63.7 (C$^{30}$), 62.0 (C$^{28}$), 57.4 (C$^{27}$), 53.0 (C$^{21}$), 52.8 (C$^{14}$, 13), 37.1 (C$^{26}$), 41.5 (C$^{31}$) 35.2 (C$^{25}$), 30.5 (C$^{24}$), 30.1 (C$^{23}$), 25.6, 25.4 (C$^{1-4}$).

Mass Spectrum ESI-MS calculated for C$_{31}$H$_{43}$BN$_{4}$O$_{4}$S [M + H]$^+$ 579.3171, found 579.3316. HPLC: Rt = 7.5 min.

IR spectrum: 2973 cm$^{-1}$, 1704 cm$^{-1}$, 1610 cm$^{-1}$, 1566 cm$^{-1}$, 1510 cm$^{-1}$, 1566 cm$^{-1}$, 1510 cm$^{-1}$, 1357 cm$^{-1}$, 1164 cm$^{-1}$, 1141 cm$^{-1}$, 1084 cm$^{-1}$, 857 cm$^{-1}$, 658 cm$^{-1}$.

(f) Synthesis of 2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-4-(3-(4-((5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)methyl)benzyl)-3-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)thioureido)benzoic acid (7)

![Chemical structure](image)

**Figure 6.** 2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-4-(3-(4-((5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)methyl)benzyl)-3-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)thioureido)benzoic acid

231 mg (0.44 mmol) compound 6 was deprotected in 1 mL TFA to remove the boc group, mixed with 172 mg (0.44 mmol) FITC in 10 mL of DMF, 4 eq. of TEA was added consequently. The mixture was stirred overnight at room temperature covered
with aluminium foil. The mixture was separated via extraction with DCM and water, products were isolated in organic phase, the DCM was evaporated and 41 mg scarlet solid was yielded. (4.4% yield)

$^1$HNMR (300 MHz, CD$_3$OD, 298 K) $\delta$ = 7.65 (d, 2H, H$^{25,27}$, J=7.83 Hz), 7.28 (m, 5H, H$^1,3,4,24,28$), 6.53 (m, 10H, H$^8,9,11,15,17,18,37,38,40,41$), 4.95 (s, 2H, H$^{22}$), 4.37 (m, 1H, H$^{51}$), 4.25 (d, 2H, H$^{15}$, J=8.21Hz), 4.14 (m, 1H, H$^{49}$), 3.98 (s, 2H, H$^{42}$), 3.07 (m, 1H, H$^{48}$), 2.79 (d, 2H, H$^{52}$, J=4.93Hz), 2.14 (m, 8H, H$^{44-47}$), 1.27 (m, 12H, H$^{30-33}$).

$^{13}$C NMR ((CD$_3$)$_2$OD, 300MHz, 298 K) $\delta$ = 172.4 (C$^{21}$), 169.3 (C$^{43}$), 169.0 (C$^{20}$), 163.1 (C$^{50}$), 162.6 (C$^{10}$), 157.5 (C$^{12}$), 156.1 (C$^{13}$), 152.7 (C$^2$), 146.4 (C$^{43}$), 142.9 (C$^{16}$), 141.6 (C$^{14}$), 141.0 (C$^{39}$), 139.0 (C$^{26}$), 138.5 (C$^{23}$), 134.9 (C$^{18}$), 134.6 (C$^{36}$), 129.5 (C$^8$), 128.8 (C$^4$), 128.4 (C$^{17}$), 127.4 (C$^{24,28}$), 127.2 (C$^{19}$), 126.6 (C$^{38}$), 126.4 (C$^{40}$), 126.0 (C$^{25}$), 125.9(C$^{27}$), 125.2 (C$^{15}$), 83.6 (C$^{29,34}$), 35.5(C$^{49,51}$), 33.7 (C$^{22,35}$), 28.5 (C$^{42}$), 28.4 (C$^{52}$), 25.7 (C$^{44}$), 25.3 (C$^{47}$), 25.0 (C$^{46}$), 24.8(C$^{15}$), 24.4 (C$^{30-33}$).

Mass Spectrum ESI-MS calculated for C$_{39}$H$_{37}$N$_5$O$_7$S$_2$ [M – H] $^-$ 966.3383, found 966.3471. HPLC R$_t$= 8.9 minutes

IR spectrum: 3390 cm$^{-1}$, 1735 cm$^{-1}$, 1659 cm$^{-1}$, 1500 cm$^{-1}$, 1450 cm$^{-1}$, 1357 cm$^{-1}$, 1250 cm$^{-1}$, 1206 cm$^{-1}$, 1174 cm$^{-1}$, 1164 cm$^{-1}$, 845 cm$^{-1}$, 657 cm$^{-1}$.

Figure 6.7. Structure of 4-[[4-[[[(tert-butoxy) carbonyl] amino} methyl] phenyl] methyl] carbamothioyl] amino]-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl) benzoic acid

196 mg (0.5 mmol) FITC and 118 mg (0.5 mmol) boc protected p-xylylenediamine were dissolved in 10 mL of DMF, 4 eq. of TEA (146.83 µL) was added consequently. The mixture was stirred overnight at room temperature and covered with aluminium foil. The product was evaporated and 200 mg of yellow solid yielded. (63% yield)

$^1$H NMR (300 MHz, (CD$_3$)$_2$OS, 298 K) δ = 8.29 (s, 1H, H$_1$), 7.79 (d, 1H, H$_3$, J=8.90 Hz), 7.30 (d, 2H, H$_{25,27}$, J=8.22 Hz.), 7.21 (d, 2H, H$_{24,28}$, J=7.95 Hz), 7.18 (d, 1H, H$_4$, J=7.95 Hz), 6.70 (m, 4H, H$_8,9,11,18$), 6.60 (m, 2H, H$_{15,17}$), 4.75 (s, 2H, H$_{29}$), 4.12 (s, 2H, H$_{22}$), 1.34 (s, 9H, H$_{32,33,34}$).

$^{13}$C NMR ((CD$_3$)$_2$OS, 300MHz, 298 K) δ =181.1 (C$_{21}$), 169.1 (C$_{20}$), 160.0 (C$_2$), 156.3 (C$_{30}$), 152.3 (C$_{13}$), 141.6 (C$_{16}$), 139.4 (C$_{26}$), 137.4 (C$_{23}$), 130.0 (C$_{4,8}$), 129.6(C$_{4,8}$), 127.7 (C$_{25,27}$), 127.3 (C$_{25,27}$), 127.2 (C$_{24,28}$), 127.0 (C$_{17}$), 117.2 (C$_{15}$), 113.0 (C$_{3,9}$), 102.7 (C$_{1,11}$), 48.1 (C$_{22}$), 43.5 (C$_{29}$), 36.6 (C$_{31}$), 28.7 (C$_{32,33,34}$).

Mass Spectrum ESI-MS calculated for C$_{39}$H$_{37}$N$_5$O$_7$S$_2$ [M - H] $^-$ 624.1882, found 624.1861. HPLC R$_t$ = 9.0 minutes

IR spectrum: 3256 cm$^{-1}$, 1751 cm$^{-1}$, 1696 cm$^{-1}$, 1608 cm$^{-1}$, 1535 cm$^{-1}$, 1247 cm$^{-1}$, 1164 cm$^{-1}$.

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Synthesis of 2-((6-hydroxy-3-oxo-3H-xanthen-9-yl)-4-(3-(4-(((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)methyl)benzyl)thioureido) benzoic acid (9)

Figure 6.8. Structure of 2-((6-hydroxy-3-oxo-3H-xanthen-9-yl)-4-(3-(4-(((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)methyl)benzyl)thioureido) benzoic acid

376 mg (0.5 mmol) of compound 8 was added to a solution of 2 mmol TFA and 6 mL DCM, the mixture was stirred at room temperature for two hours. Then solvent was evaporated and 263 mg yellow solid was yielded. Subsequently, 263 mg (0.5 mmol) de-protected compound 8 was mixed with 171 mg (0.5 mmol) biotin-NHS were dissolved into 6 mL DMF, the mixture was stirred overnight at room temperature. Evaporate solvent to yield 117 mg scarlet solid (compound 9). (31% yield)

$^1$H NMR (300 MHz, CD$_3$OD, 298 K) $\delta =$ 10.70 (s, 1H, H$^{20}$), 9.03 (s, 1H, H$^8$), 8.84 (s, 1H, H$^6$), 8.03 (s, 1H, H$^{15}$), 7.75 (d, 1H, H$^{17}$, J = 8.64 Hz), 7.40 – 7.07 (m, 4H, H$^{24,25,27,28}$), 6.93 (d, 1H, H$^{18}$, J = 8.40 Hz), 6.63 (bd, 2H, H$^{1,11}$, J$^{orth} = 9.34$ Hz), 6.47 (s, 1H, H$^4$), 6.41 (s, 1H, H$^5$), 6.02 (dd, 2H, H$^{3,9}$, J$^{orth} = 9.34$ Hz, J$^{meta} = 1.66$ Hz), 5.92 (s, 2H, H$^{11}$), 4.70 (m, 2H, H$^{22}$), 4.27 (m, 1H, H$^{38}$), 4.21 (d, 2H, H$^{29}$, J = 5.36 Hz), 4.10 (m, 1H, H$^{36}$), 3.07 (m, 1H, H$^{35}$), 2.80-2.55 (dd, 2H, H$^{30}$, J = 5.02 Hz), 2.12 (t, 2H, H$^{34}$), 1.71 (m, 6H, H$^{31,32,33}$). H$^4$ and H$^8$ are chemically inequivalent. However, due to the frequency of the spectrometer and a possible dynamic tautomeric equilibrium involving FITC unit and affecting its protons in the NMR time scale. These resonances appear as magnetic equivalent leading to a broad doublet peak.
\textbf{13C NMR} ((CD$_3$)$_2$OS, 300MHz, 298 K) \(\delta = 181.2\ (C^{20}),\ 172.6\ (C^{21}),\ 170.2\ (C^{30}),\ 163.8\ (C^{37}),\ 162.9\ (C^{2,10})\ 158.8\ (C^{19}),\ 141.4\ (C^{16}),\ 139.2\ (C^{26}),\ 138.7(C^{23}),\ 130.0\ (C^{4,8}),\ 129.4\ (C^{18}),\ 127.5\ (C^{24,25}),\ 124.3\ (C^{15}),\ 123.2\ (C^{17}),\ 123.1\ (C^{3,9}),\ 122.7\ (C^{27,28}),\ 102.9\ (C^{1,11}),\ 61.4\ (C^{38}),\ 59.6\ (C^{36}),\ 55.8\ (C^{35}),\ 46.8\ (C^{22}),\ 42.5\ (C^{39}),\ 42.2\ (C^{29}),\ 35.4\ (C^{34}),\ 29.5\ (C^{31}),\ 28.6\ (C^{32}),\ 23.4\ (C^{33}).

Mass Spectrum ESI-MS calculated for C$_{39}$H$_{37}$N$_5$O$_7$S$_2$ [M - H] $-$ 750.2062, found 750.2128. HPLC R$_t$ = 8.7 minutes.

IR spectrum: 3244 cm$^{-1}$, 1751 cm$^{-1}$, 1632 cm$^{-1}$, 1608 cm$^{-1}$, 1535 cm$^{-1}$, 1247 cm$^{-1}$, 1166 cm$^{-1}$, 849 cm$^{-1}$.

(i) Synthesis of 4-(tetramethyl-1, 3, 2-dioxaborolan-2-yl) benzaldehyde (10)

\begin{center}
\textbf{Figure 6. 9.} Structure of 4-(tetramethyl-1,3,2-dioxaborolan-2-yl)benzaldehyde
\end{center}

149 mg (1 mmol) 1, 4-formyl-phenyl-Boronic acid and 118 mg (1 mmol) pinacol were added to 10 mL of THF and 10 mL Toluene. The mixture was concentrated to dryness under reduced pressure and this procedure (solvents addition/evaporation) was repeated three times. (81.9% yield).

\textbf{1H NMR} (300 MHz, CDCl$_3$, 298 K) \(\delta = 10.05\ (s, 1H, H^1),\ 7.98 - 7.79\ (m, 4H, H^{3,4,6,7}, J= 8.2\ Hz),\ 1.35(s, 12H, H^{10,11,12,13}).\)

\textbf{13C NMR} (300 MHz, (CD$_3$)$_2$OS, 298K) \(\delta = 194.8\ (C^1),\ 136.6\ (C^{5,7}),\ 130.1\ (C^{4,6}),\ 127.0\ (C^2),\ 86.0\ (C^{8,9}),\ 25.6\ (C^{10-13}).\) The peak of C$^5$ is broadened by the quadrupole relaxation effect caused by the boron group attached, hence no peak has been detected in this NMR time scale by using a 300MHz spectrometer at 298 K.
Mass Spectrum ESI-MS calculated for C_{13}H_{17}BO_{3} [M + H] + 255.1116, found 255.1164.

HPLC R_t = 6.6 minutes.

IR spectrum: 1662 cm\(^{-1}\), 1564 cm\(^{-1}\), 1505 cm\(^{-1}\), 1339 cm\(^{-1}\), 1200 cm\(^{-1}\), 1184 cm\(^{-1}\), 1167 cm\(^{-1}\), 600 cm\(^{-1}\).

(j) Synthesis of tert-butyl N-\{4-[4-(tetramethyl-1, 3, 2-dioxaborolan-2-yl) phenyl] methyl\} amino) methyl\} phenyl\} methyl carbamate (11)

![Figure 6. 10. Structure of tert-butyl N-\{4-[4-(tetramethyl-1, 3, 2-dioxaborolan-2-yl) phenyl] methyl\} amino) methyl\} phenyl\} methyl carbamate](image)

142 mg (0.6 mmol) Boc protected p-xylylenediamine and 139 mg (0.6 mmol) pinacol protected boronic acid were added into 18 mL DCM, 178 mg (0.6 mmol) Sodium triacetoxyborohydride was subsequently added. The mixture was stirred overnight at room temperature under nitrogen conditions. Then MilliQ water was added to eliminate Sodium triacetoxyborohydride and compound 11 was washed by water. DCM was evaporated to yield 202 mg of white solid (73% yield).

\(^1\)H NMR (300 MHz, CDCl\(_3\), 298K) \(\delta = \)
7.69 (d, 2H, H\(^8\), \(^{12}\), J=8.17 Hz), 7.33 (d, 2H, H\(^9\), \(^{11}\), J=8.17 Hz, overlapping with H\(^8\) and H\(^{12}\)), 7.28 (d, 2H, H\(^{17}\), J=8.17 Hz, overlapping with H\(^8\) and H\(^{12}\)) and 7.15 (d, 2H, H\(^{16}\), \(^{20}\), J=8.17 Hz, overlapping with H\(^{17}\) and H\(^{19}\)), 4.85 (s, 1H, H\(^b\)), 4.32 (d, 2H, H\(^{21}\), J=6.52Hz), 3.75 (bs, 2H, H\(^{14}\)), 3.46 (bs, 2H, H\(^{13}\)), 1.39 (s, 9H, H\(^{24,25,26}\)), 1.27 (s, 12H, H\(^{14}\)).
\textbf{13C NMR} (300 MHz, (CD\textsubscript{3})\textsubscript{2}OS, 298K) \(\delta = 193.9\) (C\textsubscript{22}), 143.0 (C\textsubscript{18}), 135.3 (C\textsubscript{10}), 132.8 (C\textsubscript{15}), 131.9 (C\textsubscript{8,12}), 129.0 (C\textsubscript{9}), 128.9 (C\textsubscript{11}), 128.4 (C\textsubscript{16,20}), 127.3 (C\textsubscript{17}), 127.2 (C\textsubscript{19}), 84.0 (C\textsubscript{5,6}), 78.4 (C\textsubscript{23}) 58.0 (C\textsubscript{13,14}), 28.4 (C\textsubscript{1-4,24-26}),

Mass Spectrum ESI-MS calculated for C\textsubscript{26}H\textsubscript{37}BN\textsubscript{2}O\textsubscript{4} [M + Na] \(^+\) 453.2920, found 453.2966. HPLC R\(_t\) = 5.1 minutes.

IR spectrum: 2980 cm\(^{-1}\), 1695 cm\(^{-1}\), 1564 cm\(^{-1}\), 1508 cm\(^{-1}\), 1200 cm\(^{-1}\), 600 cm\(^{-1}\).

(k) Synthesis of 4-[[4-[[[(\text{tert}-butylamino)methyl]phenyl]methyl][4-(tetramethyl-1,3,2 - dioxaborolan-2-yl)phenyl][methyl]]carbamothioyl]amino]-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid (12)

196 mg (0.26 mmol) FITC and 118 mg (0.26 mmol) boc protected p-xylylenediamine were dissolved in 10 mL of DMF, 4 eq. of TEA (146.8 \(\mu\)L) was added consequently. The mixture was stirred overnight at room temperature and covered with aluminium foil. The product was evaporated and yielded 102 mg yellow solid. (46.7\% yield)

\textbf{\textsuperscript{1}HNMR} (300 MHz, CD\textsubscript{3}OD, 298K) \(\delta = 7.89\) (d, 1H, H\textsuperscript{1}, J=1.61 Hz), 7.79-7.10 (m, 6H, H\textsuperscript{2,3,11,12,13,14}), 6.69-6.50 (m, 10H, H\textsuperscript{4,5,6,7,8,9,20,21,22,23}), 5.11 (s, 2H, H\textsuperscript{15}), 4.23 (s, 2H, H\textsuperscript{10}), 3.87 (s, 2H, H\textsuperscript{19}), 1.43 (s, 9H, H\textsuperscript{16-18}), 1.27 (s, 12H, H\textsuperscript{24-27}).
Mass Spectrum ESI-MS calculated for C_{47}H_{48}BN_{3}O_{9}S [M - H] - 840.3132, found 840.3195. HPLC R_t = 9.2 minutes.

(1) Synthesis of 4-(5,5-difluoro-1,3,7,9-tetramethyl-5H-4λ^4,5λ^5-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-10-yl)-N-(4-((5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)methyl)benzyl)-N-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)benzamide (13)

Figure 6.12. Structure of 4-(5,5-difluoro-1,3,7,9-tetramethyl-5H-4λ^4,5λ^5-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-10-yl)-N-(4-((5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)methyl)benzyl)-N-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)benzamide

57 mg (0.16 mmol) bodipy and 68 mg (0.16 mmol) compound 6 were dissolved in 6 mL of DMF, 4 eq. of TEA was added subsequently. The mixture was stirred overnight at room temperature and covered with aluminium foil. Subsequently, the mixture was separated via extraction with DCM and water, products were isolated in organic phase, the DCM was evaporated and yielded a red solid.

^1H NMR (300 MHz, (CD3)2OS, 298K) δ = 8.18-7.01 (m, 12H, H^{15,16,18, 19, 23, 24, 26, 27, 35,36, 38, 39}), 6.82 (s, 1H, H^{11}), 5.98 (s, 1H, H^{5}), 4.31 (m, 1H, H^{48}), 4.24 (d, 2H, H^{41}, J = 3.3 Hz), 4.12 (m, 1H, H^{49}), 4.10 (m, 2H, H^{34}), 3.41 (m, 2H, H^{29}), 3.17-0.70 (m, 11H, H^{43-47, 50}), 1.32 (s, 6H, H^{1, 13}), 1.23 (s, 12H, H^{29-32}), 1.10 (s, 6H, H^{5,10}).
**Chapter 6. Experimental section**

**13C NMR** (300 MHz, DMSO-d$_6$, 298K) $\delta$ = 211.8 (C$_{42}$), 211.1 (C$_{20}$), 210.5 (C$_{51}$), 144.7 (C$_{14}$), 140.1 (C$_4$), 136.6 (C$_{22}$), 136.2 (C$_{37}$), 130.6 (C$_{23,24,25,27}$), 129.7 (C$_{35,36,38,39}$), 129.1 (C$_{15,16,18,19}$), 126.5 (C$_{12}$), 85.0 (C$_{28,33}$), 63.7 (C$_{48}$), 59.3 (C$_{49}$), 57.4 (C$_{47}$), 45.3 (C$_{34}$), 44.4 (C$_{21}$), 41.5 (C$_{41}$), 37.1 (C$_{50}$), 35.8 (C$_{43}$), 29.8 (C$_{45}$), 27.2 (C$_{44}$), 26.5 (C$_{46}$), 25.4 (C$_{29-32}$), 15.2 (C$_{5,10}$), 11.9 (C$_{1,13}$).

Mass Spectrum ESI-MS calculated for C$_{51}$H$_{60}$B$_2$F$_2$N$_6$O$_5$S $[M-2H]$ $^2-$ 463.2177, found 463.2174. HPLC $t_R$ = 10.1 minutes.

IR spectrum: 3244 cm$^{-1}$, 1698 cm$^{-1}$, 1632 cm$^{-1}$, 1557 cm$^{-1}$, 1545 cm$^{-1}$, 1359 cm$^{-1}$, 1260 cm$^{-1}$, 1166 cm$^{-1}$, 1087 cm$^{-1}$, 1017 cm$^{-1}$, 849 cm$^{-1}$, 796 cm$^{-1}$.

(m) Synthesis of 2-oxo-N-(4-((5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)methyl)benzyl)-N-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)-2H-chromene-3-carboxamide (15)

![Figure 6. 13. Structure of 2-oxo-N-(4-((5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)methyl)benzyl)-N-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)-2H-chromene-3-carboxamide](image)

42 mg (0.22 mmol) Coumarin-3-carboxylic acid and 127 mg (0.22 mmol) compound 6 were dissolved in 6 mL of DMF, with 4 eq. (123.3 µL) of TEA subsequently added. The mixture was stirred overnight at room temperature and covered with aluminium foil. Consequently, the mixture was separated via extraction with DCM and water, products were isolated in the organic phase, the DCM was evaporated and 52 mg of yellowish solid was yielded (31% yield).
**1H NMR** (300 MHz, (CD$_3$)$_2$OS, 298K) δ = 8.07-6.99 (m, 13H, H$_1$, 2, 3, 7, 9, 13, 14, 16, 17, 26, 27, 29, 30), 4.31 (m, 1H, H$_{38}$), 4.23(m, 4H, H$_{24,31}$), 4.15 (m, 1H, H$_{39}$), 3.46 (s, 2H, H$_{11}$), 1.96-0.63 (m, 23H, H$_{19-22,33-37,40}$).

**13C NMR** (DMSO-d$_6$, 500MHz, 298 K): δ = 206.2 (C$_{32}$), 172.0 (C$_{41}$), 141.8 (C$_5$), 137.7 (C$_4$), 136.0 (C$_{28}$), 134.3 (C$_{25}$), 134.0, 133.7 (C$_{14,16}$), 133.6 (C$_{13,17}$), 128.0 (C$_{26,30}$), 127.9 (C$_{27,29}$), 127.2 (C$_2$), 126.8 (C$_9$), 126.6 (C$_1$), 83.9 (C$_{23}$), 83.1 (C$_{18}$), 82.7, 33.0 (C$_{35}$), 24.6 (C$_{34}$), 23.9 (C$_{36}$), 23.8 (C$_{19-22}$).

Mass Spectrum ESI-MS calculated for C$_{41}$H$_{47}$BN$_4$O$_7$S [M + H] $^+$ 750.3331, found 750.3338. HPLC R$_t$= 12.0 minutes.

IR spectrum: 1699 cm$^{-1}$, 1611 cm$^{-1}$, 1565 cm$^{-1}$, 1515 cm$^{-1}$, 1355 cm$^{-1}$, 1164 cm$^{-1}$, 1143 cm$^{-1}$, 1086 cm$^{-1}$, 1020 cm$^{-1}$, 857 cm$^{-1}$, 658 cm$^{-1}$.

### 6.16. Fluorescence spectroscopy test

**Fluorescence spectroscopy titration:**

1×10$^{-6}$ mol of compound 7 was dissolved into 50 mL methanol (HPLC) solvent to give 2×10$^{-5}$ M compound 7 stock solution. From this stock, 1 mL was taken and dissolved into 49 mL of MilliQ water solution to make 4×10$^{-7}$ M solution. 1 mg of β-D-glucan was weighed and dissolved into 1 mL DMSO at 50 ºC.

The fluorimeter was switched on and warmed for 30 min before use. The spectra were recorded with a 488 nm excitation wavelength. A blank reading for the pure solvent mixture used was taken, then for the stock solution and the glucan solution was added cumulatively, one aliquot per every 10 minutes, as it is shown in the following table. Because β-D-glucan is a polysaccharide, the number of glucose repeating units is known to vary in different glucan chains. Therefore, average molecule weight of glucan used in this project is 2 kDa, which was the molecular weight provided by the supplier.
Table 6.1. Volume of β-D-glucan (1 mg/mL in DMSO) titrated

<table>
<thead>
<tr>
<th>Volume (µL)</th>
<th>Total conc. (M)</th>
<th>Volume (µL)</th>
<th>Total conc. (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>6</td>
<td>1×10⁻⁷</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
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<tr>
<td>5</td>
<td>50</td>
<td>10</td>
<td>7×10⁻⁷</td>
</tr>
</tbody>
</table>

All the results are fitted by the equation:

\[ I = I_0 + I_f K_D^{-1} [C] / [1 + K_D^{-1} [C]] \]

I stands for overall intensity, \( I_0 \) and \( I_f \) is initial and final intensity respectively, \([C]\) means concentration of compound and \( K_D^{-1} \) is the binding constant.

**Kinetic stability of fluorescence compound:**

4 mg of compound 7 was dissolved in 1 mL of methanol (4 mM), 0.1 mL of the compound 7 solution was diluted into 3.9 mL of water (0.1 mM). The fluorescence of solution was measured every 30 minutes in 4.5 hours.

**Kinetic stability of fluorescence compound in different medium:**

4 mg of compound 7 was dissolved in 1 mL of methanol (4 mM), 0.1 mL of the compound 7 solution was diluted into 9.9 mL of methanol (0.04 mM), then mix solution one to one with water, serum medium or serum free medium respectively.

**Qualitative avidin binding tests for the fluorescence sensors:**

1 mg avidin was dissolved into 1 mL of water, which made avidin solution to react with 10 µg of biotin. 4.0 mg of compound 7 was dissolved into 1 mL of methanol (4 mM), and then diluted 1 in 100 into water. 3.0 mg of compound 9 was dissolved into 1 mL of methanol (4 mM), and then diluted 1 in 100 into water. 0.5 mL of compound 7 or compound 9 was mixed with 0.5 mL of water to give samples 1 and sample 2, 0.5 mL
of compound 7 or compound 9 was mixed with 0.5 mL of avidin solution (1 mg/ml in water) to give samples 3 and 4.

**Direct interaction between fluorescent sensors with purified SWNTs:**
4 mg (4 mM) of compound 7 was dissolved into 1 mL of methanol (4 mM stock solution) and then solution was diluted 500 µL in 1500 µl water (Compound 7 solution). 500 µL compound 7 stock solution was dissolved into 1 mL of water, and then add 500 µL of 1mg/mL SWNTs dispersion (Compound 7 @SWNTs dispersion).

### 6.17. Cellular culturing methods

Cell lines involved in this project include cervical cancer cells (HeLa) and epithelial fibroblast cells (FEK-4), breast cancer cells (MCF-7) and prostate cancer cells (PC-3). All cells lines were inherited from Rex Tyrrell group or provided in Rutherford Appleton Laboratory (RAL). Cells were normally frozen at -196 °C under liquid nitrogen condition until required, then cells would be thrown quickly and incubated at 37 °C under 5% carbon dioxide environment. Different cells required different solvent system to grow, for example, HeLa and FEK-4 cells were usually incubated in an Eagle’s Minimum Essential Medium (EMEM), whilst Dulbecco’s Modified Eagle’s Medium (DMEM) were suitable for MCF-7 cells growing and PC-3 cells could be cultured in Roswell Park Memorial Institute (RPMI) medium. All media contained activated foetal calf serum (FCS) (10% for HeLa, PC-3 and MCF-7 and 15% for FEK-4), 0.5% penicillin/streptomycin (10,000 IU mL-1/10,000 mg mL-1) and 2.5% L-Glutamine.

### 6.18. Cells thawing

A vial of frozen cells were taken from the liquid nitrogen storage and placed in a 37 °C water bath. Immediately after all the ice had thawed, the cells were transferred into a centrifuge tube with 10 mL of serum media. Unless otherwise stated, serum media contained 10% foetal calf serum (FCS) for cancer cell lines and 15% FCS for healthy
cell lines. The centrifuge tube was then spun at 1000 rotations per minute (rpm) for 5 minutes to precipitate the cells. The supernatant would subsequently be aspirated and refilled with 10 mL serum media. Finally, all cells with fresh media were transferred into a T75 cell culture flask and incubated at 37 °C.

6.19. Cells splitting

Cell splitting was the fundamental part of the cell growing experiment, as it was essential to split cells once or twice per week depending on the confluence of the cells in the flask, in order to harvest cells for the research and to leave space for more cells to grow.

The surplus supernatant contained dead cell matter and excess proteins were aspirated. All attached cells were washed by 10 mL phosphate buffer saline (PBS) twice, (all solvents, buffer solutions and media were warmed to 37 °C in the water bath prior to addition) 3 mL of trypsin - PBS solution (0.25% trypsin) was subsequently loaded and incubated at 37 °C for 5 minutes. After trypsinisation, 5 mL serum medium was added to neutralise the excess trypsin and solution was centrifuged at 1000 rpm for 5 minutes. Afterwards, the supernatant was aspirated and re-suspended with 5 mL serum medium. Cells were counted in a haemocytometer and seeded appropriately for different applications.

6.20. Cell freezing

After cell splitting, the excess cells could be frozen for future use. 0.5 million – 1 million cells were kept in a vial with 2 mL mixture solution of 50% serum medium, 40% foetal calf serum and 10% DMSO. There were two ways to freeze cells; A Thermo Scientific™ Mr. Frosty™ Freezing Container was used in the direct method, vials were placed in the container and left container at -80 °C freezer overnight, cells would be cooling at rate of -1 °C / minute and be ready to stay in a liquid nitrogen environment. If there was no freezing container, an indirect method was applied instead; vials were placed in the 4°C fridge for 20 min. Then vials were transferred into -20°C freezer for
30 min. Subsequently, vials were moved into -80°C freezer for 24 hours. Finally, vials would be sent to liquid nitrogen for long term storage.

6.21. MTT assays

After cell splitting, cells (3×10³ cells per well) were seeded on a sterile 96 well plate and incubated for 48 hours to adhere. Varieties of compound were subsequently loaded at different concentration into wells and cultured for another 48 hours. The Concentration used ranged between 250 μM (1% DMSO, 99% Eagle’s Modified Essential Medium (10% FCS, or 15 % FCS as per cell line) and 100 nM as they were shown in Table 6.1. Subsequently, cells were washed three times with PBS and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was added (0.5 mg/mL, 10% serum free medium (SFM)) followed by a two hour incubation. After aspiration, 100 μl of DMSO was added and 96 well plates were read by an ELISA plate reader, Molecular Devices Versa Max (BN02877). The absorption wavelength was at 570 nm and 630 nm wavelength was used as a reference. For establishing the correct protocols, a library of known compounds with solubility similarities and which also carries the N/S motifs were analysed.

![Chemical structures](image.png)

**Figure 6.14.** Compounds 16a-c, 17a-b were antihypoxia reagent tested in MTT assays (samples were provided by Professor Golzar Hossain, from University of Dhaka in Bangladesh)
Figure 6.15. Complexes 18a-c, 19a-c were thiosemicarbazone antihypoxia reagents examined by MTT assays (samples were provided by Rory Arrowsmith, from University of Bath).

Standard conditions of the MTT assays was established based on several classes of anticancer reagent provided by Prof. Golzar Hossain, from University of Dhaka in Bangladesh and Rory Arrowsmith (Pascu group, University of Bath). As it was shown in figure 6.14 and 6.15, both compound 16-17 (organic compounds) and compound 18-19 (bisthiosemicarbazone complexes) series are known to have potential to target hypoxic regions, a trait commonly associated with later stages of cancer. The reason compound 16-17 was chosen was because these compounds all carried −NC=SN− groups, which was similar to compound 7. In addition, the qualities of compounds were enough for sufficient repeats of the experiment. Compound 18-19 was chosen as a comparison of compound 16-17, in order to investigate whether the protocol established was suitable for the general use of the MTT assays. As a result, the following conditions were subsequently established, and for all new compounds developed in this project.
Table 6.2. Concentrations of different samples in the MTT assay

<table>
<thead>
<tr>
<th>Control (DMSO)</th>
<th>Sample 1</th>
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Table 6.3. Concentrations of different SWNTs and their composites in the MTT assay

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<th>Control (DMSO)</th>
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<th>Sample 2</th>
<th>Sample 3</th>
<th>Sample 4</th>
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In the next stage, several compounds and composites were tested in the previously established procedure, including individual boronic acid attached fluorophores (Figure 6.16), SWNTs in different stages (raw, purified and β-D-glucan coated) (Figure 6.17) and fluorophore @glucan @SWNTs. All compounds and composites were dissolved or dispersed into DMSO to make a stock solution (dispersion), then diluted into various concentrations.

Figure 6.16. Structure of biotin-boronic acid fluorescent probes with different fluorophores
Figure 6.17. Structure of SWNTs at different stages: (a) Raw SWNTs, (b) Purified SWNTs, (c) β-d-glucan coated SWNTs and (d) Fluorescent probe attached β-d-glucan SWNTs nanohybrids.

6.22. Confocal microscopy

Fluorescence microscopy images were captured on a Nikon eclipse TE2000 epi-fluorescence or a Zeiss LSM510META microscope at University of Bath, whereas at the Rutherford Appleton Laboratory a modified Nikon TE2000-U was utilised. HeLa, PC-3 or MCF-7 cells were cultured as above and plated in a glass bottomed petri dish (35 mm diameter and 1.5 mm thickness) at 1.5×10^5 - 2.5×10^5 cells per dish depending on the cell line and culture time. In general, HeLa cells required 12 hours to attach onto the dish, whereas MCF-7 and PC-3 needed 48 hours to adhere. Prior to the microscopy observation, cultured cells were washed with Hank’s solution (HBSS) five times and refilled with 990 µL of serum free medium. Subsequently, a small volume of compound (10 µL) in DMSO was loaded to make the final volume 1 mL at the appropriate concentration. The final concentration on the plate were usually at 100 nM with 1% - 5% DMSO depending on the compound solubility. After 15 min or 1 hour incubation at 37 °C, cells were washed with HBSS three times and refilled with fresh SFM (1 mL). Confocal images were captured immediately afterwards.

Concentration dependent confocal imaging protocols

HeLa cells were cultured as above and plated in a glass bottomed petri dish at 1.5×10^5 cells per dish. Prior to the microscopy observation, cultured cells were washed with HBSS five times and refilled with 990 µL of serum free medium (room temperature).
Subsequently, a small volume of compound (10 µL) in DMSO was loaded to make the final concentration 1 nM, 10 nM, 50 nM or 100 nM with 1% DMSO. After 15 min or 1 hour at 37 °C, cells were washed with HBSS three times and refilled with fresh SFM (1 mL). Confocal images were captured immediately afterwards.

Temperature dependent confocal imaging methods
HeLa cells were cultured as above and plated in a glass bottomed petri dish at 1.5×10^5 cells per dish. Prior to the microscopy observation, cultured cells were washed with HBSS five times and refilled with 990 µL of cold serum free medium (4°C). Subsequently, a small volume of compound (10 µL) in DMSO was loaded to make the final concentration at 100 nM with 1% DMSO. After 15 min or 1 hour incubation at 4 °C in the fridge, cells were washed with HBSS three times and refilled with fresh cold SFM (1 mL). Confocal images were captured immediately afterwards.

Time dependent confocal imaging methods
HeLa cells were cultured as above and plated in a glass bottomed petri dish at 1.5×10^5 cells per dish. Prior the microscopy observation, cultured cells were washed with HBSS five times and refilled with 990 µL of serum free medium (room temperature). Subsequently, a small volume of compound (10 µL) in DMSO was loaded to make the final concentration 100 nM with 1% DMSO. After 15 min, 30 min, 1 hour, 3 hour or overnight incubation at 37 °C, cells were washed with HBSS three times and refilled with fresh SFM (1 mL). Confocal images were captured immediately afterwards.

Alternative method to wash cells
HeLa cells were cultured as above and plated in a glass bottomed petri dish at 1.5×10^5 cells per dish. Prior to the microscopy observation, cultured cells were washed with phosphate buffered saline (PBS) five times and refilled with 990 µL of serum free medium. Subsequently, a small volume of compound (10 µL) in DMSO was loaded to make the final concentration 100 nM with 1% DMSO. After 15 min incubation at 37 °C, cells were washed with PBS three times and refilled with PBS (1 mL). Confocal images were captured immediately afterward.
Chapter 6. Experimental section

Cell fixation with methanol
The cell plate was washed by PBS two times, then 1 mL of 10% methanol / 90% water was added and left for five minutes, afterward, supernatant was removed. 1 mL of 30% methanol / 70% water mixture was subsequently loaded. Cells were incubated for five minutes and the supernatant was removed. The plate was refilled with 1 mL of 50% methanol / 50% water mixture and after five minutes the supernatant was removed. Next, 1 mL of 80% methanol / 20% water mixture was added to the plate, left for five minutes and the supernatant was removed. Finally, the sample was left on the beach to dry in air.

6.23. Fluorescence lifetime imaging microscopy (FLIM)
Lifetime measurements were recorded using time-correlated single photon counting with an excitation wavelength of 910 nm and the emission measured at 360-580 nm. The measurement of compounds were carried out in DMSO/ water solvent with concentration of 10 mM. Emission spectral detection was carried out using an acton Research Component 275 spectrograph and an Andor iDus 740-BU CCD camera.

Cell uptake studies were performed using HeLa, PC-3 and CHO cells. Cells were plated on a glass petri dishes and left cells to attach for 12-24 hours depend on the growth rate. Control FLIM were recorded before the addition of compound. Plates were then mounted on the microscope stage and kept at 37ºC.

Compounds were dissolved in DMSO and added to cells to achieve final concentration of 100µM in EMEM/RPMI medium containing 1% of DMSO. Cells were incubated for 15 minutes prior to confocal imaging, FLIM was carried out immediately after the confocal imaging with the same area as the confocal imaging.

The fluorescence decay curve is well fitted to a multiexponential function as:

\[ I(t) = \sum a_i \exp\left(\frac{-t}{\tau_i}\right) \]
6.24. Co-localisation studies

There were varieties of tracking dyes used to localise distribution of samples via confocal microscopy; all dyes in the following studies were purchased from Invitrogen. Cells were cultured in Eagle’s Minimum Essential Medium (EMEM) or Roswell Park Memorial Institute (RPMI)-1640 medium using standard protocols described above. Prior to addition of any commercial co-localisation dye, cells were washed five times with HBSS. Protocols were adapted from Invitrogen.1-4

Hoechst solution (Nuclear DNA dye):

![Figure 6. 18. Structure for Hoechst® nucleus stain from Invitrogen](image)

The 10 mg/mL Hoechst original solution was prepared by dissolving 10mg Hoechst 33342 (Invitrogen) in 1 mL of sterile MilliQ water, 10 μL of the original solution was dissolved into 990 μL sterile water to make a 100 μg/mL stock solution. Cells were then washed five times with HBSS, and then refilled with 990 μL SFM. 10 μL of stock solution was then added to the 990 μL SFM. Cells were then incubated for 20 to 30 minutes. Subsequently, cells were washed three times with HBSS and refilled with 1 mL SFM to take control images. Then, cells were washed and the compounds of interest for co-localisation studies were loaded. After 15 min incubation with the compound, cells were washed three times with HBSS and refilled with fresh SFM (1 mL). Confocal images were recorded immediately afterwards.

Mitotracker (mitochondrial dye)
There were two mitotracker dyes used (*Invitrogen*) in this group’s research, one was mitotracker Red (Figure 6.19 20) and the other one was mitotracker Green (Figure 6.19 21), both dyes could label mitochondria with the former excited by red light at 543 nm and the later excited at 488 nm, which was a green light. In each case, to prepare stock solutions, to the as-bought 50 μg of mitotrackers, 69 μL DMSO was added to mitotracker red and 74 μL DMSO to mitotracker green each resulting in a 1 mM stock solution.

![Figure 6.19. Structure of mitotracker dyes used](image)

Subsequently, 20 μL of the 1 mM stock solution was diluted into 980 μL of DMSO to make 20 μM solution. Prior to the test, cells were washed five times with HBSS, then the cell plate was refilled with 990 μL SFM. To this, 10 μL of 20 μM solution was added. Cells were incubated for 15 to 45 minutes and subsequently washed three times with HBSS. The dishes were then refilled to 1 mL fresh SFM to take control images of the cells with dye-stained mitochondria. Then, cells were washed and the imaging probe of interest for the co-localisation studies was loaded. After 15 min incubation with the compound, cells were washed three times with HBSS and refilled with fresh SFM (1 mL). Confocal images were recorded immediately afterwards.
Lysotracker (lysosomes dye):

As was for the lysotracker dye, the group also uses two different dyes: Lysotracker red (Figure 6.20 22-a) and Lysotracker green (Figure 6.20 22-b). The excitation wavelength for each Lysotracker was 577 nm and 488 nm respectively. The stock solution available from the supplier (Invitrogen) is 1 mM. For co-localisation experiments, 20 μL of this stock was dissolved into 980 μL DMSO to obtain 20 μM diluted solutions ready for use. Cells were washed five times with HBSS, and then plates were refilled with 990 μL SFM. 10 μL of the diluted stock solution was prepared as above and were added to the 990 μL SFM to make a 200 nM final concentration on the cell plate. Cells were incubated for 30 to 120 minutes then washed three times with HBSS. Plates were refilled with 1 mL SFM to take control images of lysosomes stained cells. Then, cells were washed with HBSS and refilled with fresh SFM and the desired compound for co-localisation images were loaded. After 15 min incubation with the compound, cells were washed three times with HBSS and refilled with fresh SFM (1 mL). Confocal images were recorded immediately afterwards.

ER tracker (stains the endoplasmic reticulum):

The original solution provided by the supplier (Invitrogen) was 1 mM. From this 10 μL was diluted with 90 μL DMSO to obtain 100 μM stock solutions. Then, cells were washed five times with HBSS and plates were refilled with 990 μL SFM. Subsequently, 10 μL of the diluted stock solution (prepared as above) was added to make 1 μM
solutions on the cell plate. Cells were incubated for 15 to 30 minutes and subsequently cells were washed three times with HBSS. The plates were refilled with 1 mL SFM to take control images of ER (endoplasmic reticulum) -stained cells. Finally, cells were washed and the compound of interest for co-localisation studies was loaded. After 15 min incubation with the compound, cells were washed three times with HBSS and refilled with fresh SFM (1 mL). Confocal images were recorded immediately afterwards.

![Figure 6.21. Structure of ER tracker dyes used](image)

6.25. Epifluorescence microscopy

Epifluorescence imaging was performed on a Nikon Eclipse TE2000-E epifluorescence microscopy. This was equipped with a mercury lamp (Nikon HG-100W, Tokyo, Japan) as the excitation source, and a high-definition cooled colour digital camera (DXM 1200C, with 12.6-mega output pixels). Fluorescence images were captured at four different channels, including, DAPI (blue) channel: $\lambda_{\text{ex}} = 340-380$ nm, $\lambda_{\text{em}} = 435-485$ nm. GFP-L (green) channel: $\lambda_{\text{ex}} = 460-500$ nm, $\lambda_{\text{em}} = 510$ nm long pass. CY3 HYQ (orange) channel: $\lambda_{\text{ex}} = 530-560$ nm, $\lambda_{\text{em}} = 573-648$ nm. CY5 HYQ (red) channel: $\lambda_{\text{ex}} = 590-650$ nm, $\lambda_{\text{em}} = 663-738$ nm. Pictures were collected and analysed via NIS-elements software package.
6.26. Reference for chapter six


Chapter 7. Conclusions and proposals for future work

In this research project, a novel synthetic pathway for boronic acid-based fluorescence sensors was designed and several new fluorophores were synthesised. A biotin-xylylenediamine-boronic acid linker was established as the most promising scaffold. Three fluorophores: FITC, Bodipy and coumarin were functionalised onto this linker respectively. These fluorescent molecules were synthesised and characterised successfully by spectroscopic techniques, but due to the presence of the boronic acid, all of the final products were difficult to purify through column chromatography. Hence, a low yield was one of the major challenges encountered in these synthesis. These fluorescent sensors were examined via fluorescence and UV-Vis spectroscopies, which allowed kinetic stability tests and binding studies to be performed. SWNTs-based hybrids incorporating these fluorophores were synthesised, characterised by microscopies such as TEM and SEM, and investigated in vitro via MTT assays, confocal microscopy and fluorescence-lifetime imaging microscopy (FLIM).

At the start, the purification of arc-made SWNTs through well-established solvent oxidation methods was performed and it was found that the literature published methods are not ideal to generate entirely defectless SWNTs strands, as it was previously predicted, and as is necessary, for any biomedical application. Although the methods available are sufficiently proficient at removing the metal particles present as contaminants (e.g. Fe, Ni, Y catalysts), it remains challenging to eliminate the carbonaceous structures, attached to the surface of these SWNTs strands, such as amorphous carbon and carbon nanoparticles, without damaging the structure of SWNTs. Further improvements were introduced here, but the remains scope for improvement for the mass production of such defect-free species in high yield needed for commercial applications. Commercially available, already pre-modified, CVD-made SWNTs became available instead as a part of an on-going collaboration involving the Oxford Nanotube Group and Thomas Swann & Co. Ltd. Those samples were readily purified by a steam-based gas phase oxidation methods and have been proven hereby that they are appropriate to be used in vitro on basic of investigation research purposes through
Chapter 7. Conclusions and proposals for future work

TEM, Raman spectra and MTT assays. There were two methods applied in this work to functionalise the purified SWNTs in H₂O, the non-covalent functionalisation of SWNTs with β-D-glucan or interior cavity filling of SWNTs with metal ions as models for metal radioisotopes incorporation. Both methods were successfully established and resulting nano-hybrid characterised via TEM, EDX analysis and Raman spectroscopy. However, the resolution of TEM micrograph obtained so far with available instrumentation was not high enough for imaging the soft material wrapped around the SWNTs strands and β-D-glucan was deteriorated under the electron beam, forming an aggregation in the dry state. Under TEM, it was difficult to find an individual SWNT or several SWNT bundles from the film resulting from β-D-glucan aggregation. Higher resolution TEM imaging of metal salt filled SWNTs still needed to clearly demonstrate the expected filling and estimate the filling yield in the presence of glucan. Nevertheless, despite difficulty with TEM imaging, both EDX analysis and Raman showed that the wrapping and filling of SWNTs was successfully achieved.

Several MTT assays have been carried out to determine the cytotoxicity of varieties of compounds as well as nano-composites of relevance to this work. The library tested included SWNTs, functionalised SWNTs and the fluorescent sensor synthesised. In fact, SWNTs were found to be toxic materials via the biological assays, but after purification and functionalisation with β-D-glucan, the cytotoxicity dropped significantly and thus the viability of these systems as nanomedicines increased considerably. As for the fluorescent sensors used in this research, the cytotoxicity varied across these series and the majority of these sensors were only cytotoxic at high concentration (5×10⁻⁶ mg/mL). Most of the fluorescent compounds synthesised were non-toxic at the concentration used in the confocal microscopy and could be tolerated in the living cell experiments under the dilution of the experiments. The results were extremely promising indicating that most of the fluorescent sensors in assays are suitable for imaging experiments in cells, and matched expectations with errors within acceptable ranges (ρ < 0.05).

Confocal images the fluorescent sensors synthesised were recorded, but only fluorescein- (FITC), coumarin- and bodipy-based boronic acid fluorescent sensors showed interesting results in terms of cellular uptake and cell stability, since these three
systems covered a wide range of the fluorescence emission spectrum. Hence, these three fluorophore were selected to incorporate with design of the new fluorescent sensors. There were a variety of fluorescent sensors imaged in different cell lines, under different ambient conditions, under laser scanning confocal microscopy and FLIM (single photon and two photon excitation. In confocal microscopy, FITC based fluorescent sensors were shown to have a good penetrability through the cell membrane and nuclear membrane, but most of FITC-based fluorescent sensors stayed in the cytoplasm. Both Hoechst and lysotracker co-localisation experiments were performed to confirm the presence of FITC-based fluorescent molecules in the nucleus and cytoplasm respectively. Biocompatible SWNTs functionalised with fluorescent sensors were subsequently examined. As a result, β-D-glucan with a FITC fluorescent sensor tag was added to two different cell lines. Both cells types showed a reasonable uptake of these composites and fluorescence imaging showed that there were fewer composites absorbed by the cell nucleus than by the cytoplasm. However, SWNTs@ glucan@ FITC fluorescent sensor images showed a clear penetration of both the cell membrane and nuclear membrane, strong fluorescent signals were detected from the entirety of the cells, with slight disruption of the cell homeostasis. Subsequently, it found that incubation time and temperature are also important factors affecting the results of confocal imaging experiments. The increment of incubation time significantly augmented both the fluorescent signals and toxicity in cells. Conversely, cells cultured at lower temperatures could considerably reduce the uptake rate of fluorescent compounds and inhibited the damage caused by these compounds. As for the FLIM experiments, the fluorescence lifetime and component distributions were performed for different fluorescent sensors, with a variety of solvents or cells under different conditions. In TCSPC, the fluorescence lifetime were appreciated on single components or two components exponential fitting functions and the two lifetime components were close to evenly separated in solutions (DMSO). In FLIM, the majority of the fluorescence lifetimes were presented only two-component functions, with the components distributions being rather variable. The chi-squared values were, however, within the acceptable range (basically, $\chi^2<1.5$).
In conclusion, novel boronic acid-based fluorescent sensors incorporating biotin as the targeting biomolecule were designed, synthesised and characterised. Robust MTT assay and confocal imaging protocols were established successfully based on a large number of iterative experiments on a number varieties of anticancer drugs and fluorescent sensors which were incorporated to verify the robustness of the method. As made fluorescent sensors have been proven to be useful and biocompatible in the in vitro experiment based on confocal imaging via established MTT assays and the confocal imaging processes designed. In the same way, commercial steam-purified SWNTs were functionalised by β-D-glucan through a non-covalent wrapping method in aqueous media. After characterisation via TEM, SEM and Raman spectroscopy, these glucan@ SWNTs and fluorescent sensor @ glucan @ SWNTs nanohybrid have been delivered to HeLa cells to investigate cytotoxicity effect and obtain confocal images of their bio-localisation. On the other hand, purified SWNTs have been used to encapsulate small ions in water, such as Zr$^{4+}$, Na$^+$ and Cu$^{2+}$, of relevance to radiochemistry with unusual metallic radioisotopes and a protocol of filling, followed by wrapping for SWNTs was subsequently established for SWNTs.

In future work, more fluorophores could be tagged onto the biotin-xylylenediamine-boronic acid linkers designed to form new fluorescent sensors, giving a broader selection of fluorescent sensors. SWNTs can be filled with radiolabelled metal ions, which could be used in applications such as positron emission tomography (PET), to investigate the distribution of radiolabelled SWNTs in living animal experiments. As for MTT assays, time-dependent assays or assays in additional cell lines, including in healthy cells (e.g. a FEK-4 cell line), could be carried to make all data more comparable. Synthesised and characterised fluorescent sensors could also be radiolabelled in order to be traceable in tissue via PET, compared with optical multimodality imaging techniques, which could make the fluorescent sensors usable in both in vivo and in vitro imaging.
Appendix A: control confocal image of different cell lines

(Appendix A. 1) Confocal microscopy images of CHO cells treated with 10 µL DMSO (1% DMSO), incubated at 37 °C for 15 minutes. The sample was excited at 488 nm wavelength and long-pass filtered at 450 nm, 515 nm and 605 nm. Image (a) was merged image of images (b), (c), (d) and (e). Image (b) was blue fluorescence channel. Image (c) was green fluorescence channel. Image (d) was red fluorescence channel. Image (e) represented for image took from DIC. Scale bar: 50 µm.
Appendix A. 2. Confocal microscopy images of HeLa cells treated with 10 µL DMSO (1% DMSO), incubated at 37 °C for 15 minutes. The sample was excited at 488 nm wavelength and long-pass filtered at 450 nm, 515 nm and 605 nm. Image (a) was merged image of images (b), (c), (d) and (e). Image (b) was blue fluorescence channel Image, (c) was green fluorescence channel. Image (d) was red fluorescence channel. Image (e) represented for image took from DIC. Scale bar: 50 µm.
Appendix A. 3. Confocal microscopy images of PC-3 cells treated with 10 µL DMSO (1% DMSO), incubated at 37 °C for 15 minutes. The sample was excited at 488 nm wavelength and long-pass filtered at 450 nm, 515 nm and 605 nm. Image (a) was merged image of images (b), (c), (d) and (e). Image (b) was blue fluorescence channel Image, (c) was green fluorescence channel. Image (d) was red fluorescence channel, Image (e) represented for image took from DIC. Scale bar: 50 µm.
Appendix B. Confocal microscopy images of CHO cells treated with 1 µg/mL Hoechst 33342 (Compound 19) (1% water), incubated at 37 ºC for 15 minutes. The sample was excited at 405 nm wavelength and long-pass filtered at 450 nm, 515 nm and 605 nm. Image (a) was merged image of images (b), (c), (d) and (e). Image (b) was blue fluorescence channel Image, (c) was green fluorescence channel. Image (d) was red fluorescence channel, Image (e) represented for image took from DIC. Scale bar: 20 µm.
Appendix B. 2. Confocal microscopy images of HeLa cells treated with 1 µg/mL Hoechst 33342 (Compound 19) (1% water), incubated at 37 ºC for 15 minutes. The sample was excited at 405 nm wavelength and long-pass filtered at 450 nm, 515 nm and 605 nm. Image (a) was merged image of images (b), (c), (d) and (e). Image (b) was blue fluorescence channel Image, (c) was green fluorescence channel. Image (d) was red fluorescence channel, Image (e) represented for image took from DIC. Scale bar: 20 µm.
Appendix B. 3. Confocal microscopy images of HeLa cells treated with 0.2 µM lysosome red tracker (Compound 22-a) (1% DMSO), incubated at 37 °C for 60 minutes. The sample was excited at 543 nm wavelength and long-pass filtered at 450 nm, 515 nm and 605 nm. Image (a) was merged image of images (b), (c), (d) and (e). Image (b) was blue fluorescence channel Image, (c) was green fluorescence channel. Image (d) was red fluorescence channel, Image (e) represented for image took from DIC. Scale bar: 20 µm.
**Appendix B. 4.** Confocal microscopy images of HeLa cells treated with 0.2 µM Mitochondria red tracker (Compound 21-a) (1% DMSO), incubated at 37 ºC for 30 minutes. The sample was excited at 543 nm wavelength and long-pass filtered at 450 nm, 515 nm and 605 nm. Image (a) was merged image of images (b), (c), (d) and (e). Image (b) was blue fluorescence channel Image, (c) was green fluorescence channel. Image (d) was red fluorescence channel, Image (e) represented for image took from DIC. Scale bar: 20 µm.
Appendix B. 5. Confocal microscopy images of HeLa cells treated with 0.2 µM Mitochondria green tracker (Compound 21-b) (1% DMSO), incubated at 37 ºC for 30 minutes. The sample was excited at 488 nm wavelength and long-pass filtered at 450 nm, 515 nm and 605 nm. Image (a) was merged image of images (b), (c), (d) and (e). Image (b) was blue fluorescence channel Image, (c) was green fluorescence channel. Image (d) was red fluorescence channel, Image (e) represented for image took from DIC. Scale bar: 20 µm.
Appendix C: library of compounds used to establish the protocol of MTT assay.

16a

16b

16c

17a

17b

Appendix C. 1. Dose response curves of HeLa cells treated with compound 16a over 48 hours.

Appendix C. 2. Dose response curve of HeLa cells treated with compound 16b over 48 hours, EC$_{50} = 1.63 \times 10^{-6}$ M.
Appendix C. 3. Dose response curve of HeLa cells treated with compound 16c over 48 hours, EC$_{50}$ = 3.08 × 10$^{-6}$ M.

Appendix C. 4. Dose response curve of HeLa cells treated with compound 17a over 48 hours, EC$_{50}$ = 2.23 × 10$^{-7}$ M.
Appendix C. 5. Dose response curve of HeLa cells treated with compound 17b over 48 hours, EC$_{50}$ = 1.02 × 10$^{-6}$ M.

Appendix D: NMR spectra of synthesised compounds

Appendix D. 1. $^1$H COSY spectrum of compound 7 in deuterated methanol
Appendix D. 1H COSY spectrum of compound 8 in $d_6$-DMSO

Appendix D. 13C NMR spectrum of compound 8 in $d_6$-DMSO
Appendix D. 4. $^1$H, $^{13}$C HMBC spectrum of compound 8 in $d_6$-DMSO

Appendix D. 5. $^1$H COSY spectrum of compound 9 in deuterated methanol
Appendix D. 6. $^{13}$C NMR spectrum of compound 9 in d$_6$-DMSO

Appendix D. 7. $^1$H, $^{13}$C HMBC spectrum of compound 9 in d$_6$-DMSO
Appendix D. 13C NMR spectrum of compound 10 in d6-DMSO

Appendix D. 13C NMR spectrum of compound 11 in d6-DMSO
Appendix D. 10. $^1$H, $^{13}$C HMBC spectrum of compound 11 in d$_6$-DMSO

Appendix E: Mass spectrometry result of synthesised compounds

Appendix E. 1. The ESI/MS spectrum of compound 2, showing peak at m/z 365.2005 and corresponding to [M + Na]$^+$. 

Appendix E. 2. The ESI/MS spectrum of compound 3, showing peak at m/z 559.3679 and corresponding to [M + H]$^+$. 

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Appendix E. 3. The ESI/MS spectrum of compound 4, showing peak at \( m/z \) 969.4586 and corresponding to \([M + Na-H]^+\).

Appendix E. 4. The ESI/MS spectrum of compound 5, showing peak at \( m/z \) 485.2193 and corresponding to \([M + H]^+\).

Appendix E. 5. The ESI/MS spectrum of compound 6, showing peak at \( m/z \) 579.3316 and corresponding to \([M + H]^+\).
Appendix E. 6. The ESI/MS spectrum of compound 7, showing peak at (a) \( m/z \) 966.3471 and corresponding to \([M - H]^+\), data acquired at University of Bath (b) \( m/z \) 968.90 and corresponding to \([M + H]^+\), data acquired in Chemistry Research Laboratory (CRL) University of Oxford.

Appendix E. 7. The ESI/MS spectrum of compound 8, showing peak at \( m/z \) 624.1861 and corresponding to \([M - H]^+\).

Appendix E. 8. The ESI/MS spectrum of compound 9, showing peak at \( m/z \) 750.2128 and corresponding to \([M - H]^+\).

xviii
Appendix E. 9. The ESI/MS spectrum of compound 10, showing peak at \( m/z \) 255.1164 and corresponding to \([M + Na]^+\).

Appendix E. 10. The ESI/MS spectrum of compound 11, showing peak at \( m/z \) 453.2966 and corresponding to \([M + Na]^+\).

Appendix E. 11. The ESI/MS spectrum of compound 12, showing peak at \( m/z \) 840.3195 and corresponding to \([M - H]^−\).

Appendix E. 12. The ESI/MS spectrum of compound 13, showing peak at \( m/z \) 463.2174 and corresponding to \([M - 2H]^−\).
Appendix E. 13. The ESI/MS spectrum of compound 15, showing peak at \( m/z \) 751.3338 and corresponding to \([M + H]^+\).

Appendix F: Inferred spectrum of synthesised compounds

<table>
<thead>
<tr>
<th>Assignment</th>
<th>Range (cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>biotin ureido C=O</td>
<td>1711</td>
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<tr>
<td>-NH-C=O</td>
<td>1645</td>
</tr>
<tr>
<td>N-H bend with C-N stretch</td>
<td>1552</td>
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<td>cycloalkane</td>
<td>1061</td>
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Appendix

Appendix F. 1. Infrared spectra of compound 1

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<td>1695</td>
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<td>Alkane chain</td>
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Appendix F. 2. Infrared spectra of compound 5
## Appendix F. 3. Infrared spectra of compound 6

<table>
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<td>1610</td>
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<tr>
<td>Ar C-C</td>
<td>1510;1566</td>
</tr>
<tr>
<td>B-O</td>
<td>1357</td>
</tr>
<tr>
<td>C-N</td>
<td>1164</td>
</tr>
<tr>
<td>-C(CH(_3))(_2)</td>
<td>1084;1141</td>
</tr>
<tr>
<td>Alkane chain</td>
<td>857</td>
</tr>
<tr>
<td>B-C</td>
<td>658</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Assignment</th>
<th>Range (cm(^{-1}))</th>
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<tr>
<td>Ar C-H</td>
<td>2973</td>
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<tr>
<td>biotin ureido C=O</td>
<td>1704</td>
</tr>
<tr>
<td>-NH-C=O</td>
<td>1610</td>
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<tr>
<td>Ar C-C</td>
<td>1510;1566</td>
</tr>
<tr>
<td>B-O</td>
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<tr>
<td>C-N</td>
<td>1164</td>
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<tr>
<td>-C(CH(_3))(_2)</td>
<td>1084;1141</td>
</tr>
<tr>
<td>Alkane chain</td>
<td>857</td>
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<tr>
<td>B-C</td>
<td>658</td>
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Appendix F. 4. Infrared spectra of compound 7
Appendix F. 5. Infrared spectra of compound 8
## Appendix F.6: Infrared spectra of compound 9

<table>
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<tr>
<td>-NH-C=O</td>
<td>1632</td>
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<tr>
<td>Ar C-C</td>
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<td>C=S</td>
<td>1247</td>
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<tr>
<td>C-N</td>
<td>1166</td>
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<tr>
<td>Alkane chain</td>
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</table>

![Infrared spectra graph]

The graph shows the normalised absorbance (% on the y-axis) against the wavelength (cm\(^{-1}\)) on the x-axis. The absorbance peaks correspond to the assigned functional groups and their characteristic frequencies.
Appendix F. 7. Infrared spectra of compound 10

<table>
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<td>-C(CH₃)₂</td>
<td>1167:1184</td>
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<tr>
<td>B-O</td>
<td>1339</td>
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<td>B-C</td>
<td>600-1200</td>
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### Appendix F. 8. Infrared spectra of compound 11

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<tr>
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<tr>
<td>Ar C-C</td>
<td>1508;1564</td>
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<td>-C(CH$_3$)$_2$</td>
<td>1086;1140</td>
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<tr>
<td>B-O</td>
<td>1357</td>
</tr>
<tr>
<td>B-C</td>
<td>600-1200</td>
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</table>

![Infrared spectrum graph]
<table>
<thead>
<tr>
<th>Assignment</th>
<th>Range (cm(^{-1}))</th>
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<tbody>
<tr>
<td>COO-H</td>
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</tr>
<tr>
<td>biotin ureido C=O</td>
<td>1698</td>
</tr>
<tr>
<td>-NH-C=O</td>
<td>1632</td>
</tr>
<tr>
<td>Ar C=C</td>
<td>1545;1557</td>
</tr>
<tr>
<td>B-O</td>
<td>1359</td>
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<tr>
<td>C=S</td>
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<tr>
<td>C-N</td>
<td>1166</td>
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<td>B-F</td>
<td>1017;1087</td>
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**Appendix F. 9.** Infrared spectra of compound 13
## Assignment

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<td>-NH-C=O</td>
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<td>B-O</td>
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<td>-C(CH₃)₂</td>
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<td>Alkane chain</td>
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<td>B-C</td>
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**Appendix F. 10.** Infrared spectra of compound 15

![Infrared spectra graph]

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xxix
Appendix G: UV-Vis spectra of as-made compounds

Appendix G. 1. UV-Vis spectrum of compound 7 in methanol at 4×10^{-7} M.

Appendix G. 2. UV-Vis spectrum of compound 9 in methanol at 4×10^{-7} M.
Appendix G. 3. UV-Vis spectrum of compound 13 in methanol at $2 \times 10^{-6}$ M.

Appendix G. 4. UV-Vis spectrum of compound 15 in methanol at $4 \times 10^{-3}$ M.
Appendix H: Fluorescent spectra of synthesised compounds

Appendix H. 1. Fluorescence spectrum of compound 7 in methanol at $4\times10^{-7}$ M.

Appendix H. 2. Fluorescence spectrum of compound 9 in methanol at $4\times10^{-7}$ M.
Appendix H. 3. Fluorescence spectrum of compound 13 in methanol at $2 \times 10^{-6}$ M.

Appendix H. 4. Fluorescence spectrum of compound 15 in methanol at $4 \times 10^{-3}$ M.