

**POLYTECHNIQUE MONTRÉAL**

affiliée à l'Université de Montréal

**Real-Time Confocal Raman Imaging of Multi-Walled Carbon Nanotubes in  
Live Prostate Cancer Spheroids to Enhance Photothermal Cancer Therapy**

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Institut de génie biomédical

Mémoire présenté en vue de l'obtention du diplôme de *Maîtrise ès sciences appliquées*

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Ce mémoire intitulé :

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présenté par **Jun Hui YEOH JUN HUI**

en vue de l'obtention du diplôme de *Maîtrise ès sciences appliquées*

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**DEDICATION**

*To my family who always supported me,*

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## RÉSUMÉ

Le cancer de la prostate est la troisième cause de décès par cancer au Canada. La prostatectomie et la radiothérapie externe sont les traitements de référence pour le cancer de la prostate. Cependant, ces traitements entraînent de nombreux effets secondaires. Ablation focale au laser, une méthode de traitement émergente du cancer de la prostate. En particulier, la thérapie photothermique (PTT) suscite de nombreux intérêts car il s'agit d'une méthode non-invasive. Depuis la dernière décennie, le développement rapide des MWCNT a favorisé une augmentation significative de l'intérêt pour les MWCNT en tant qu'agents photothermiques. Une étude récente a montré que le MWCNT combiné au laser 1064 nm peut élever la température locale des cellules tumorales cibles pour induire l'apoptose, sans qu'il soit nécessaire d'augmenter la puissance d'irradiation du laser, minimisant ainsi les dommages thermiques autour des tissus sains. Malgré sa croissance, le MWCNT est toujours confronté à un défi de taille. En particulier, le manque de caractérisation des MWCNT dans les cellules entrave son développement pour la thérapie photothermique in vivo. La caractérisation des MWCNT dans les cellules est importante car elle révèle les interactions de liaison des MWCNT avec les cellules tumorales. Une mauvaise interaction de liaison du MWCNT et des cellules peut diminuer l'efficacité du MWCNT en tant qu'agent photothermique. Pour évaluer les interactions de liaison des MWCNT et des cellules, une microscopie confocale Raman est proposée ici comme méthode pour caractériser les MWCNT dans les cellules. La microscopie confocale Raman a pu capturer l'empreinte moléculaire complète du MWCNT dans les cellules et distinguer son empreinte moléculaire du MWCNT des cellules. Les changements dans les fréquences de bande et l'intensité des bandes MWCNT proéminentes ainsi que des bandes d'acides nucléiques et de protéines offrent un aperçu des interactions de liaison des MWCNT et des cellules. En sondant les interactions du MWCNT et des cellules, il révèle une empreinte digitale unique relative au CNT-PEP (liaison spécifique au PSMA) et au CNT-PEG (contrôle). Ces empreintes digitales uniques nous permettront de vérifier la liaison entre le CNT-PEP et les cellules du tissu tumoral cible. Les concentrations minimales détectables de CNT-PEP ont été identifiées à partir de cette étude nous permettront de définir la plage de concentrations pour déterminer la concentration maximale détectable de CNT-PEP. Les deux concentrations sont nécessaires pour tracer la courbe d'étalonnage qui sera utilisée pour quantifier la concentration réelle de CNT-PEP présente sur la tumeur cible in vivo.

## ABSTRACT

Prostate cancer is the third leading cause of cancer death in Canada. Prostatectomy and external radiation therapy are gold standard treatments for prostate cancer. However, these treatments have numerous side effects. Focal laser ablation, an emerging treatment method for prostate cancer. In particular, photothermal therapy (PTT) has drawn numerous interests because it is a non-invasive method. Since the last decade, rapid development of MWCNT has promoted a significant increase of interests in MWCNT as photothermal agents. Recent study showed that MWCNT combined with 1064nm laser can elevate the local temperature of the target tumor cells to induce apoptosis, without the need to increase the laser irradiation power, minimizing thermal damage around healthy tissue. Despite of its growth, MWCNT are still facing a significant challenge. Particularly, the lack of characterization of MWCNT in cells, is hindering its development for in vivo photothermal therapy. The characterization of MWCNT in cells is important because it reveals the binding interactions of MWCNT with tumor cells. A poor binding interaction of MWCNT and cells can lessen the efficacy of MWCNT as a photothermal agent. To assess the binding interactions of MWCNT and cells, a confocal Raman microscopy was proposed here as the method to characterize MWCNT in cells. Confocal Raman microscopy was able to capture the full molecular fingerprint of MWCNT in cells and distinguish its molecular fingerprint of MWCNT from cells. The changes in band frequencies and intensity of prominent MWCNT bands along with bands of nucleic acids and proteins, offers insights into the binding interactions of MWCNT and cells. By probing the interactions of MWCNT and cells, it reveals unique fingerprint pertaining to CNT-PEP (specific binding to PSMA) and CNT-PEG (control). These unique fingerprints will allow us to verify the binding between CNT-PEP and cells in the target tumor tissue. The minimal detectable concentrations of CNT-PEP were identified from this study will allow us to define the range of concentrations to determine the maximum detectable concentration of CNT-PEP. Both concentrations are necessary for plotting the calibration curve that will be used to quantify the actual concentration of CNT-PEP present on the target tumor in vivo.

|                                                                                              |     |
|----------------------------------------------------------------------------------------------|-----|
| DEDICATION .....                                                                             | III |
| ACKNOWLEDGEMENTS .....                                                                       | IV  |
| RÉSUMÉ.....                                                                                  | V   |
| ABSTRACT .....                                                                               | VI  |
| TABLE OF CONTENTS .....                                                                      | VII |
| LIST OF TABLES .....                                                                         | X   |
| LIST OF FIGURES.....                                                                         | XI  |
| LIST OF SYMBOLS AND ABBREVIATIONS.....                                                       | XV  |
| CHAPTER 1 INTRODUCTION.....                                                                  | 1   |
| 1.1 Prostate Cancer: Overview and Treatment Options .....                                    | 1   |
| 1.2 Photothermal Therapy for Prostate Cancer: Role of Multi-Walled Carbon Nanotubes ..       | 3   |
| 1.3 Lack of Characterization of Multi-Walled Carbon Nanotubes in Cells .....                 | 6   |
| 1.4 Hypothesis and Objectives .....                                                          | 6   |
| 1.5 Organization of This Document .....                                                      | 9   |
| CHAPTER 2 REVIEW OF LITERATURE.....                                                          | 10  |
| 2.1 Confocal Raman Microscopy .....                                                          | 10  |
| 2.1.1 Raman spectroscopy.....                                                                | 10  |
| 2.1.2 Confocal Raman microscopy: integrate Raman spectroscopy with confocal microscopy ..... | 16  |
| 2.2 Multi-Walled Carbon Nanotubes: Structure, Thermal Properties, and Functionalization..... | 21  |
| 2.3 Raman Characterization of MWCNT .....                                                    | 25  |
| 2.3.1 Raman scattering in MWCNT.....                                                         | 25  |
| 2.3.2 Interpretation of Raman spectra of MWCNT .....                                         | 28  |

|                                                                                                                                  |                                                                                                                   |    |
|----------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------|----|
| 2.4                                                                                                                              | Raman Characterization of Cells: Biological Molecules .....                                                       | 32 |
| 2.4.1                                                                                                                            | Raman scattering of biological molecules .....                                                                    | 32 |
| 2.4.2                                                                                                                            | Interpretation of Raman spectra of biological molecules.....                                                      | 33 |
| 2.5                                                                                                                              | Raman Characterization of MWCNT in Cells .....                                                                    | 35 |
| CHAPTER 3 OPTIMIZATION OF CONFOCAL RAMAN MICROSCOPE FOR IMAGING<br>MULTI-WALLED CARBON NANOTUBES IN LIVE PROSTATE SPHEROIDS..... |                                                                                                                   | 37 |
| 3.1                                                                                                                              | Introduction and Project Goals.....                                                                               | 37 |
| 3.2                                                                                                                              | Methodology .....                                                                                                 | 37 |
| 3.2.1                                                                                                                            | Confocal Raman microscope system .....                                                                            | 37 |
| 3.2.2                                                                                                                            | Synthesis of functionalized and non-functionalized MWCNT .....                                                    | 38 |
| 3.2.3                                                                                                                            | Protocol for cell culture.....                                                                                    | 39 |
| 3.2.4                                                                                                                            | Protocol for spheroid generation .....                                                                            | 39 |
| 3.2.5                                                                                                                            | Optimization of experimental protocol for characterizing live spheroids.....                                      | 40 |
| 3.2.6                                                                                                                            | Technique for mapping LNCaP spheroid.....                                                                         | 40 |
| 3.2.7                                                                                                                            | Incubation of CNT samples in spheroids .....                                                                      | 42 |
| 3.2.7                                                                                                                            | Sample preparation before measurements.....                                                                       | 42 |
| 3.2.8                                                                                                                            | Procedures for Raman spectral acquisition .....                                                                   | 42 |
| 3.2.9                                                                                                                            | Data processing for Raman spectra .....                                                                           | 43 |
| 3.2.10                                                                                                                           | Generation of figures for results.....                                                                            | 44 |
| 3.3                                                                                                                              | Results .....                                                                                                     | 49 |
| 3.3.1                                                                                                                            | Preliminary study: characterize effect of functionalization on MWCNT .....                                        | 49 |
| 3.3.2                                                                                                                            | Characterize the molecular fingerprint of prostate cancer cells using LNCaP spheroids<br>as a model.....          | 52 |
| 3.3.3                                                                                                                            | Characterization of CNT in LNCaP spheroids: probe interactions of multi-walled carbon<br>nanotubes and cells..... | 57 |

|              |                                                                                                                |    |
|--------------|----------------------------------------------------------------------------------------------------------------|----|
| 3.4          | Discussions.....                                                                                               | 65 |
| 3.4.1        | Preliminary study: characterize effect of functionalization on MWCNT .....                                     | 65 |
| 3.4.2        | Characterize the molecular fingerprint of prostate cancer cells using LNCaP spheroids as a model.....          | 66 |
| 3.4.3        | Characterization of CNT in LNCaP spheroids: probe interactions of multi-walled carbon nanotubes and cells..... | 68 |
| CHAPTER 4    | CONCLUSION AND FUTURE RESEARCH .....                                                                           | 73 |
| BIBLIOGRAPHY | .....                                                                                                          | 77 |

## LIST OF TABLES

|                                                                                                                                            |    |
|--------------------------------------------------------------------------------------------------------------------------------------------|----|
| Table 3.1 Sample calculation for MWCNT-GNR shown in Figure 3.5. ....                                                                       | 45 |
| Table 3.2 Raman intensity of D-band and G-band of different concentrations of CNT-PEP and<br>CNT-PEG from Figure 3.11 and Figure 3.12..... | 48 |
| Table 3.3 Prominent Raman bands identified from Figure 3.4.....                                                                            | 51 |
| Table 3.4 Prominent Raman bands identified from Figure 3.6b and Figure 3.7b.....                                                           | 55 |
| Table 3.5 Prominent Raman bands identified from Figure 3.9b, 3.10, 3.11, and 3.12.....                                                     | 60 |

## LIST OF FIGURES

|                                                                                                                                                                                                                                                                                                                            |    |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Figure 1.1 Anatomy of the prostate. Inset figure showing four different zones of the prostate gland (central, peripheral, fibromuscular, transitional) (Adapted from [5]). .....                                                                                                                                           | 1  |
| Figure 1.2 External radiation therapy uses a machine to generate high doses of radiation at tumor sites to destroy cancer cells or shrink tumors (Adapted from [9]). .....                                                                                                                                                 | 2  |
| Figure 1.3 Illustration of focal laser ablation or focal therapy. High intensity light is delivered via an optical fiber needle inside a patient's body, at the specific site of a prostate tumor (Adapted from [12]). .....                                                                                               | 3  |
| Figure 1.4 Photothermal ablation of a tumor tissue using carbon nanotubes as photothermal agent. Upon laser irradiation, MWCNT (black cylindrical tube) attached onto the membrane of tumor cells transport heat energy through phonon conduction, inducing apoptosis (cell death) (Adapted from [20]). .....              | 5  |
| Figure 2.1 Representation of elastic scattering (Rayleigh) and inelastic scattering (Stokes and Anti-Stokes).....                                                                                                                                                                                                          | 11 |
| Figure 2.2 Representation of four vibrational modes of CO <sub>2</sub> a) symmetric stretch (Raman active); b) asymmetric stretch (Raman inactive); and c) Two modes of bending (Raman inactive).....                                                                                                                      | 12 |
| Figure 2.3 Jablonski energy level diagram of Rayleigh, Stokes, and Anti-Stokes scattering.....                                                                                                                                                                                                                             | 14 |
| Figure 2.4 Typical Raman spectrum (Anti-Stokes, Rayleigh, Stokes) .....                                                                                                                                                                                                                                                    | 16 |
| Figure 2.5 Optical layout of a confocal Raman microscope (Adapted from [32]). .....                                                                                                                                                                                                                                        | 18 |
| Figure 2.6 Illustrates the separation of Airy disk and Rayleigh criterion a) the separation between the two disks exceed their radii, thus they are resolvable; b) Two disks are not individually resolvable by Rayleigh criterion; c) two disks overlap and appear as one poorly resolved disk (Adapted from [33]). ..... | 19 |
| Figure 2.7 a) Without a slit, in-focus Raman scatter (red) and out-of-focus Raman scatter (blue) enter the spectrometer; b) Slit blocking out-of-focus Raman scatter (blue) and only allow in-focus Raman scatter (red) entering the spectrometer.....                                                                     | 20 |

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |    |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Figure 2.8 Illustrates that distance of spacing affects the number of spectra shown on a CCD detector. Farther spacings of line gratings increases the number of spectra (Adapted from [34])...                                                                                                                                                                                                                                                                                                 | 21 |
| Figure 2.9 a) Illustration of the formation of SWCNT and MWCNT from graphene (Adapted from [33]); b) Schematic diagram showing how a hexagonal sheet of graphene is rolled to form a MWCNT via armchair or zig-zag configurations (Adapted from [38]).                                                                                                                                                                                                                                          | 22 |
| Figure 2.10 Illustration of the MWCNT conjugated with functional groups (gold), linker (dark red), cross-linker (purple), and antibodies(y-shaped) onto the surface of carbon nanotubes. Antigens (bright red) are captured by the antibodies (Adapted from [35])                                                                                                                                                                                                                               | 25 |
| Figure 2.11 Raman scattering process of MWCNT a) Light irradiation on aromatic rings of MWCNT; b) Energy band diagram of MWCNT. Raman scattering occurs when excited electron in conduction band of MWCNT scattered by emitting a phonon (Stokes) or absorbing a phonon (Anti-Stokes) before relaxes back to valence band; c) Two types of scattering that can occur: Intravalley scattering (within same conduction band valley) or intervalley scattering (different conduction band valley). | 27 |
| Figure 2.12 Raman spectra of different carbon forms: graphite, graphene, SWCNT, DWCNT, and MWCNT (Adapted from [47]).                                                                                                                                                                                                                                                                                                                                                                           | 29 |
| Figure 2.13 Raman spectra of a) pristine CNTs, $I_D/I_G = 0.8$ ; b) COOH functionalized CNTs, $I_D/I_G = 1.2$ ; c) functionalized CNTs, $I_D/I_G = 1.4$ (Adapted from [47]).                                                                                                                                                                                                                                                                                                                    | 30 |
| Figure 2.14 Raman spectra of a) pristine multi-walled carbon nanotubes at different excitation laser powers (excitation wavelength 441.6 nm); b) pristine multi-walled carbon nanotubes and composite films containing different wt% loading obtained at laser power of 0.55W (Adapted from [37]).                                                                                                                                                                                              | 31 |
| Figure 2.15 Vibrational modes of the backbone of nucleic acids a) $PO_2^-$ symmetric stretching with the band near $1096\text{ cm}^{-1}$ ; b) O-P-O symmetric stretching near $783\text{ cm}^{-1}$ ; c) Raman scattering of biological molecules: (1) Stretching distorts the electron clouds causing a change in polarizability; (2) Change in polarizability induce an electric dipole; (3) Induced dipole that oscillates at a frequency emits radiation (Raman scattering) along an angle.  | 33 |

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |    |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Figure 2.16 Raman spectra of SWCNT and CNTs coated with PEG in 2D cell culture in vitro, OVCAR8 ovarian cancer cells were subjected to Raman analysis to detect the presence of nanotubes using a handheld portable iRaman spectrometer with a 785 nm laser source. (a) Untreated cells, serving as controls, did not show any characteristic carbon nanotubes peaks. The SWCNT (b) and CNTs (C) treated cells indeed showed the characteristic G peaks (Adapted from [56]). . . . .                                   | 35 |
| Figure 2.17 Raman probing of SWCNT-PL-PEG-NH <sub>2</sub> and CNTs-PL-PEG-NH <sub>2</sub> in 3D spheroids. OVCAR8 ovarian cancer cell spheroids (a) treated with LP dispersed SWCNTs (B, D) and CNTs (C, E) labeled with Qdots (red), for 3 h and washed were subjected to Raman analysis to detect the presence of nanotubes using a handheld portable iRaman spectrometer with a 785 nm laser source. The SWCNT (F) and CNTs (G) treated cells indeed showed the characteristic G peaks (Adapted from [56]). . . . . | 36 |
| Figure 3.1 Confocal Raman Microscope (Adapted from [59]) . . . . .                                                                                                                                                                                                                                                                                                                                                                                                                                                     | 38 |
| Figure 3.2 Confocal microscopy image of a) LNCaP spheroid before measurement; b) LNCaP spheroid mapped point-by-point; c) LNCaP spheroid mapped using point-by-point in a raster scan. . . . .                                                                                                                                                                                                                                                                                                                         | 41 |
| Figure 3.3 Raman signal processing workflow. . . . .                                                                                                                                                                                                                                                                                                                                                                                                                                                                   | 44 |
| Figure 3.4 Raman spectra of functionalized CNTs (COOH) and non-functionalized CNTs (TSHR ab, IgG ab, GNR). All CNTs have the same concentration of 0.025mg/mL. Prominent Raman peaks were identified as shown in Table 3.3. . . . .                                                                                                                                                                                                                                                                                    | 50 |
| Figure 3.5 The effect of functionalization on the D/G ratio of MWCNT. . . . .                                                                                                                                                                                                                                                                                                                                                                                                                                          | 52 |
| Figure 3.6 a) Confocal microscopy image of LNCaP spheroid; b) Raman spectra of LNCaP spheroids acquired at laser power of 150mW, 30mW, and 3mW are plotted in the same graph. . . . .                                                                                                                                                                                                                                                                                                                                  | 53 |
| Figure 3.7 a) Confocal microscopy images of different sizes of LNCaP spheroid i) Seeding density = $0.016 \times 10^6$ cells/cm <sup>2</sup> and spheroid diameter of 300 $\mu$ m, ii) Seeding Density = $0.0031 \times 10^6$ cells/cm <sup>2</sup> and spheroid diameter of 100 $\mu$ m, and iii) $0.0016 \times 10^6$ cells/cm <sup>2</sup> and spheroid diameter of 50 $\mu$ m; (b) Raman spectra of LNCaP spheroids of different sizes are all plotted on the same graph. . . . .                                  | 56 |

|                                                                                                                                                                                                                                                                                                                                                                                                                                      |    |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Figure 3.8 Confocal microscopy image of LNCaP cells with 0.1mg/mL of CNT a) before laser irradiation; b) after laser irradiation. ....                                                                                                                                                                                                                                                                                               | 57 |
| Figure 3.9 (a) Confocal microscopy image showing that CNT can be detected by locating dark areas on the spheroid; (b) Raman spectra of LNCaP with carbon nanotubes and LNCaP without carbon nanotubes are plotted on the same graph. Each spectrum corresponds to selected areas shown in Figure 3.9a. Refer to Table 3.5 for the wavenumber and molecular bond assignment of prominent peaks (a, b, c, d, e, f, g, h, i, j, k)..... | 59 |
| Figure 3.10 Molecular fingerprint of CNT in LNCaP spheroids. Raman spectra of different concentrations of CNT are plotted on the same graph and LNCaP is included here as a control. The prominent bands (a, c, d, e, i, g, j) vary from low to high concentration of CNT.....                                                                                                                                                       | 61 |
| Figure 3.11 Molecular fingerprint of CNT-PEP (targeting PSMA) in LNCaP spheroid. Raman spectra of different concentrations of CNT-PEP are plotted on the same graph and LNCaP is included here as a control. The prominent bands (a, c, i, g, j) vary from low to high concentration of CNT-PEP. 0.02mg/mL was dropped from the experiment due to instrumentation error during measurements. ....                                    | 63 |
| Figure 3.12 Molecular fingerprint of CNT-PEG (non-specific to PSMA) in LNCaP spheroid. Raman spectra of different concentrations of CNT-PEG are plotted on the same graph and LNCaP is included here as a control. The prominent bands (a, c, i, g, j) vary from low to high concentration of CNT-PEG. 0.02mg/mL was dropped from the experiment due to instrumentation error during measurements. ....                              | 64 |
| Figure 3.13 Linear Regression a) Comparing the D bands of CNT-PEP and CNT-PEG at $1360\text{ cm}^{-1}$ ; (b) Comparing the G bands of CNT-PEP and CNT-PEG at $1610\text{ cm}^{-1}$ . Note that 0.02mg/mL was excluded from the linear regression analysis due to instrumentation error during measurements. ....                                                                                                                     | 65 |
| Figure 4.1 Illustration of Proposed Grand Project Phases (Preliminary, Phase I, Phase II, Phase III) (Adapted from [1], [2], [3]).....                                                                                                                                                                                                                                                                                               | 75 |
| Figure 4.2 Illustration of Proposed Integration System (Raman/Fluorescence Imaging and Photothermal Ablation) for Phase II (Adapted from [1] and [2]).....                                                                                                                                                                                                                                                                           | 76 |

## LIST OF SYMBOLS AND ABBREVIATIONS

|         |                                                                                       |
|---------|---------------------------------------------------------------------------------------|
| CNT     | COOH-functionalized multi-walled carbon nanotubes                                     |
| CNT-PEG | COOH functionalized multi-walled carbon nanotubes conjugated with polyethylene glycol |
| CNT-PEP | COOH functionalized multi-walled carbon nanotubes conjugated with peptidomimetic      |
| COOH    | Carboxylic acids                                                                      |
| EDC     | 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide                                        |
| GNR     | Gold nanorods                                                                         |
| IgG     | Immunoglobulin G                                                                      |
| LNCaP   | Lymph node carcinoma of the prostate                                                  |
| LRO     | Laboratory of Radiological Optics                                                     |
| MRI     | Magnetic resonance imaging technology                                                 |
| MWCNT   | Multi-walled carbon nanotubes                                                         |
| NHS     | N-hydrocysuccinimide                                                                  |
| NIR     | Near-infrared                                                                         |
| PEG     | polyethylene glycol                                                                   |
| PEP     | peptidomimetic                                                                        |
| PSMA    | Prostate-specific membrane antigens                                                   |
| PTT     | Photothermal therapy                                                                  |
| RBM     | Radial breathing mode                                                                 |
| SWCNT   | Single-walled carbon nanotubes                                                        |
| SWIR    | Short-wave infrared                                                                   |
| TSHR    | Thyroid stimulating hormone receptor                                                  |

## CHAPTER 1 INTRODUCTION

### 1.1 Prostate Cancer: Overview and Treatment Options

Prostate cancer is the third leading cause of cancer death in Canada and accounted for 20% of all estimated new cancer cases in men in 2022 [4]. On average, 67 men are diagnosed with prostate cancer in a day with a mortality rate of approximately 20% [4]. Prostate cancer is confined to the prostate gland which is a male reproductive organ located below the bladder. It contributes to semen secretions and help to maintain sperm viability [5]. The prostate gland can be divided into four different zones such as peripheral, central, fibromuscular stroma, and transitional, as shown in Figure 1.1. Normal development and maintenance of prostate gland depends on androgens (hormones) and androgen receptors (hormone receptor) [6]. However, both androgens and androgen receptors also contribute to the growth of prostate cancer cells in the peripheral zone [5, 6]. In prostate cancer, these androgen receptors activate the expression of prostate-specific membrane antigens (PSMA) that drive cancer growth. High levels of PSMA have been detected in secretory cells of prostate cancer in the epithelium [7]. Therefore, PSMA and androgen receptors have been used as biomarkers for prostate cancer in imaging and treatment.

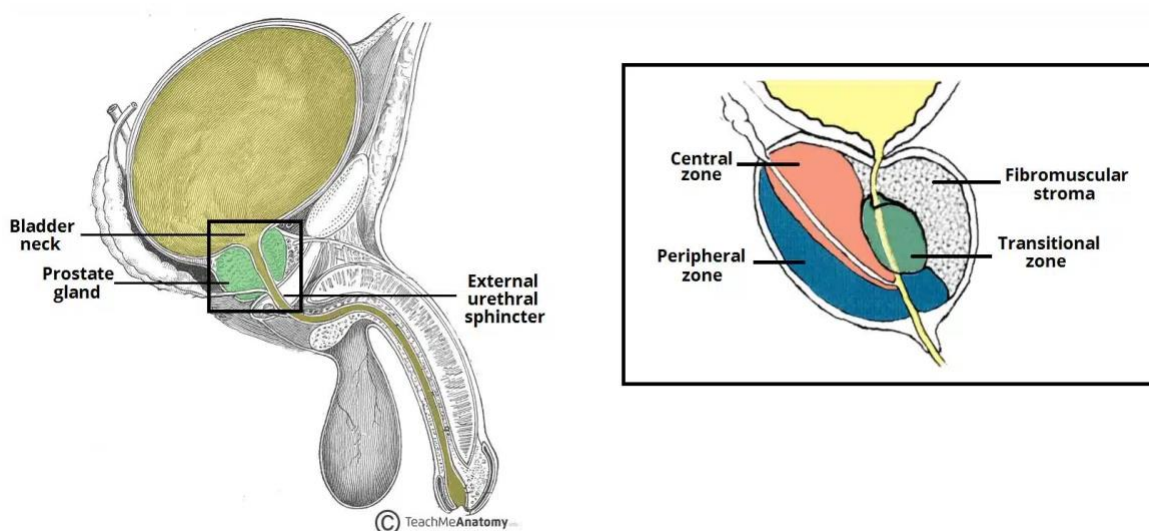


Figure 1.1 Anatomy of the prostate. Inset figure showing four different zones of the prostate gland (central, peripheral, fibromuscular, transitional) (Adapted from [8]).

Prostate cancer can be divided into three different stages (localized, locally advanced, metastatic) depending where the cancer has spread. Cancer that has spread to other organs are known as metastatic. Locally advanced refers to cancer that has spread outside of prostate to nearby tissues. For cancer that has not spread outside of the prostate gland (localized prostate cancer), prostatectomy and external radiation therapy continue to be the gold standard for treatment of prostate cancer [9]. Prostatectomy is a surgical procedure in which partial or the entire prostate gland is removed. External radiation therapy is a type of therapy that destroy cancer cells by generating high doses of radiation at tumor sites as shown in Figure 1.2. However, a substantial number of patients who went through these treatments have reported significant side effects such as incontinence and erectile dysfunction [9]. In one study of 1288 men who received radical prostatectomy, 55% of patients reported incontinence after 12 post-treatment [10]. About 46% of patients from the same study also reported erectile dysfunction after 12-months post-treatment [10]. Another study found an increase of, 60% of patients reported impotence after radical prostatectomy and 20% patients reported impotence for external radiation therapy [11]. There is an urgent need for better therapies with fewer complications.

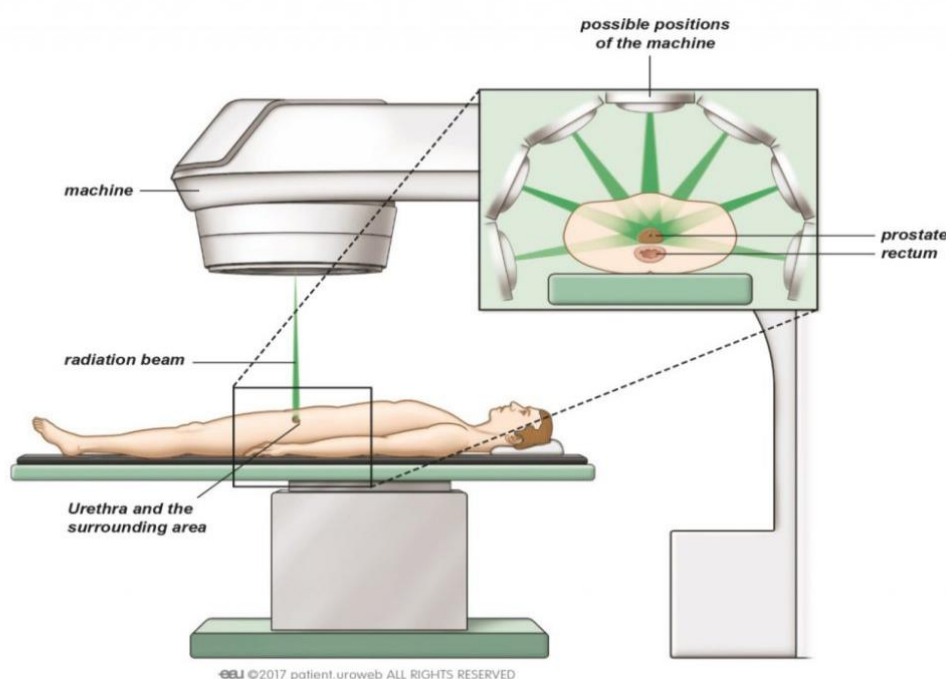


Figure 1.2 External radiation therapy uses a machine to generate high doses of radiation at tumor sites to destroy cancer cells or shrink tumors (Adapted from [12]).

In recent years, focal laser ablation known as focal therapy, has become an emerging treatment method for prostate cancer. It is a procedure that inserts an optical laser fiber needle inside a patient's body. The laser fibers deliver radiation energy at specific areas of the prostate tumor to destroy cancer cells, as shown in Figure 1.3. It is based on the use of the photothermal effect, in which light energy is converted into thermal energy [13]. The absorption of light in tumor tissue induces a local elevation in temperature and thermal damage destroys cancer cells in the tumor [13]. The thermal effect depends on the amount of heat energy of light and the depth of light distribution [13]. Wavelengths between 590 - 1064 nm are commonly used to achieve a deep tissue penetration [13]. Light is delivered via quartz fibers of diameter from 300 to 600  $\mu\text{m}$  and a needle can provide a large ablative area up to 50mm [13]. The risk of urinary incontinence and erectile dysfunction was found to be lower in patients who received focal laser ablation, in comparison to those who underwent whole gland treatment [14].

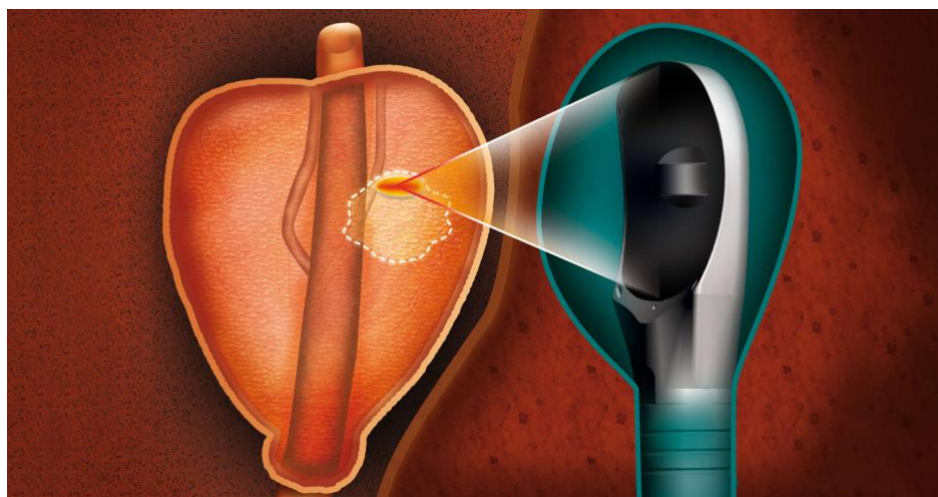


Figure 1.3 Illustration of focal laser ablation or focal therapy. High intensity light is delivered via an optical fiber needle inside a patient's body, at the specific site of a prostate tumor (Adapted from [12]).

## 1.2 Photothermal Therapy for Prostate Cancer: Role of Multi-Walled Carbon Nanotubes

Two most common focal laser ablations are photothermal and photodynamic therapy. Among the two therapies, photothermal therapy (PTT) has drawn interests because it is highly effective

(localize heating) and non-invasive method [15]. All cells including cancer cells are sensitive to temperature changes. Under the appropriate temperatures, cancer cell death can be induced either through apoptosis or necrosis. Apoptosis is preferable than necrosis because reduce inflammatory response [16], reducing the risk of cancer recurrence and metastasis. Apoptosis occurs in the range of 43°C - 50°C [13]. The concept of photothermal therapy is based on photothermal agents that convert light energy into heat, induces localized heating around tissue without causing excessive thermal damage to surrounding healthy tissues, thus reduces side effects significantly [15]. PTT agents can be modulated to induce excessive heating to trigger apoptosis in tumors [17].

Since the inception of nanotechnology, there have been numerous efforts to develop nanomaterials (nanoclusters, nanoshell, carbon nanotubes) as photothermal agents. The greatest benefit of using nanomaterials for focal laser ablation is the ability to modulate its thermal properties to maintain appropriate temperatures to induce apoptosis uniformly [18]. Gold nanoclusters were used in combination with infrared light, observed significant reduction in tumor volume of prostate mice within 15 days [19]. In a clinical pilot study, gold-nanoshell mediated photothermal ablation was successfully achieved in 94% of prostate cancer patients, without serious complications or deleterious changes in genitourinary function [20]. In the last decade, there has been a growing interest in carbon nanotubes such as single-walled carbon nanotubes (SWCNT) and multi-walled carbon nanotubes (MWCNT) as photothermal agents. MWCNT and SWCNT offer higher thermal conductivity than other nanomaterial, at  $3000 \frac{W}{m K}$  and  $2000 \frac{W}{m K}$  respectively [37]. MWCNT has strong optical absorbance at visible and near-infrared wavelengths, reduces light attenuation and scattering in skin and subcutaneous tissue significantly [19]. MWCNT absorbs near-infrared (NIR) radiation better than SWCNT because they have more available electrons for absorption per particle (contributed by walls of nanotubes) and possess greater mass [19]. When MWCNT is coupled with NIR light, molecules of MWCNT that interacts with light experience vibrational motion. The energy of that molecular vibration is known as phonon. The heat energy is generated through the overall kinetic energy of molecules due to molecular motion. As temperature increase, the frequency of phonon that propagates within the lattice structure of MWCNT also increases and so is the heat conductivity of MWCNT. By maintaining the heat conductivity across MWCNT, it raises local temperature at the target tumor until it reaches the temperature of apoptosis to destroy

cancer cells [18]. Recent study has shown a significant temperature elevation and decreased in cell viability were achieved, when 1064nm laser were used in combination with MWCNT to induce apoptosis in prostate cancer cells (PC3). PC3 were irradiated at  $15.3 \frac{W}{cm^2}$  for 1.5 minutes with MWCNT, increased temperature of the sample by  $30^{\circ}C$ . In contrast, cells without MWCNT only increased the temperature of sample by  $5^{\circ}C$ . When heating duration was increased to 5 minutes, temperature elevations of both samples increased to  $43^{\circ}C$  and  $7^{\circ}C$ . The combination of laser heating at  $15.3 \frac{W}{cm^2}$  with MWCNT for 5 minutes showed low cell viability and downregulation of heat shock protein, indicating the cancer cell death [20]. However, laser irradiation without MWCNT would need to be increased to  $50.9 \frac{W}{cm^2}$  for 10 mins to achieve a similar thermal dose and cell viability [21]. Another study showed that multi-walled carbon nanotubes conjugated with prostate-specific membrane antigens antibodies enhance the nearfield cell ablation effect in LNCaP cells [22]. Cell ablation increased from  $38.8\% \pm 4.3\%$  to  $71.2\% \pm 4.7\%$  when the concentration of multi-walled carbon nanotubes increases [22].

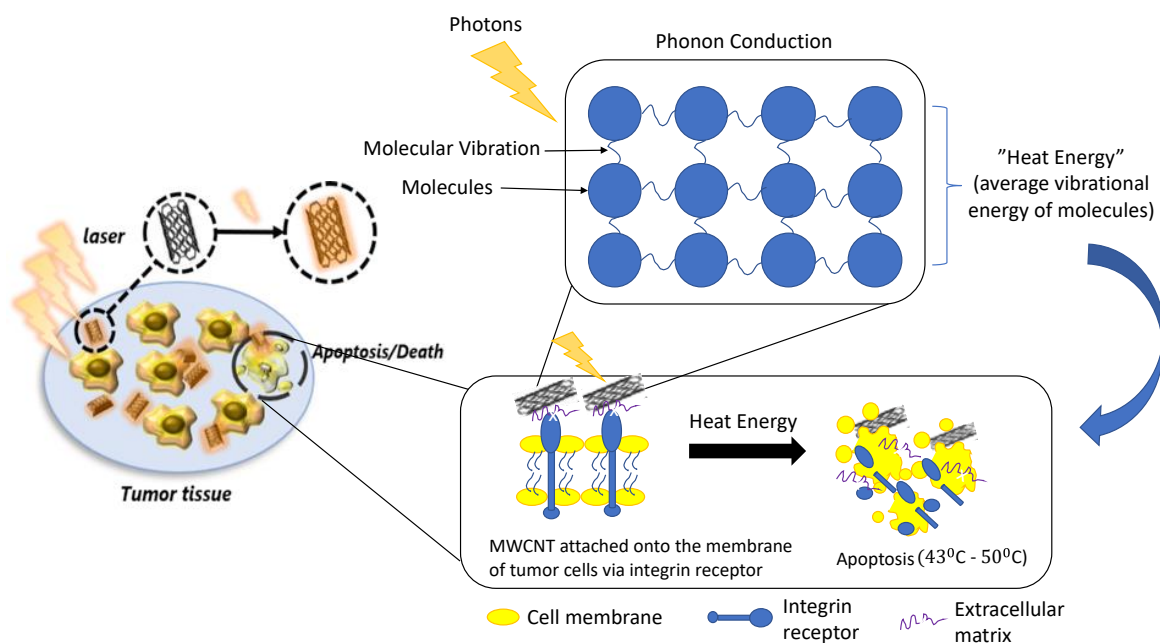


Figure 1.4 Photothermal ablation of a tumor tissue using carbon nanotubes as photothermal agent. Upon laser irradiation, MWCNT (black cylindrical tube) attached onto the membrane of tumor cells transport heat energy through phonon conduction, inducing apoptosis (cell death) (Adapted from [23]).

### **1.3 Lack of Characterization of Multi-Walled Carbon Nanotubes in Cells**

Most studies that have been discussed so far, demonstrated the potential of MWCNT as photothermal agents for photothermal ablation. When laser light is directed at the targeted tumor, photons interact with MWCNT as well as the cells, contributing to different bands in the spectra. Numerous studies characterized nanoparticles in cells, however, very few studies characterize MWCNT in cells. Cai et al. [24] characterized the physical interactions between carbon nanotubes and cells using flow cytometry. They observed an increase in side scattering signals, where the amplitude is proportional to the amount of carbon nanotubes that are associated with cells [24]. The time constant observed from complexes of carbon nanotubes and cells indicates a high level of pore formation in cell membranes [24]. The drawback of flow cytometry is it can only detect and analyze one cell at a time, limiting its ability to characterize cells. There is an urgent need for more characterization of MWCNT in cells. The characterization studies reveal the interactions of carbon nanotubes and cells such as receptor binding, antigen-antibody recognition, and internalization [24]. Furthermore, interactions of carbon nanotubes and cells have also been correlated to nanomaterial toxicity and biocompatibility [24]. In this case, an optical method that can effectively characterize MWCNT in cells is Raman spectroscopy. It is a non-destructive label-free technique that provides information about molecular interactions and chemical structure. It has been employed in analytical sciences for purity determination of carbon nanotubes [25]. To obtain a full molecular profile of MWCNT in cells, a confocal Raman microscopy is advantageous over conventional Raman spectroscopy. Confocal Raman microscopy combines high-resolution confocal imaging with Raman spectroscopy to map sample in lateral (X, Y) and depth (Z) axes. It can generate three dimensional Raman spectral maps to capture the molecular fingerprint of carbon nanotubes and cells, enabling to understand the interactions of carbon nanotubes and cells.

### **1.4 Hypothesis and Objectives**

This research project was conducted at the Laboratory of Radiological Optics (LRO). LRO has been dedicated to the development and application of Raman spectroscopy technologies for prostate cancer. Our group developed an image guided Raman spectroscopy navigation system, combining spectral and MRI-radiomics features, to improve prostate cancer detection [26]. We demonstrated that a Raman spectroscopy system can be integrated into a robotic-assisted surgical

system for real-time tissue characterization during radical prostatectomy procedures [27]. Another study demonstrated a non-destructive technique based on high-precision spatial registration of single-point optical measurements with histopathology analysis, to interrogate radical prostatectomy specimens from prostate cancer patients [28]. LRO also demonstrated that a confocal Raman microscope can be used to identify the molecular features of intraductal carcinoma in prostate tissue [29]. These works pave the way for advancing Raman spectroscopy as a probing tool for photothermal ablation of prostate tumors. The ultimate goal of the grand project, in collaboration with McGill university, is to enhance photothermal therapy using MWCNT to elevate the temperature of tumor tissue to induce apoptosis. To improve its efficacy, MWCNT can be conjugated with a cyanine tag such as ICG to image and locate the target tumor. The versatility of MWCNT sparks the idea of integrating Raman spectroscopy into the photothermal therapy as a probing tool. Raman spectroscopy can be used to quantify the actual concentration of MWCNT at the target tumor to ensure that the heat generated is sufficient to induce apoptosis and not overheating the tissue, thereby minimizes thermal damage to healthy tissue. The overall aim of this thesis, as part of the preliminary phase of the grand project, is to obtain the molecular profiles of MWCNT in prostate cancer cells which consist of information related to the interaction of MWCNT and cells. By elucidating these interactions, unique Raman fingerprint of MWCNT in cells can be identified as well as the minimum detectable concentrations. Based on the minimum detectable concentration, MWCNT will be tested with higher concentrations. From here, a calibration concentration curve can be plotted to determine the maximum and minimum detectable concentrations. The molecular fingerprint of MWCNT will be used to confirm its binding at target tumor and the calibration curve will be used to quantify the actual concentration of MWCNT at target tumor. Currently, our group is developing an imaging system that will be equipped with two lasers (785nm and 1064nm) and an InGaAs camera for detecting near-infrared wavelengths (800 – 2500nm). We envision a system integrating fluorescence/Raman imaging (locating target tumor) with photothermal ablation of tumor under the same platform. The idea is to inject a solution containing ligand conjugating MWCNT tagged with a fluorescence probe (ICG) into mice or patients. 785nm will be used to excite the fluorescence emission of ICG to visualize the target tumor and a portable Raman probe will be used to confirm its binding and to determine the amount of MWCNT present at the target tumor. Finally, the laser ablation will be performed using 1064nm to generate heat through MWCNT at the target tumor to destroy cancer cells.

Two hypotheses were formulated as part of this thesis for the preliminary phase of the project,

Hypothesis 1: Confocal Raman microscopy can be used to obtain the molecular fingerprint of multi-walled carbon nanotubes in cells

Hypothesis 2: The molecular fingerprint of multi-walled carbon nanotubes in live cell aggregates (spheroids) obtained by Confocal Raman microscopy, can reveal the information relating to interactions of carbon nanotubes and cells.

Thus, the following objectives were established to test these hypotheses,

Objective 1: Characterize effect of functionalization on MWCNT.

- a) To compare the molecular fingerprint of functionalized and non-functionalized MWCNT.
- b) To analyze the effect of functionalization on MWCNT.

Objective 2: Characterize the molecular fingerprint of prostate cancer cells using LNCaP spheroids as a model.

- a) To optimize laser irradiation power for detecting prominent biomolecules associated with LNCaP cells.
- b) To optimize the size of spheroid towards the detection of the molecular fingerprint of LNCaP cells.

Objective 3: Characterize CNT in LNCaP spheroids to probe the interactions of multi-walled carbon nanotubes and cells.

- a) To optimize laser irradiation power for characterizing CNT in LNCaP spheroids.
- b) To distinguish prominent Raman bands of CNT associated with LNCaP cells.
- c) To obtain the molecular fingerprint of CNT in LNCaP spheroids.

- d) To compare the molecular fingerprint of CNT conjugated with peptidomimetic (CNT-PEP) and polyethylene glycol (CNT-PEG) in LNCaP spheroids.

## **1.5 Organization of This Document**

This document consists of 4 chapters including an introduction. The introduction provides an overview of prostate cancer and current treatment approaches, as well as the hypothesis and research objectives of the thesis. Chapter 2 presents a review of literature covering the current state of confocal Raman microscopy, fundamentals of MWCNT, and characterization of MWCNT. Chapter 3 presents a proof-of-concept study demonstrating how a confocal Raman microscope can be optimized for imaging and characterizing CNT in live spheroids as well as probing the interactions of multi-walled carbon nanotubes and cells. Finally, chapter 4 concludes by highlighting the findings, addressing challenges during these studies, and proposing recommendations for future research directions.

## CHAPTER 2 REVIEW OF LITERATURE

This chapter consists of a brief overview of the current state of art in confocal Raman microscopy and its application in the characterization of MWCNT. The first section will cover the basic concepts of confocal Raman microscopy, including Raman spectroscopy and confocal microscopy. The second section will introduce readers to MWCNT, providing a basic understanding of its structure, properties, and functionalization. The third section will review studies on Raman characterization of MWCNT and discuss the prominent bands of MWCNT.

### 2.1 Confocal Raman Microscopy

#### 2.1.1 Raman spectroscopy

Raman spectroscopy is a spectroscopic technique that utilizes scattered light to determine the vibrational modes (vibrational Raman spectroscopy) or rotational modes (rotational Raman spectroscopy) of molecules. Rotational modes of molecules are pronounced when molecules in gas phase. For this review, the discussion is focused on vibrational modes of molecules which contributes significantly to the scattering processes observed in carbon nanotubes (crystalline solids) and biological molecules (molecular solids). Raman spectroscopy is named after an Indian physicist, Chandrasekara Raman, who first observed Raman scattering in 1928 with K.S.Krishnan [30]. Similar to other spectroscopic techniques, Raman spectroscopy consists of an excitation light sources (laser), filter (notch or edge), and a detector (CCD) for collecting scattered light. When an incident photon with an energy ( $E_{\text{incident}}$ ) interacts with a sample, it can either be reflected, absorbed, transmitted or scattered. For scattered photon, most energy of photon absorbed by the molecule is reemitted in the form elastic scattering with the same energy as the incident photon. This type of scattering is known as Rayleigh scattering ( $E_{\text{scattered}} = E_{\text{incident}}$ ). However, a small fraction of energy is reemitted in the form of inelastic scattering at high or lower energy than the incident photon [30], as show in Figure 2.1. Inelastic scattering (Stokes or Anti-Stokes) is commonly referred to as Raman scattering or Raman effect. Two theories are established to explain Raman scattering. Raman scattering caused by vibrational modes of molecules can be described by classical theory (molecular polarizability). Other properties of Raman scattering such as Raman intensities and relative dominance of Stokes Raman scattering over Anti-Stokes can be explained by quantum mechanics (transition between states).

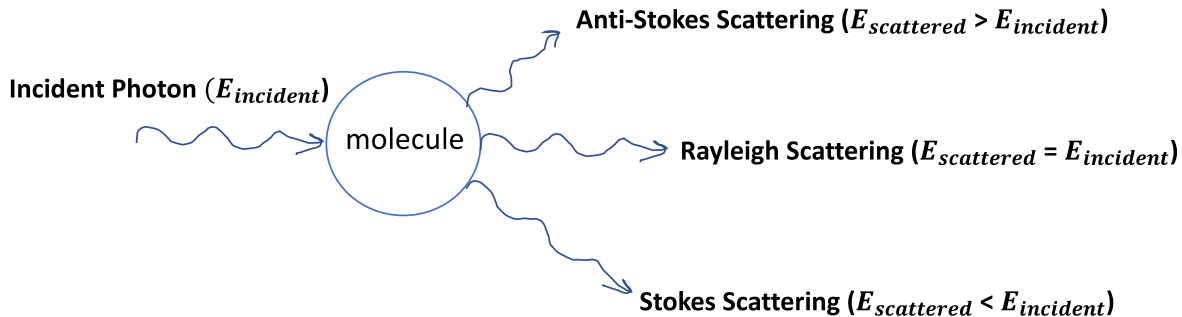


Figure 2.1 Representation of elastic scattering (Rayleigh) and inelastic scattering (Stokes and Anti-Stokes).

### 2.1.1.1 Classical Theory of Raman scattering: Molecular Polarizability

In 1934, George Placzek presented the classical derivation of Raman effect based on the process of an oscillating electric field interacting with polarizable molecule. When an electric field with amplitude  $E_0$  oscillate with frequency  $\omega_{rad}$  induces an electric dipole  $\mu$  proportional to the polarizability  $\alpha$  of the molecule, it can be expressed as equation 2.1. If the polarizability of the molecule changes during molecular vibrations and the vibration has frequency  $\omega_{vib}$  that changes the displacement  $x$  between atoms, the polarizability can be expand in Taylor series to obtain the equation 2.2.  $\phi$  is the phase of the vibration,  $t$  is time, and  $\frac{\partial \alpha}{\partial x_{x=0}}$  is the derivative of molecule's polarizability. Applying equation 2.2 to equation 2.1 which can be rewrite as equation 2.3. The Raman scattering occurs when the induced dipole that oscillates with a frequency  $\omega^4$  emits radiation along an angle  $\theta$  as described in equation 2.4. The intensity of Raman scattering decreases with the fourth power of the incident wavelength.

$$\mu = \alpha E_0 \sin(\omega_{rad} t) \quad (2.1)$$

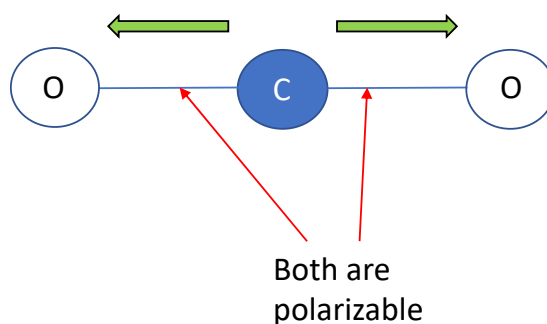
$$\alpha = \alpha_0 + \frac{\partial \alpha}{\partial x_{x=0}} x_0 \sin(\omega_{vib} t + \phi) \quad (2.2)$$

$$\mu = \alpha_0 E_0 \sin(\omega_{rad} t) + \frac{E_0}{2} \frac{\partial \alpha}{\partial x_{x=0}} x_0 (\cos [(\omega_{rad} - \omega_{vib})t - \phi] - \cos[(\omega_{rad} + \omega_{vib})t + \phi]) \quad (2.3)$$

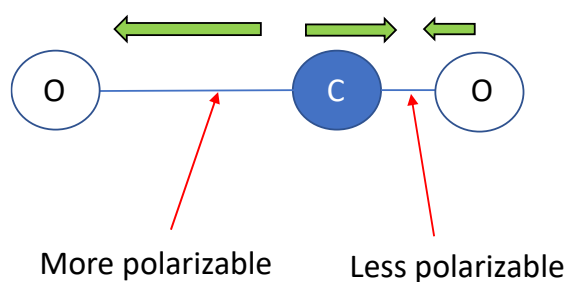
$$I = \frac{\omega^4 \mu_0^2 \sin^2 \theta}{32 \pi^2 \epsilon_0 c^3} \quad (2.4)$$

Equation 2.3 reveals different light scattering phenomena. For Rayleigh scattering,  $\sin(\omega_{rad}t)$  term describes the scattered radiation with a frequency equal to incident light. The term  $(\cos [(\omega_{rad} - \omega_{vib})t - \phi])$  corresponds to Stokes scattering, describes a decrease in frequency of scattered radiation, indicating the scattered radiation has lost energy equal to the molecular vibrations. The term  $(\cos [(\omega_{rad} + \omega_{vib})t + \phi])$  that corresponds to Anti-Stokes describes an increase in frequency of scattered radiation. For Stokes and Anti-Stokes Raman scattering to occur, the derivatives of the molecule's polarizability must be non-zero ( $\frac{\partial \alpha}{\partial x_{x=0}} \neq 0$ ) [28]. In other words, the molecular vibration must undergo a change in the polarizability of the molecule for Stokes or Anti-Stokes to occur. There is a set of selection rules to determine if a vibrational transition cause a change in the polarizability of the molecule. For illustration, carbon dioxide ( $CO_2$ ) is used as an example.  $CO_2$  is a linear molecule and has four possible vibrational modes (stretching and bending) as shown in Figure 2.2. However, not all modes of its vibrations are Raman active. To be Raman active, the electron clouds of the molecule must be distorted during vibrations. Symmetric stretch of  $CO_2$  involves changes in bond length when both oxygen atom are pulled away from the carbon atom, electron clouds are distorted, thus bonds are said to be more polarizable and this mode of vibration is Raman active. In contrast, the bending of  $CO_2$  is not Raman active because electron clouds remain undistorted due to no changes in bond length, thus the polarizability of the molecule remains unchanged. The asymmetric stretching is also not Raman active because one bond is stretched while the other bond is compressed. The change in length of the longer bond cancels out the shorter bond such that overall polarizability of the molecule does not change. It is important to note that not all bending mode of a molecule is Raman inactive and it depends on the geometries of the molecules. The concept of molecular polarizability is crucial for understanding inelastic scattering of biological molecules (lipids, proteins, and nucleic acids).

a)



b)



c)

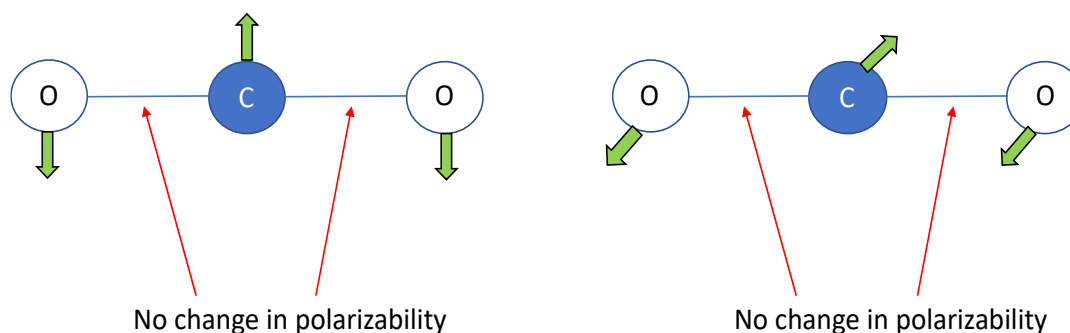


Figure 2.2 Representation of four vibrational modes of  $CO_2$  a) symmetric stretch (Raman active); b) asymmetric stretch (Raman inactive); and c) Two modes of bending (Raman inactive).

### 2.1.1.2 Quantum Mechanical Description of Raman scattering: Transition between States

Quantum mechanics describes Raman scattering as a transition between states. The transition between states can be explained by Jablonski energy level diagram of Rayleigh, Stokes, Anti-Stokes scattering as shown in Figure 2.3. When an incident photon of a certain energy  $E_{incident}$  interacts with a molecule, the  $E_{incident}$  is absorbed by the molecule, excites it from ground vibrational state (ground state) to a virtual state. From the virtual state, the molecule decays by releasing the scattered photon with an energy  $E_{scattered}$ . The type of scattering will depend on the energy change between scattered and incident photon. Most molecules will decay back to the ground vibrational state. The scattered photon produced will be equal in energy (wavelength) to the incident photon, this type of scattering is known as Rayleigh scattering [31]. However, molecules may also decay into an excited vibrational state. The scattered photon can lose energy equal to that of the vibrational state and increase in wavelength, known as Stokes scattering [31]. If the molecule is already in a vibrationally excited state before the photon incident on it, the virtual state

produced by this interaction has more energy. The photon scattered by the molecule gains energy and decreases in wavelength, this is referred to as Anti-Stokes scattering [31].

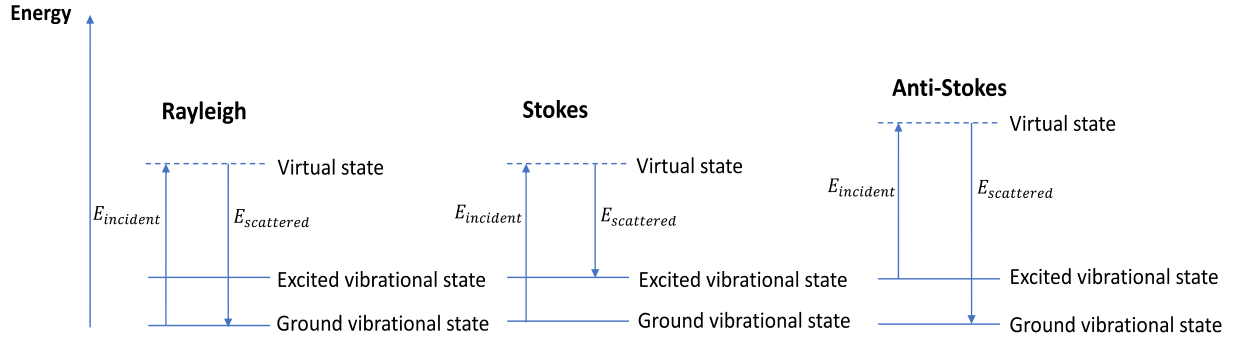


Figure 2.3 Jablonski energy level diagram of Rayleigh, Stokes, and Anti-Stokes scattering.

In quantum mechanics, the Fermi's golden rule provide a mathematical description of the transition rate between two states mediated by a transition dipole [31]. Similar to dipole moments, transition dipole refers to a difference in charge from one region of a molecule to another upon excitation of electron. By applying Fermi's golden rule to this transition, the rate of Raman scattering can be written as equation 2.5.  $e >$  is the arbitrary excited electronic state,  $\delta(\epsilon_f)$  is the density of final states,  $\omega_{rad}$  is the frequency of incident light,  $\omega_{ei}$  is the frequency of ground to excited state, and  $\Gamma_e$  is the lifetime of excited state before decaying to initial state. Raman scattering cross-section  $\sigma'$  can be used to express the intensity of scattered light as a function of the incident irradiation  $J$  and number of molecules irradiated as shown in equation 2.6.

$$R_{|i>\rightarrow|f>} \approx |\langle f | d_{|i>\rightarrow|f>} | i \rangle|^2 \approx \sum_e \left| \frac{\langle f | \mu_{ef} | e \rangle \langle e | \mu_{ie} | i \rangle}{\omega_{rad} - \omega_{ei} + i\Gamma_e} \right|^2 |E_0|^2 \delta(\epsilon_f) \quad (2.5)$$

$$I = \sigma' J N \quad (2.6)$$

Equation 2.5 shows that the rate of transition  $R_{|i>\rightarrow|f>}$  (from initial state  $|i >$  to final state  $|f >$ ) is proportional to electric amplitude of incident light  $|E_0|^2$ , indicating that Raman scattering scales linearly with the incident intensity [31]. Two process of Raman scattering can be seen in the numerator of equation 2.5. The term  $\langle e | \mu_{ie} | i \rangle$  describes the transition from the ground state to

the virtual state, and  $\langle f | \mu_{ef} | e \rangle$  describes the transition from the virtual state to the final state. Molecule is not stable in this virtual state, so scattering process occurs rapidly [31]. Most molecule tend to occupied the lowest possible vibrational state which explains why Stokes scattering is dominant over Anti-Stokes which requires molecule to be already in an excited vibrational state when incident photon arrives. Equation 2.5 also shows that Raman scattering relies on a transition through a virtual state facilitated by transition dipole  $d_{|i\rangle \rightarrow |f\rangle}$ . When an electron is excited from ground state to a virtual state, the charge distribution of a molecule changes from one region to another, known as transition dipole. The selection rules for Raman-active vibrational modes in quantum mechanics are based on transition dipole,  $\mu_{ef}$  or  $\mu_{ie}$ . Vibrational mode that contains one or more quadratic components are likely to induce transition dipole and describes as Raman-active. For instance, the symmetric stretch of  $CO_2$  is Raman active because it transforms as two quadratic components  $x^2+y^2$ , and  $z^2$ . In crystalline solids like carbon nanotubes, Raman scattering occurs through transition between states are pronounced.

### 2.1.1.3 Raman Spectra

Raman spectroscopy detects the energy shift between the incident and scattered photons associated with the Stokes and Anti-Stokes transitions. The energy shift between incident and scattered photons, commonly known as Raman shift, is measured in wavenumber which have units of inverse length ( $cm^{-1}$ ) and is described by equation 2.7. A typical Raman spectrum is shown in Figure 2.4, where Raman intensity is plotted against wavenumber. The spectrum is symmetric to the Rayleigh line which has zero Raman shift. It splits the Stokes (positive Raman shift) and Anti-Stokes line (negative Raman shift) apart. The Stokes line has a greater intensity than the Anti-Stokes line. Normally, only Stokes scattering is measured in Raman spectra which explains why most spectrum display positive Raman shift. The Raman shift observed corresponds to the type of bond and coordination of the molecules involved in the scattering process, containing encoded vibrational molecular information which is often described as the molecular fingerprint of the molecule [32].

$$Raman\ shift = \frac{1}{\lambda_{incident}} - \frac{1}{\lambda_{scattered}} \quad (2.7)$$

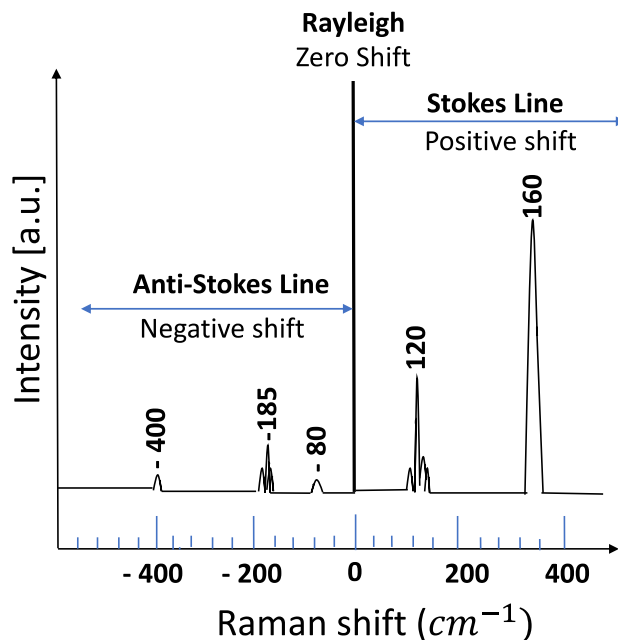


Figure 2.4 Typical Raman spectrum (Anti-Stokes, Rayleigh, Stokes).

### 2.1.2 Confocal Raman microscopy: integrate Raman spectroscopy with confocal microscopy

Raman spectroscopy is a powerful analytical technique that has been applied to various fields from chemical analysis to clinical diagnosis. More importantly, it is label-free and non-destructive, offers insight into the structure and composition of tissues and cells at molecular level [33]. The major drawback of Raman spectroscopy is that the scattering cross section is very small, results in very weak levels of Raman scattering in comparison to Rayleigh scattering. Approximately, one photon is Raman scattered for  $10^6 - 10^{10}$  excitation photons incident on the sample. Endogenous fluorescence from biomolecules, on the other hand, can be six orders of magnitude higher than Raman scattering signals [33]. If a sample shows significant fluorescence, the Raman intensity can be overpowered by fluorescence intensity, making it difficult to isolate Raman contributions [33]. Therefore, it can be useful to reduce the fluorescence background, either by using an excitation wavelength that minimizes background fluorescence from the sample or by reducing the detection volume [33]. It is not always possible to find such excitation wavelengths due to the high efficiency of the fluorescence process compared to Raman scattering [33]. In these circumstances, a confocal

pinhole or slit aperture could limit the collection volume to the exact area of interest in the focal plane, reducing the fluorescence signal from the sample surrounding the focal plane, thereby improving the quality of Raman spectrum generated [34]. A confocal setup also allows an optical cross-section analysis of biological samples in three dimensions without cutting the sample in half, making it suitable for characterizing cell aggregates or tissue samples [30]. The versatility of confocal setup led to the development of the confocal Raman microscope.

Confocal Raman microscopy is a non-destructive technique that combines Raman spectroscopy and confocal microscopy for the visualization of molecular information over a defined sample area [30]. Figure 2.5 shows an optical layout of Renishaw inVia confocal Raman microscope system consisting of an excitation laser, a microscope (objective lens), a spectrometer (Rayleigh filters, slit, diffraction gratings), and a charge-coupled device (CCD) detector. When the collimated light beam from a laser is reflected by beam splitter mirrors, it is focused down onto the sample through the objective lens of the microscope. Light interacts with the chemical bond of the sample and a small fraction of Rayleigh and Raman scatter induced from the sample, which are then collected through the objective lens, and transmits them through the beam splitter mirrors and passes through the Rayleigh filters to remove the Rayleigh scatter. The remaining Raman scatter is guided by a pre-slit lens and goes through the slit, where it is diffracted. Diffracted Raman scatter is then guided by a post-slit lens before entering the diffraction gratings. In the diffraction gratings, the wavelength of scatter is separated and the spectrum is detected by an array detector (charge-coupled device). During Raman mapping, the sample is scanned in the XYZ directions on a motorized microscope stage and acquires an array of Raman spectra which are then used to generate three dimensional Raman spectral maps of the sample.

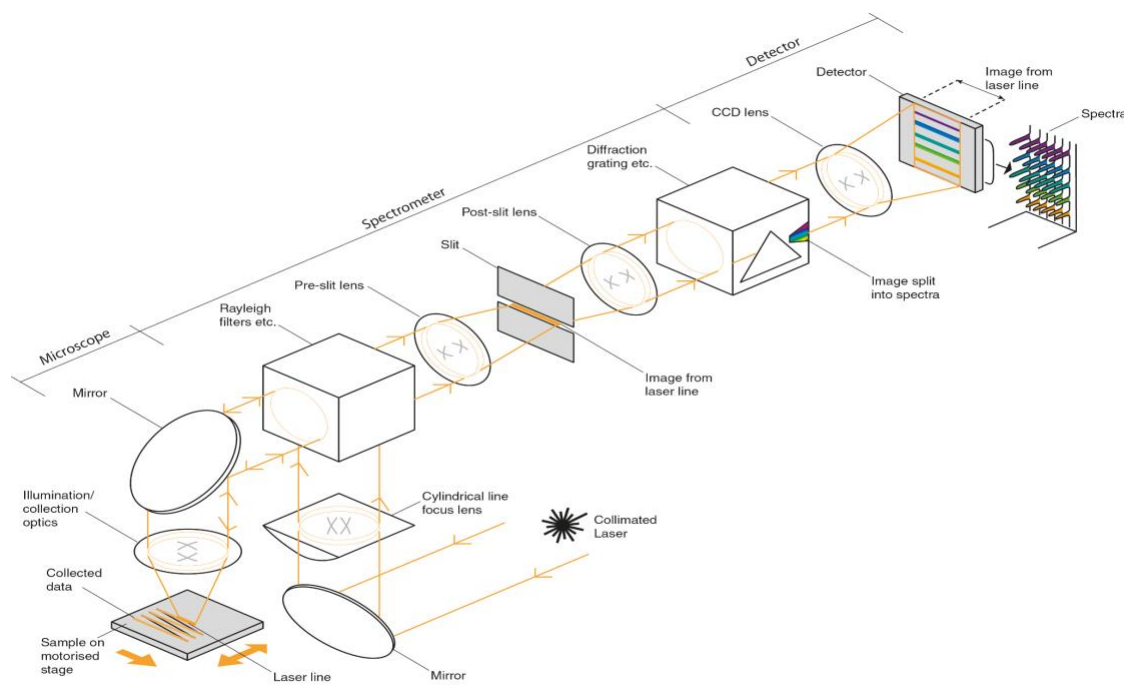


Figure 2.5 Optical layout of a confocal Raman microscope (Adapted from [35]).

Components that make up a confocal Raman microscope system are essentially the laser, numerical aperture, slit, diffraction gratings, and charge-coupled device (CCD). Laser is used in confocal Raman microscopy to provide high intensity light to increase Raman scattering. Based on classical scattering theory, the intensity of scattered light (Raman intensity) is proportional to the negative fourth power of the excitation wavelength ( $\lambda$ ) of the laser as described in equation 2.8. Therefore, the Raman intensity increases as the excitation wavelength reduces. However, a shorter excitation wavelength, with increasing frequency and energy, could increase thermal absorption, thereby increasing the risk of sample damages. When light scattered from the sample is collected through the objective lens, it passes through the numerical aperture of the objective lens, where diffraction occurs. The resulting diffraction pattern is shown in Figure 2.6. A bright region in the center along with a series of concentric rings of decreasing intensity surround it known as Airy disk. This mosaic of Airy disks of the image is what is perceived by our eyes as light and dark. When the separation distance between two disks exceeded their radii, two individual peaks are formed as shown in Figure 2.6a, indicating that two images are resolvable. But as the gap between the two disks shrinks, two disks will start to approach each other as show in Figure 2.6b, before reaching the Rayleigh

limit. Rayleigh criterion requires that the distance between two-point sources of light to be farther apart so that the disks are resolvable. If two disks continue to approach, eventually the two disks overlap and peaks will merge into as shown in Figure 2.6c, resulting in two poorly resolved images. The numerical aperture of the objective along with the laser excitation wavelength determine the laser spot size as shown in equation 2.9. In theory, a higher excitation wavelength ( $\lambda$ ) yields a larger laser spot size. As the numerical aperture (NA) increases, the Airy disk becomes smaller and resolution of image detail increases, described by equation 2.10.

$$I \propto \lambda^{-4} \quad (2.8)$$

$$\text{Laser spot size} = \frac{1.22\lambda}{NA} \quad (2.9)$$

$$\text{Spatial resolution} = \frac{0.61\lambda}{NA} \quad (2.10)$$

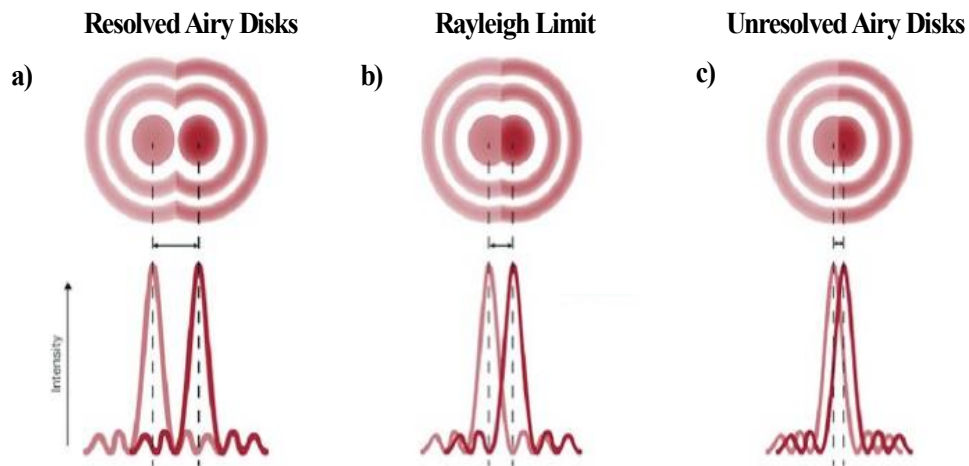
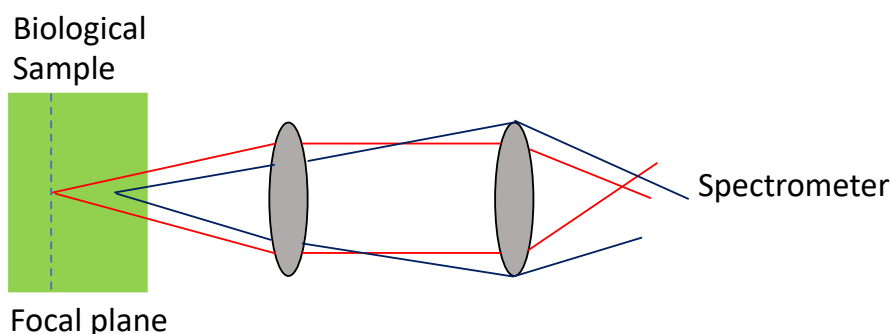


Figure 2.6 Illustrates the separation of Airy disk and Rayleigh criterion a) the separation between the two disks exceed their radii, thus they are resolvable; b) Two disks are not individually resolvable by Rayleigh criterion; c) two disks overlap and appear as one poorly resolved disk (Adapted from [36]).

Slit or confocal pinhole is the most prominent feature of a confocal microscope. Without a slit, both the in-focus Raman scatter (red) and out-of-focus Raman scatter (blue) enter the spectrometer

and would be detected by the CCD as shown in Figure 2.7a. On the other hand, the slit blocks the out-of-focus Raman scatter from entering the spectrometer as shown in Figure 2.7b. The slit serves as a spatial filter in the axial (Z) direction by blocking the Raman scatter from above and below the focal plane, allowing three-dimensional mapping. When a slit limits the light from entering, it also reduces the fluorescence background by blocking out-of-focus fluorescence. The pre-slit and post-slit lenses are used here to guide the light towards the entrance slit.

a)



b)

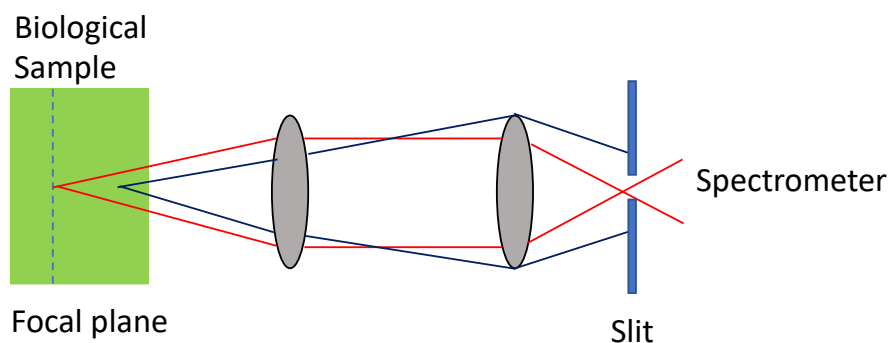


Figure 2.7 a) Without a slit, in-focus Raman scatter (red) and out-of-focus Raman scatter (blue) enter the spectrometer; b) Slit blocking out-of-focus Raman scatter (blue) and only allow in-focus Raman scatter (red) entering the spectrometer.

At the diffraction grating, Raman scatter is separated based on wavelengths and spreads evenly across the entire image plane on an array detector (CCD). The diffracted light causes destructive interference and reduces intensity, creating patterns of light and dark dots, as shown in Figure 2.8.

The number of patterns of light and dark dots also indicates the number of spectra. In Figure 2.8, the farther spacings of line gratings generate more Raman spectra. During Raman mapping, the diffraction grating scans across the detector in a series of steps. Charge generated on the CCD camera moves across the detector simultaneously with spectrum movement, this is called the extended scanning. All collected spectra will then be used to generate three dimensional Raman spectral maps of the sample.

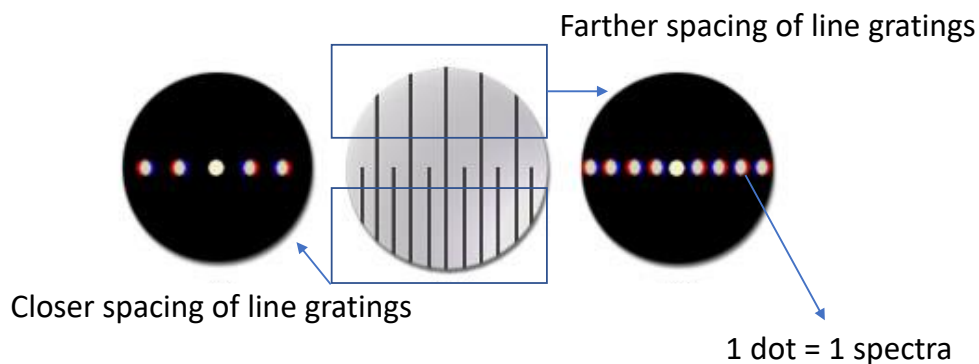


Figure 2.8 Illustrates that distance of spacing affects the number of spectra shown on a CCD detector. Farther spacings of line gratings increases the number of spectra (Adapted from [37]).

## 2.2 Multi-Walled Carbon Nanotubes: Structure, Thermal Properties, and Functionalization

Carbon atoms are remarkable elements that have the capability to bond together in various ways, forming allotropes. The properties of allotropes are attributed by their structures and can differ from one form of carbon to another. Graphene and carbon nanotubes are among the most common allotropes in the field of nanotechnology. Graphene is composed of a single sheet of carbon atoms tightly bound in a hexagonal honeycomb lattice [38]. The unique structure of graphene provides the building block for graphite and carbon nanotubes. Most common carbon nanotubes are single-walled carbon nanotubes (SWCNT) and multi-walled carbon nanotubes (MWCNT).

**Structure:** SWCNT are basically a graphene sheet seamlessly rolled up into a tube as shown in Figure 2.9a. Whereas MWCNT are made up of graphite, seamlessly rolled into a concentric tubular structure as shown in Figure 2.9a. The number of walls of MWCNT may vary, resulting in larger

diameter in comparison to SWCNT. Typically, the diameter of MWCNT ranges from 7 to 100nm, while the diameter of SWCNT ranges from 0.5 to 2.5nm. All the carbon atoms are in equivalent positions in hexagonal aromatic rings, except the atoms at the tips of the carbon nanotubes [39]. The carbon atoms in the rings are held together by alkene bonds (C=C), not confined within a single plane, due to hybridization with some  $sp^3$  character [39]. The  $sp^3$  bonding makes the surface of SWCNT or MWCNT more reactive than the planar graphene [39]. The length of SWCNT or MWCNT can vary from nanometer (nm) to micrometer ( $\mu\text{m}$ ) [39]. The atomic structure of MWCNT or SWCNT defined in terms of tube chirality ( $n, m$ ), specified by chiral vector ( $c_h = na_1 + ma_2$ ) and angle. Figure 2.9b shows how the graphite can be rolled into MWCNT through two common configurations, armchair ( $n=m$ , angle =  $30^\circ$ ) or zig-zag ( $n=0$  or  $m=0$ , angle =  $0^\circ$ ) based on the geometry of the carbon bonds around the circumference of the nanotube [37]. The configuration of metallic carbon nanotubes is either zig-zag or armchair, and non-metallic tend to be zig-zag [40].

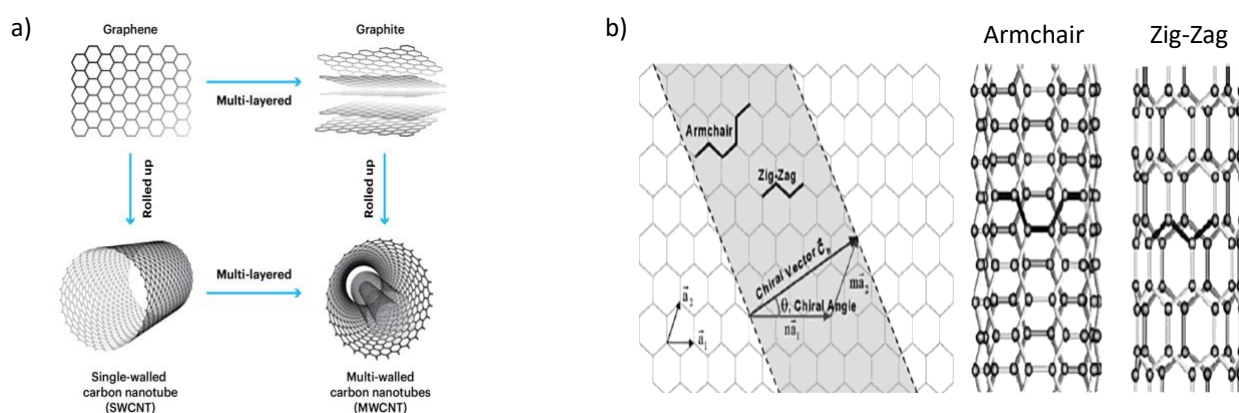


Figure 2.9 a) Illustration of the formation of SWCNT and MWCNT from graphene (Adapted from [33]); b) Schematic diagram showing how a hexagonal sheet of graphite is rolled to form a MWCNT via armchair or zig-zag configurations (Adapted from [41]).

**Thermal Properties:** Carbon nanotubes (SWCNT or MWCNT) are crystalline solids, a symmetrical and three-dimensional arrangement of atoms or molecules in repeating pattern. When light irradiate carbon nanotubes, the lattice of atoms or molecules experience a change in vibrational motion and oscillate uniformly at a different frequency. The quanta of energy for this lattice vibrations can be described as phonon. For metallic materials, the heat conduction occurs through the collision of

free-electrons within the lattice. In the case of most carbon nanotubes, the primary mechanism that drives the thermal energy in nanotubes is the phonon conduction. Heat conduction by phonons depends on three properties of phonons: 1) volumetric heat capacity of phonon modes, 2) group velocity, and 3) mean-free-path [42]. Volumetric heat capacity of phonon modes determines the amount of heat carried by phonons based on the frequency, polarization (longitudinal or transverse), and temperature. At high temperatures, the heat capacity of each phonon mode approaches the Boltzmann constant  $k_B = 1.38 \times 10^{-23} \text{ JK}^{-1}$  [38]. The group velocity of phonons determines the speed of propagation of phonons in the materials. It is a function of phonon frequency and polarization. At low frequencies, the group velocity of phonons approaches the speed of sounds of materials. Group velocity of phonons approach zero at high frequencies. Mean-free-path of phonons describes the average distance phonons propagate in materials without being scattered [38]. Most phonon modes (acoustic or optical) are excited at high temperatures, but only a small fraction of these excited modes carry the heat [38]. Acoustic phonons at the edge or center of Brillouin zone, and optical phonons are both inefficient in carrying heat due to their low group velocity or heat capacity [38]. Mean-free-paths of these acoustic phonons are not constant and vary depend on phonon frequency. Imperfections of materials such as impurities and point defects can scatter phonons, thereby slows down the heat conduction [38].

**Thermal Conductivity:** Recent studies showed that the thermal conductivity of MWCNT at 3000  $\frac{W}{m K}$  and thermal conductivity of SWCNT at 2000  $\frac{W}{m K}$  [41]. Direct and quantitative measurement of thermal transport properties of individual nanotube remains challenging due to its nanoscale size [37]. reported on thermal conductivity of carbon nanotubes are based on theoretical simulations [37]. The thermal conductivity of carbon nanotubes depends on 1) atomic arrangement, 2) diameter and length of the tubes, 3) structural defect, and 3) functionalization. The thermal conductivity of carbon nanotubes depends on its chirality which is mainly governed by the phonon [43]. In the range of 100 to 400K, the thermal conductivities of (10,13) nanotube was lower than (11,11) and (20,0) [39], suggesting that phonon travelling in (10,13) nanotube has lower group velocity or heat capacity compared to the two types of nanotube. Besides chirality, the diameter and length of carbon nanotubes are also important. The thermal conductivity of MWCNT at room temperature increased as its diameter of wall decreased, varied from about 500  $\frac{W}{m K}$  for an outer diameter of 28nm to 2069  $\frac{W}{m K}$  for a 10nm diameter [38]. When diameter of nanotube decreases, less optical

phonon participates in the scattering process (increases phonon polarization), which in turn enhanced volumetric heat capacity and group velocity [44]. The thermal conductivity of SWCNT was found to increase with the length of the tube from 6 to 404nm [41]. For MWCNT, the thermal conductivity increases with length up to 500nm. This phenomenon can be explained by the fact the length of nanotube is correlated to the mean-free-path of heat energy carrying phonons. Structural defects of nanotubes induced by vacancy (missing atoms), strain (change in length), or grain boundary (planar defect due to misorientation of crystallites), can reduce thermal conductivity by reducing mean-free-path of phonon through scattering. At 300K, the thermal conductivity of SWCNT (same diameter and length) decreases significantly as the vacancy defect concentration, strain (%), and grain boundary increases [45]. Functionalization allows carbon nanotubes to be tailored for specific applications. They can either obtain by covalent or non-covalent method. Covalent bonds can benefit phonon transfer between nanotubes. However, this method introduces side scatters electrons and phonons that can interferes with phonon. For non-covalent functionalization, the weak interactions between the wrapping molecules and carbon nanotubes, increases thermal resistance (decrease thermal conductivity of carbon nanotubes) [38]. In addition, radiative heat transfer between nanotubes is important because nanotubes do overlap when bind onto cells. Recent study found that the heat transfer is dependent on the distance between the nanotubes and chirality [40].

Functionalization: Their distinct external surfaces open up spaces for a varied range of functional groups to be added to the tips and walls of carbon nanotubes, known as functionalization. The MWCNT can be functionalized by the addition of a chemical group, linker, cross-linker, antibodies, and antigens as shown in Figure 2.10. Functionalization increases solubility and dispersibility of MWCNT aqueous solutions, enhances their biocompatibility for photothermal applications. MWCNT can be functionalized by either covalent (carboxylic acids known as COOH) or non-covalent (polyethylene glycol known as PEG) routes onto the wall of carbon nanotubes [35]. Covalent treatment adds to either the sides or tips of carbon nanotubes. Covalent bonds act as substitution sides for functionalization, increases its solubility in water and organic solvents [43]. Hydrophilic groups introduced onto the surface make nanotubes more biocompatible and biodegradable [46]. Non-covalent treatment involves adsorption or wrapping of various groups on the surface of carbon nanotubes without disrupting the  $\pi$  system of the graphene sheets [46]. This approach is based on interactions of the hydrophobic part of the adsorbed molecules with the

side wall of nanotubes through van der Waals,  $\pi - \pi$ , CH- $\pi$ , and other interactions. The hydrophilic part of the molecules provides the aqueous solubility. The ionic molecules adsorbed on the nanotube surface prevents its aggregation by coulombic repulsion forces [43].

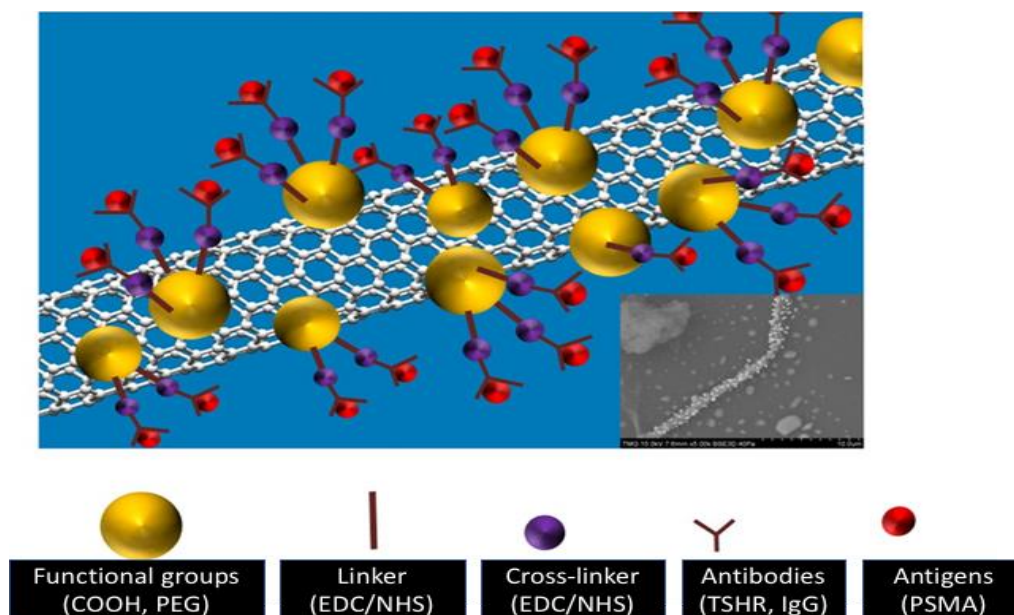


Figure 2.10 Illustration of the MWCNT conjugated with functional groups (gold), linker (dark red), cross-linker (purple), and antibodies(y-shaped) onto the surface of carbon nanotubes. Antigens (bright red) are captured by the antibodies (Adapted from [35]).

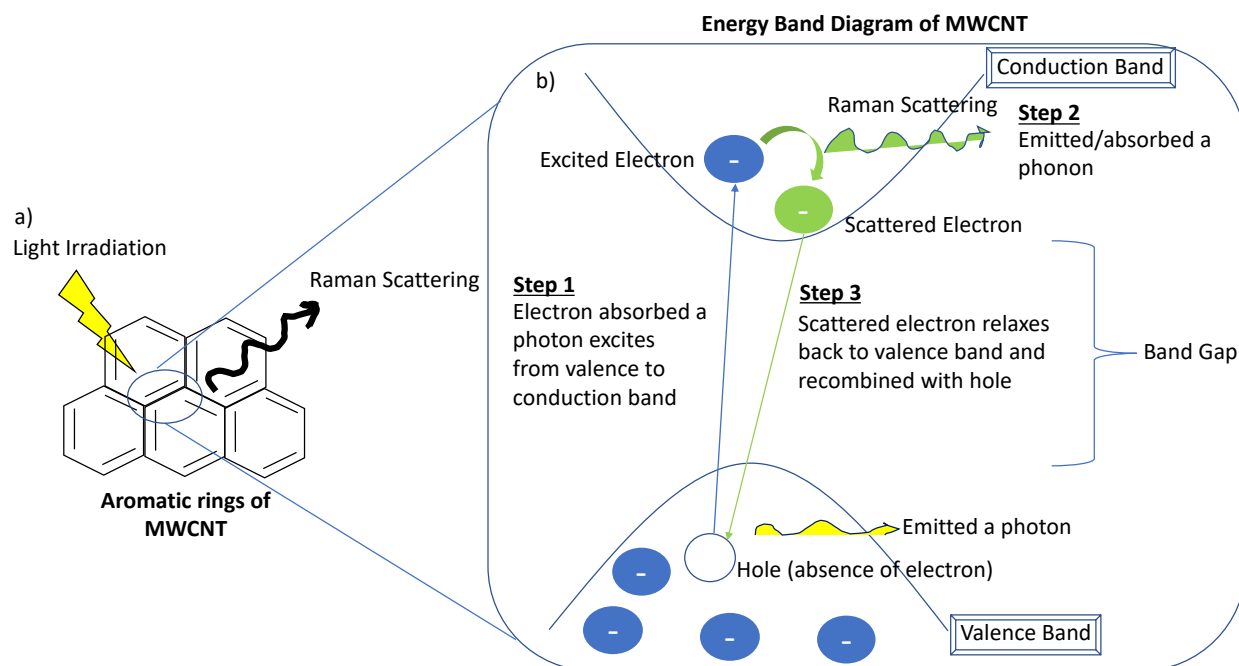
## 2.3 Raman Characterization of MWCNT

There are several techniques that have been used to characterize the degree of the MWCNT, such as Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), UV-Vis spectroscopy, and Raman spectroscopy [47]. Raman spectroscopy yields information about the purity, defects, structure, and dispersions of CNTs. This can be evaluated by examining the position of bands (Raman shift), associated vibrational features (band frequencies and widths), and intensity of bands.

### 2.3.1 Raman scattering in MWCNT

The Raman scattering occurs in carbon nanotubes can be explained using quantum mechanics. Since carbon nanotubes (SWCNT or MWCNT) are crystalline solids, we have to consider phonon

besides electron and photon. Inelastic scattering of light also involves other elementary excitation (plasmon or magnon), but Raman scattering process in MWCNT or SWCNT are contributed mostly by phonon. Hence, the following discussion will be focused on inelastic scattering of light by phonon. The optical transition in the Raman scattering process of MWCNT or SWCNT can be understood as a transition between states as described in quantum mechanics. Instead of having discrete energies as in the case of free atoms, the energy states in solids are often described as “band”. Band is formed when discrete molecular orbitals overlaps. Valence band is the outermost electron orbital of an atom, whereas the conduction band is the band of electron orbital that electrons can excite to. Upon light irradiation on MWCNT, the photons absorbed by electrons of aromatic rings as shown in Figure 2.11a. These electrons also absorb the energy of the photons, that cause them to jump from the valence band to the conduction band, leaving a hole in the valence band as shown in Figure 2.11b. The excited electron in the conduction band is then scattered by emitting phonon (Stokes) or absorbing phonon (Anti-Stokes). After scattering event, the electron relaxes to the valence band and recombine with the hole by emitting a photon. This physical phenomenon describes the scattering that occurs in carbon nanotubes, which contributes to Raman bands of MWCNT or SWCNT observed in the spectra.



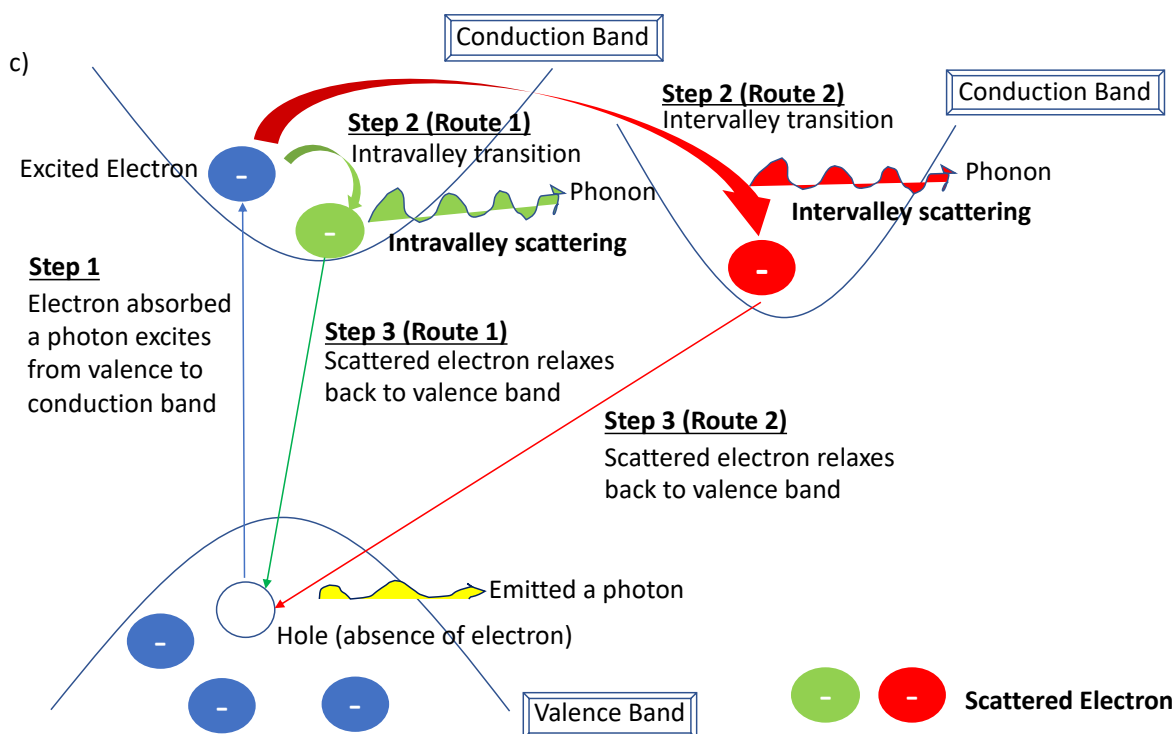


Figure 2.11 Raman scattering process of MWCNT a) Light irradiation on aromatic rings of MWCNT; b) Energy band diagram of MWCNT. Raman scattering occurs when excited electron in conduction band of MWCNT scattered by emitting a phonon (Stokes) or absorbing a phonon (Anti-Stokes) before relaxes back to valence band; c) Two types of scattering that can occur: Intravalley scattering (within same conduction band valley) or intervalley scattering (different conduction band valley) (Adapted from [53] and [84]).

Raman scattering processes of carbon nanotubes can be classified by 1) phonon modes that are involved, 2) order of scattering process, 3) Stokes or Anti-Stokes, 4) one-phonon or two-phonon, and 5) intravalley or intervalley. The phonon mode can be described as optical (vibrations with neighboring atoms in anti-phase) or acoustic (long-wavelength vibrations with neighboring atoms in phase) [46]. This mode can be predicted by group theory once the lattice structure and its symmetry are specified. Number and symmetries of Raman-active modes for different types of carbon nanotubes are described in [48]. The order of a scattering process is defined by the number in the sequence of total scattering events, including elastic scattering. Elastic scattering (Rayleigh scattering) involves a change of photon direction but no frequency shift. The lowest order process is the first-order Raman scattering process which involves one-phonon emission. The emission of

a phonon is known as Stokes shift and absorption of a phonon is described as Anti-Stokes shift, which appear in the Raman spectra, on the lower and higher frequencies respectively. Second-order scattering consists either two phonon scattering events or one-phonon and one-elastic scattering event. Two-phonon scattering events may involve same phonon modes (overtone mode) or different phonon modes (combination mode). Intravalley transition refers to process where the initial and final conduction band valley of the scattered electron is the same as shown in Figure 2.11c. Intervalley transition takes place between different conduction band valleys. In the first-order Raman process, only intravalley scattering is possible. For second-order Raman process, both intravalley and intervalley scattering are possible.

### **2.3.2 Interpretation of Raman spectra of MWCNT**

In Raman spectra of carbon nanotubes, there are three distinct peaks (radial breathing mode, D-band, G-band) as shown in Figure 2.12. Radial breathing mode and G-band are both first-order Raman scattering process. The radial breathing mode also known as RBM, involves one-phonon emission (Stokes) from the scattering of excited electron. This mode is originated out-of-plane bond-stretching, in which all the carbon atoms vibrate in phase along the radial direction. RBM typically observed in carbon nanotubes appears on the low frequency region ( $100 - 600 \text{ cm}^{-1}$ ) of the Raman spectra and is inversely proportional to the tube diameter. The intensity of RBM drops significantly as tube diameter increases as shown in Figure 2.12. The RBM bands are originated from the narrow innermost carbon nanotubes [49]. As the walls of carbon nanotubes increases, innermost tube is buried within layers of tubes bonded by van der Waals forces, hindering the photon-phonon interaction, thereby reduces the phonon emission from the innermost tube.

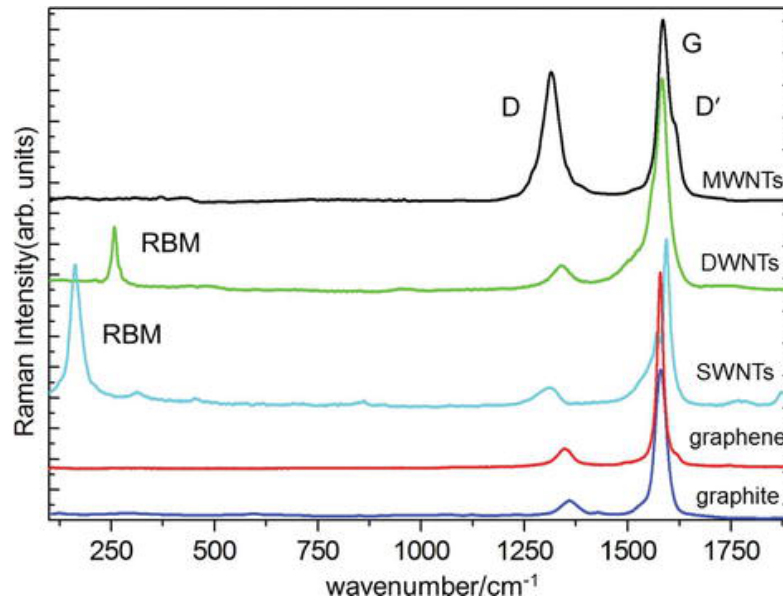


Figure 2.12 Raman spectra of different carbon forms: graphite, graphene, SWCNT, DWCNT, and MWCNT (Adapted from [50]).

Another first-order Raman scattering, G-band also involves one phonon emission (Stokes) from the scattering of excited electron and is derived from the in-plane vibration of the  $sp^2$  bonded carbon in aromatic ring of carbon nanotubes upon light irradiation. It is the primary Raman active mode in graphite and is observed in high-frequency region ( $1550 - 1600 \text{ cm}^{-1}$ ) of MWCNT. The G-band modes are composed of both G-band modes (linewidth  $4 \text{ cm}^{-1}$ ) from the innermost carbon nanotubes and graphite-like mode (linewidth  $20 \text{ cm}^{-1}$ ) from the outer cylinders in the carbon nanotubes [51]. The G-band modes has two features,  $G_+$  and  $G_-$ , that is due to confinement of the vibrational wave in the circumferential direction [52].  $G_+$  band is attributed by the atomic displacements along the carbon nanotubes axis and it is independent of the diameter. In comparison,  $G_-$  band is caused by the circumferential atomic displacement related to the diameter of carbon nanotubes. However, the assignment of  $G_+$  and  $G_-$  bands of MWCNT still remain a challenging task because the characterization of  $G_+$  and  $G_-$  bands have only been extensively focused on SWCNT. In Figure 2.12, the intensity of G-band (graphite-like mode) increases with number of walls. This band is contributed by the phonon emission from the outer cylinder. D-band is a second-order Raman scattering process that involves intervalley transition, where two phonons or one phonon and one defect participate in the scattering of the excited electron [53]. It is observed

at around  $1340\text{ cm}^{-1}$  and is strongly influenced by structural defects (geometrical distortion or atomic vacancy) in carbon nanotubes. This mode is exhibited on the edge of a graphene sheet or the open end of the carbon nanotubes where defects of tube are normally found [54]. The D-band is observed strongly in MWCNT than SWCNT, as structural defects increases with number of walls. In the case of MWCNT, structural defects on the wall of MWCNT promote the participation of one phonon and one defect in the scattering of excited electron.

The ratio of the Raman intensity of the D-band to the Raman intensity of G-band ( $\frac{I_D}{I_G}$ ) can be used to assess the quality or defect densities of carbon nanotubes as shown in Figure 2.13. The higher D/G ratio, the more defects the carbon nanotubes might have. One of the major parameters that influence the properties of the MWCNT is the distribution or dispersion of the MWCNT in conjunction with their weight percentage loading in the polymer matrix [47]. Alterations in the spectral features of the carbon nanotubes bands can be used to evaluate the dispersion of MWCNT [47]. Shifts of Raman bands of MWCNT have also been linked to residual stresses imposed by sample crystallinity or laser heating [47]. In fact, Kao et al. [55] observed a shift of a prominent carbon nanotube band (G') up to  $5\text{ cm}^{-1}$  between the pristine and composite carbon nanotubes, for a single case of 0.1% loading weight, was attributed by dispersion of carbon nanotubes [55].

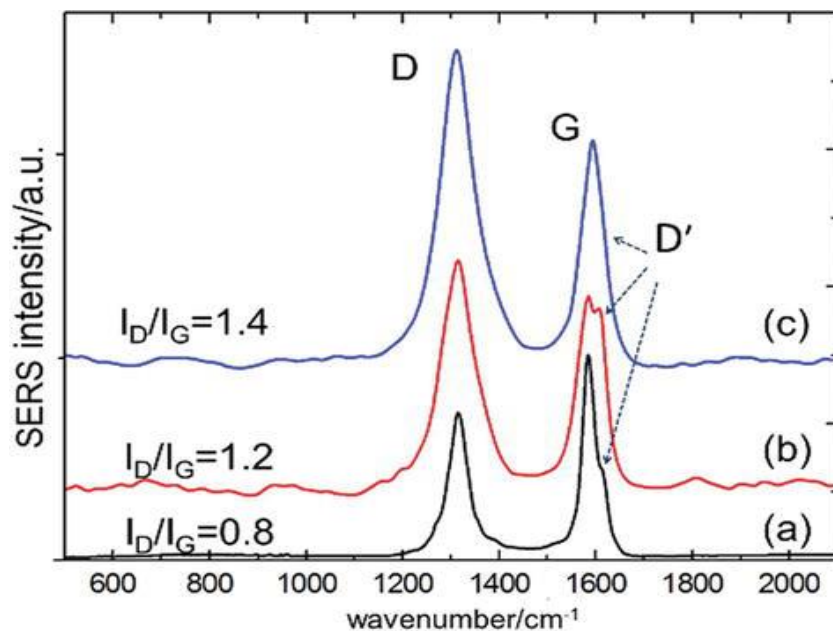


Figure 2.13 Raman spectra of a) pristine MWCNT,  $\frac{I_D}{I_G} = 0.8$ ; b) COOH functionalized MWCNT,  $\frac{I_D}{I_G} = 1.2$ ; c) functionalized MWCNT,  $\frac{I_D}{I_G} = 1.4$  (Adapted from [50]).

Bounos et al. [47] showed that the intensity of D and G-band increases with the excitation laser power as shown in Figure 2.14. The frequency of D and G-band also shifted as the laser power increased as shown in Figure 2.14a. Figure 2.14b shows that for each weight percentage loading, there is a laser power threshold (dotted line) above which the melting of the matrix of carbon nanotubes is visually and spectroscopically observed. Below that threshold (dotted line), the G-band frequency shifted with respect to the laser power. At a low laser power, the frequency of the G-band is independent of the weight percentage loading of MWCNT and remains the same as the pristine MWCNT.

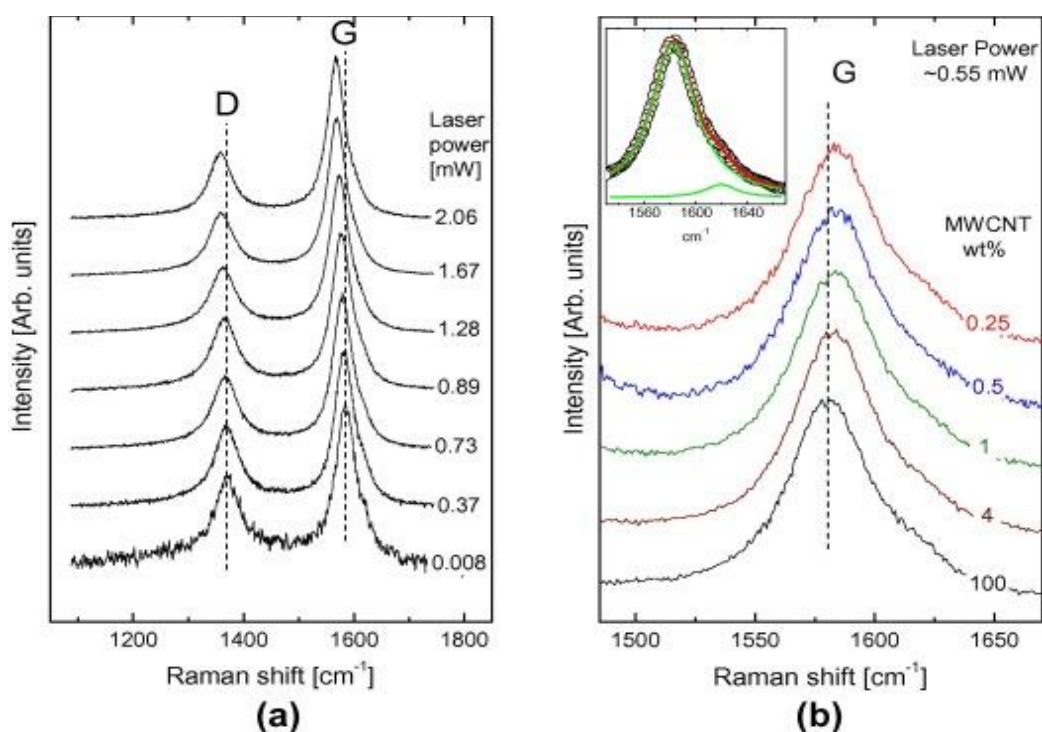


Figure 2.14 Raman spectra of a) pristine multi-walled carbon nanotubes at different excitation laser powers (excitation wavelength 441.6 nm); b) pristine multi-walled carbon nanotubes and composite films containing different wt% loading obtained at laser power of 0.55W (Adapted from [37]).

The functionalization of MWCNT changes the D and G-band features. The functional group (nitric acid) generates a small slope at the G-band of multi-walled carbon nanotubes [56]. Rebelo et al.

[5] investigated the Raman analysis of covalently diazonium functionalized multi-walled carbon nanotubes and oxidation functionalized MWCNT. There is a systematic band broadening effect and higher valley lines between the D ( $1325\text{ cm}^{-1}$ ) and G ( $1585\text{ cm}^{-1}$ ) bands, upon diazonium functionalization. The relative intensity of the D and G-bands did not change upon diazonium functionalization. It was unexpected because the chemical functionalization tends to increase D-band intensity. However, the intensity of D and G of MWCNT increased upon oxidation functionalization. The intensity of D-band increased significantly with the number of walls of multi-walled carbon nanotubes [5].

## 2.4 Raman Characterization of Cells: Biological Molecules

### 2.4.1 Raman scattering of biological molecules

The Raman spectra of cells and tissues are contributed by vibrational modes in biological molecules such as lipids, protein, and nucleic acids molecules. In essence, the Raman scattering process in cells or tissues is based on the change in polarizability during the molecular vibration. It is different from the Raman scattering process in MWCNT or SWCNT which occurs through transition between different energy states. The concept of molecular polarizability can be explained using the classical theory discussed in the earlier section. For illustration, the vibrational modes correspond to the backbone in nucleic acids is used here as an example. Figure 2.15 shows the vibrational modes of the backbone in nucleic acids observed in Raman spectra. When light irradiates cells, the energy of photons is absorbed by the backbone of nucleic acids causing the molecule to vibrate in different modes. The phosphate group of nucleic acids can either stretch symmetrically via  $PO_2^-$  ( $1096\text{ cm}^{-1}$ ) or O-P-O ( $783\text{ cm}^{-1}$ ). Both vibration modes distort the electron clouds of the molecule, induces electric dipole. Raman scattering occurs when the induced dipole oscillates with a frequency and emits radiation along an angle. The same principle also applies to other vibrational modes.

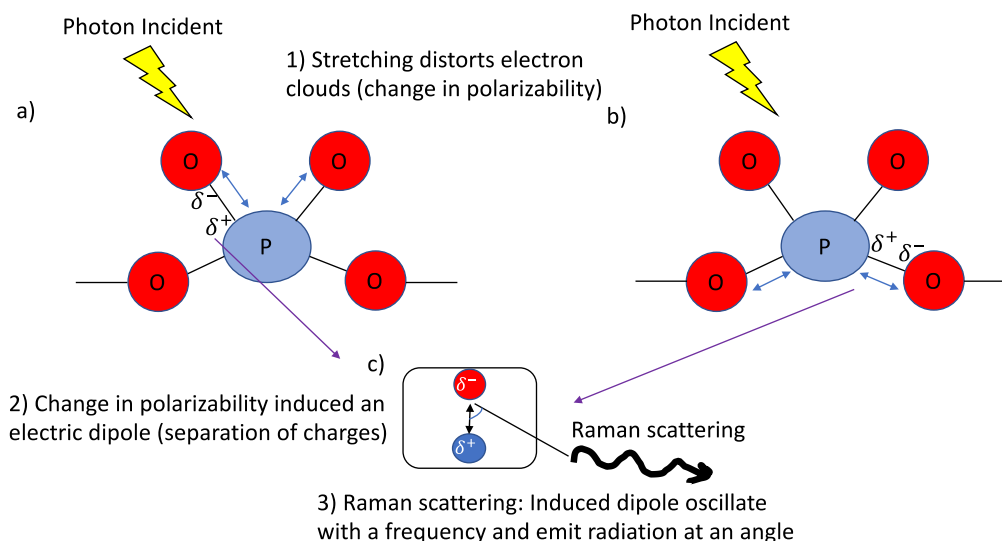


Figure 2.15 Vibrational modes of the backbone of nucleic acids a)  $PO_2^-$  symmetric stretching with the band near  $1096\text{ cm}^{-1}$ ; b) O-P-O symmetric stretching near  $783\text{ cm}^{-1}$ ; c) Raman scattering of biological molecules: (1) Stretching distorts the electron clouds causing a change in polarizability; (2) Change in polarizability induce an electric dipole; (3) Induced dipole that oscillates at a frequency emits radiation (Raman scattering) along an angle.

## 2.4.2 Interpretation of Raman spectra of biological molecules

The spectra are divided into three spectral regions (fingerprint, silent, high wavenumber). The fingerprint region covers the range between  $600$  and  $1800\text{ cm}^{-1}$ . This region consists of sharp and localized spectral features that give molecules unique “fingerprints”. Fingerprint region is contributed by major biological molecules such as nucleic acids, proteins, and lipids. The molecules that are involve in this region are larger atoms (carbon, nitrogen, oxygen) or complexes of several hydrogen atoms. Most prominent bands in nucleic acids originate from the backbone of nucleic acids which are located near  $1095$  and  $785\text{ cm}^{-1}$ . The  $1095\text{ cm}^{-1}$  band is associated with the symmetric stretching of the  $PO_2^-$  in the backbone and  $785\text{ cm}^{-1}$  is assigned to either symmetric stretching along the O-P-O backbone or ring breathing in the cytosine, thymine, and uracil bases. Since proteins have the same amino-acid backbone molecular structure, they contain a class of vibrations known as Amide modes. These modes consist of complex combinations of motions from different parts of the backbone and are not usually referred to by a specific chemical bond but rather spectroscopic label (Amide I, II and III). Amide I and III modes are commonly observed in Raman

spectra. The relatively large range of frequencies of the Amide I and III vibrations is due to varied local molecular environment in which backbone unit can be in. Many different proteins in cells or tissues have a mix of secondary structure, thus resulting in broad amide bands. In addition, majority of the side-chain groups have similar composition to many other biomolecules such as aliphatic side chains. The vibrations of this side-chains can overlap with common  $CH_2$  and  $CH_3$  bands in Raman spectra, makes it harder to be distinguished. Fortunately, side-chains do produce distinct bands in spectra. For instance, the sharp band observed in cell and tissue spectra at  $1000 - 1006\text{ cm}^{-1}$ , is assigned to the symmetric ring breathing mode of phenylalanine. Other side-chain groups that are visible are tryptophan and tyrosine. The side-chain groups can either be charged, polar, hydrophobic, or contain certain atoms such as sulfur which can form disulfide bonds. The interaction of side-chain groups and the backbone determines the secondary structure. Therefore, proteins are sensitive to the solvent and pH environment. Solvents like phosphate buffered saline (PBS) is used a lot in biological research to maintain a constant pH level of 7.4 that matches pH in our human body [55]. Generally, PBS is relatively dilute so there is less chances to form hydrogen bonding with molecules. Even if it does, its molecular fingerprint can easily distinguish from molecules due to its low Raman intensity. Water made up about 75% of the fluid outside of the cells in human body, tend to form hydrogen bonding within themselves and also with molecules, give rise to broad intense bands [55]. Fortunately, these bands are found in high-wavenumber region ( $2400 - 4000\text{ cm}^{-1}$ ), which is far from the fingerprint region [57]. Another common class of biological molecules are lipids which are common in every cell type and fatty tissue. Phospholipids (lipids with phosphate groups at their heads), are the primary molecules in membranes including the cellular, nuclear, and mitochondrial membranes. In Raman spectra, the lipid molecule is dominated by the large number of bonds in the hydrocarbon chain. The most prominent bands of lipids,  $CH_2$  scissors bending and stretching of  $C=C$  double bonds, are located near  $1440 - 1650\text{ cm}^{-1}$ . A complete list of nucleic acids, proteins, and lipids bands are described in [58]. Silent region is the intermediate region between the fingerprint and the high wavenumber region, from  $1800$  to  $2500\text{ cm}^{-1}$ . Most biological molecules do not contribute to this region. The high wavenumber region is contributed by higher energy vibrations, from  $2500$  to  $3400\text{ cm}^{-1}$ . These vibrations involve independently vibrating hydrogen atoms that requires higher energies than other bonds.

## 2.5 Raman Characterization of MWCNT in Cells

Bhirde et al. [59] were the first group to show that both SWCNT and MWCNT can be selectively irradiated and detected in cancer cells using a portable Raman instrument (785 nm laser). The SWCNT and MWCNT treated cells showed the characteristic G peaks,  $G_+$  at  $1600\text{ cm}^{-1}$  and  $G_-$  at  $2600\text{ cm}^{-1}$ , as shown in Figure 2.16. The intensity of G peaks of MWCNT was significantly lower than SWCNT due to the laser power of the portable Raman instrument.

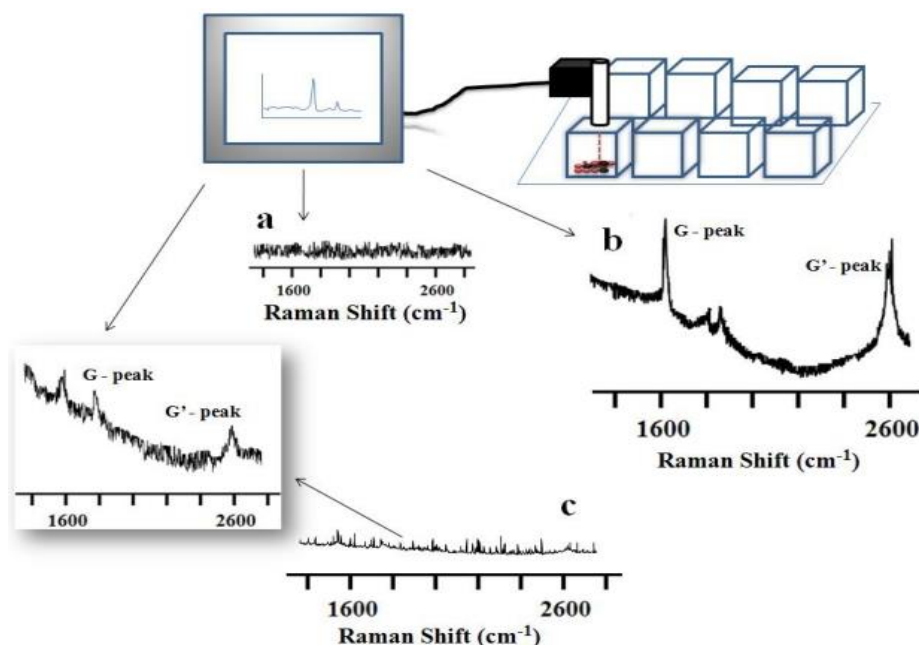


Figure 2.16 Raman spectra of SWCNT and MWCNT coated with PEG in 2D cell culture in vitro, OVCAR8 ovarian cancer cells were subjected to Raman analysis to detect the presence of nanotubes using a handheld portable iRaman spectrometer with a 785 nm laser source. (a) Untreated cells, serving as controls, did not show any characteristic carbon nanotubes peaks. The SWCNT (b) and MWCNT (c) treated cells indeed showed the characteristic G peaks (Adapted from [59]).

Similarly, they were able to detect stronger intensity of G-bands of SWCNT in comparison to MWCNT in cell aggregates or 3D spheroids as shown in Figure 2.17. There is a possibility that the probe is not detecting the MWCNT in cells. A confocal Raman microscopy will overcome this limitation by scanning the entire spheroid and generate three dimensional Raman spectral maps of

carbon nanotubes in cells. In summary, three distinct peaks (radial breathing mode, D-band, G-band) of carbon nanotubes can be used to characterize MWCNT in spheroids.

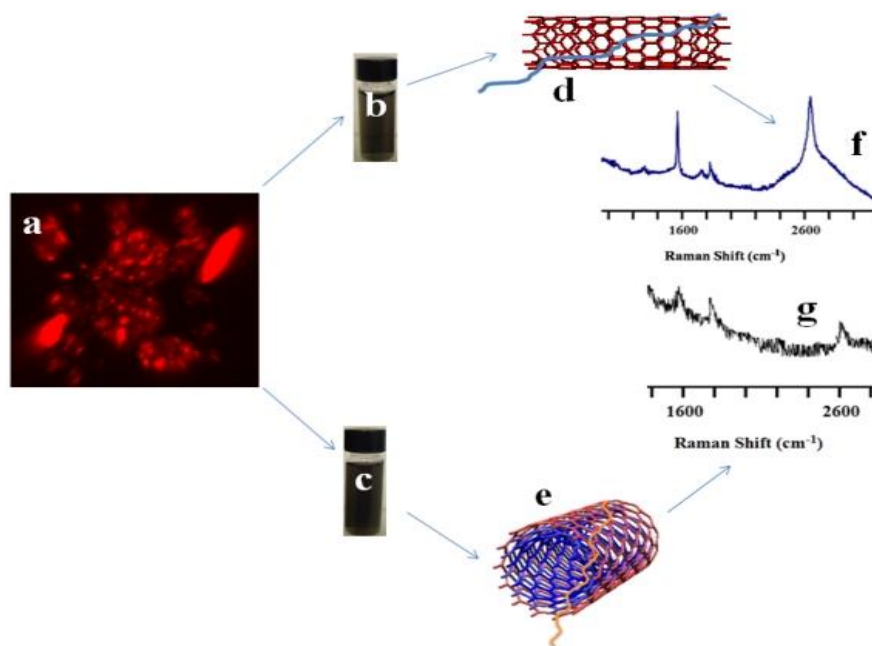


Figure 2.17 Raman probing of SWCNT-PL-PEG-NH<sub>2</sub> and MWCNT-PL-PEG-NH<sub>2</sub> in 3D spheroids. OVCAR8 ovarian cancer cell spheroids (a) treated with LP dispersed SWCNTs (B, D) and MWCNT (C, E) labeled with quantum dots (red), for 3 h and washed were subjected to Raman analysis to detect the presence of nanotubes using a handheld portable iRaman spectrometer with a 785 nm laser source. The SWCNT (F) and MWCNT (G) treated cells indeed showed the characteristic G peaks (Adapted from [59]).

## **CHAPTER 3      OPTIMIZATION      OF      CONFOCAL      RAMAN MICROSCOPE      FOR      IMAGING      MULTI-WALLED      CARBON NANOTUBES IN LIVE PROSTATE SPHEROIDS**

### **3.1 Introduction and Project Goals**

In the last decade, rapid development of nanomaterials has promoted a significant increase in photothermal and photodynamic applications [60]. Animal models have been used to test the efficacy of new photothermal or photodynamic agents, because they exhibit some aspects of the biological and anatomical nature of living organisms [22]. But there are still inherent challenges that need to be addressed. In an in vivo environment, a tumor is normally surrounded by a number of layers of cells [22]. When laser light is directed on the tumor, light would need to penetrate through these layers to activate multi-wall carbon nanotubes to induce photothermal ablation effect [22]. It is pivotal to investigate the molecular interactions between multi-walled carbon nanotubes and cells. The interactions of cells and carbon nanotubes revealed information about receptor binding, antigen-antibody recognition, and internalization [61] are all important to understand how carbon nanotubes reacted at tumor sites. Hence, LUMED collaborated with McGill Jewish General Hospitals (Dr. Miltiadis Paliouras and Dr. Mark Trifirio) on a proof of concept study. The overall objective of this proof-of-concept study is to demonstrate that a confocal Raman microscopy can be used for probing interactions of CNTs and cells. The first objective is to demonstrate how to characterize CNTs, specifically analyzing the effect of surface functionalization on CNTs. Second objective is to obtain the molecular fingerprint of the prostate cells. The third objective is to probe the interactions of CNTs and prostate cells. This chapter will include a methodology section that explains all methods or techniques used to obtain results. Result section will provide a summary of observations for each figure. The last section of this chapter will discuss results in detail by unveiling the molecular fingerprints of CNTs.

### **3.2 Methodology**

#### **3.2.1 Confocal Raman microscope system**

All Raman acquisitions were performed at CRCHUM with the confocal Raman microscope (inVia, Renishaw UK) as shown in Figure 3.1. The Raman system comprises a 300mW laser that supplies

excitation light at 785 nm, which is focused onto the sample via a microscope objective (Leica NPLAN x 50 objective). The 50X objective collects the scattered light from the sample and directs it to the spectrometer. The spectrometer rejects the unwanted portion of scattered light and separates the remainder into its constituent wavelengths. The Raman spectrum is recorded on a deep depletion charge-coupled device detector (CCD). The system is managed using Renishaw Wire software (Windows based Raman Environment Version 1.3.15) which allows the experimental parameters to be set.



Figure 3.1 Confocal Raman Microscope (Adapted from [62])

### 3.2.2 Synthesis of functionalized and non-functionalized MWCNT

All MWCNT samples were prepared at McGill Jewish General Hospital. Functionalized MWCNT were oxidized with 3:1 v/v concentrated sulfuric acid: nitric acid overnight to further introduce carboxylic bonds (COOH) on its surface. For non-functionalized multi-walled carbon nanotubes, molecules of interest such as thyroid stimulating receptor (TSHR) antibodies, immunoglobulin G (IgG) antibodies, and gold nanorods (GNR) were conjugated onto its surface with linker and cross linkers, such as carbodiimide (EDC/NHS), thiol-gold bonds, and maleimide- $PEG_{5000}$ -NH<sub>2</sub>, as shown in Figure 2.11. All the non-functionalized MWCNT were diluted to a concentration of

0.025mg/mL. Functionalized MWCNT were prepared at 0.01mg/mL (original concentration), 0.05mg/mL (2-fold dilution), and 0.05mg/mL (4-fold dilution).

For CNT-PEP and CNT-PEG, CNT (COOH functionalized multi-walled carbon nanotubes) were then treated with linker such as carbodiimide (EDC/NHS) before conjugated with PEP or PEG onto its surface using maleimide-*PEG*<sub>5000</sub>-NH<sub>2</sub> crosslinker, as shown in Figure 2.11. CNT were diluted to concentrations of 0.025mg/mL, 0.01mg/mL, 0.004mg/mL, and 0.0016mg/mL. CNT-PEP and CNT-PEG were diluted to concentrations of 0.05mg/mL, 0.04mg/mL, 0.03mg/mL, 0.02mg/mL, and 0.01mg/mL.

All the samples were stored in vials and transferred to Research Center du Chum (CRCHUM).

### 3.2.3 Protocol for cell culture

All cell culture work was performed at CRCHUM. Cell cultures were maintained according to the American Type Culture Collection (ATCC, Rockville, MD, USA) and harvested using standard cell culture standards. LNCaP (PSMA expressing) cell lines were obtained from the ATCC, Rockville, MD, USA). LNCaP cell lines were cultured in complete medium (RPMI 1640 media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin) in a standard size (100mm x 15mm) petri dish. Cell lines were incubated at 37°C and 5% CO<sub>2</sub> humidified environment. Once the cells were 70-80% confluent, medium from the dish was aspirated, the cells were washed once in 1X phosphate buffered saline (PBS) and dissociated using 2mL of TrypLE reagent (trypsin with phenol red and ethylenediaminetetraacetic acid). The TrypLE reagent was then neutralized using 10mL of complete medium and cells were counted using hemocytometer. Cells with equal or greater than 90% viability were taken for spheroid generation.

### 3.2.4 Protocol for spheroid generation

For spheroid formation, the LNCaP cells were seeded in 96-well, non-adherent plates (Nunc) coated with hydrogels at a density of  $0.0016 \times 10^6/cm^2$  to  $0.016 \times 10^6/cm^2$ . 100μL of LNCaP cells diluted in the culture medium were added to the wells in the center of plates. The empty wells at the edge of plates were filled with 200μL to prevent cells from dehydration. The plates were then placed in the incubator at 37°C and 5% CO<sub>2</sub> humidified atmosphere. The time for spheroid formation depends on the cell seeding density. Typically,  $0.0016 \times 10^6/cm^2$  will take

approximately 4-5 days,  $0.0031 \times 10^6/cm^2$  will take about 3-4 days,  $0.016 \times 10^6/cm^2$  will take about 2-3 days, for cells to form into spheroids. Spheroids were monitored daily by checking them under a bright field microscope to ensure the spheroid did not overgrow and they were healthy.

### 3.2.5 Optimization of experimental protocol for characterizing live spheroids

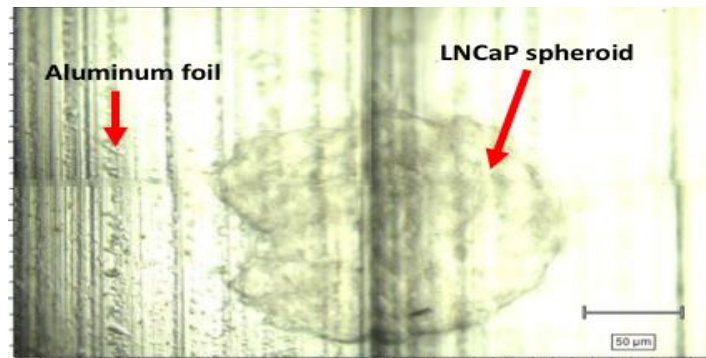
Generally, biological cells are grown either in a petri dish or culture flask which are mostly made of polystyrene. For cell culture, polystyrene is favorable because its material cost is low and it is disposable which helps to prevent cross-contamination. However, polystyrene-based materials tend to have undesirable fluorescence backgrounds that can interfere with Raman scattering. Young et.al [63] have observed autofluorescence in polystyrene sheets. Raman bands of polystyrene [64] tend to overlap with molecular fingerprints of biological cells at  $700-1600\text{ cm}^{-1}$ . Cells grown on aluminum foil yield a very low and featureless spectral background which is desirable for cell characterization [65]. In comparison, polystyrene-based petri dish has two broad bands at  $1050\text{ cm}^{-1}$  and  $1610\text{ cm}^{-1}$  [66] that will overlap with molecular peaks of biological cells. Since spheroids were grown in 96-well coated plates, we could not grow cells in aluminum due to the size of wells. Instead, we transferred spheroids onto an aluminum foil before Raman acquisition. We were able to observe a similar low and featureless background of aluminum foil.

### 3.2.6 Technique for mapping LNCaP spheroid

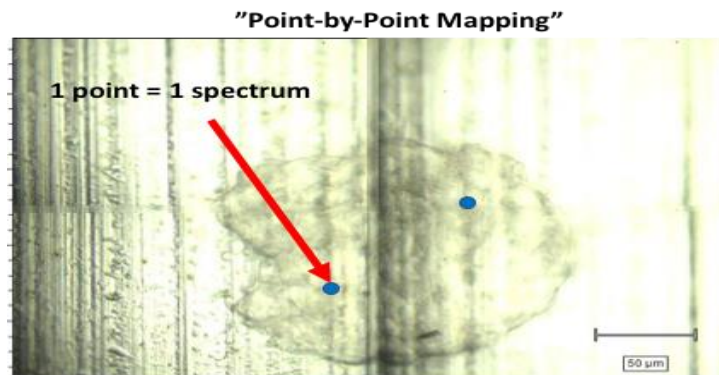
Figure 3.2a shows a confocal image taken under 50X LWD objective of the confocal Raman microscope before measurements. Figure 3.2b shows a confocal image of LNCaP spheroid mapped point-by-point. Basically, point-by-point mapping acquires spectrum for each selected point. In other words, one point produces 1 spectrum. For spheroids, point-by-point mapping was combined with raster scan as shown in Figure 3.2c, in which the entire region of the spheroid was scanned with points evenly spaced and distributed. Ideally, it would be better if the spheroid can be mapped at multiple depths to produce z-stack measurements, capturing complete molecular fingerprints of LNCaP at different depths. Due to the limitation of Renishaw inVia, the point-by-point mapping only allows the spheroid to be scanned at specific depth. To overcome the limitation, when using point-by-point mapping, focus the 50X LWD objective at the bottom of the spheroid, in this way,

it will map the spheroid at the middle layer of the spheroid. We were able to detect all prominent bands of LNCaP spheroid described in [67], when mapping at the middle layer of the spheroid.

a)



b)



c)

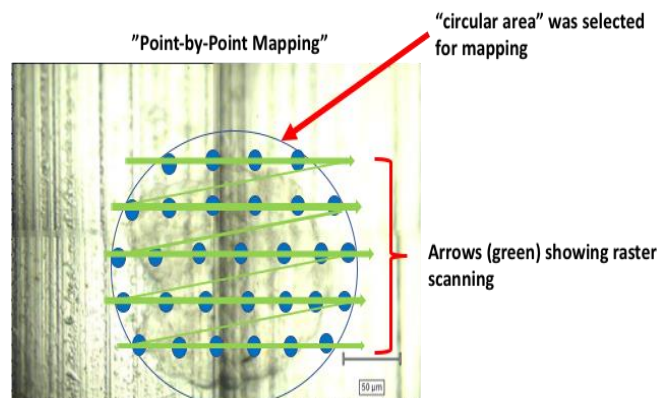


Figure 3.2 Confocal microscopy image of a) LNCaP spheroid before measurement; b) LNCaP spheroid mapped point-by-point; c) LNCaP spheroid mapped using point-by-point in a raster scan.

### **3.2.7 Incubation of CNT samples in spheroids**

On the day of incubation, spheroids were checked under a bright field microscope to ensure only those with desired size were chosen for the experiment. CNT, CNT-PEP, or CNT-PEG samples were diluted at different concentrations before adding to spheroids and incubated overnight in the incubator at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$  humidified atmosphere.

### **3.2.7 Sample preparation before measurements**

For preliminary study, 10ul of MWCNT samples from each vial were transferred carefully onto aluminum slides to dry the samples. The drying process took about 20 to 30 minutes. Once the samples were dried, we proceeded to the measurements. Before spectral acquisition, the system was turned on for 30 minutes to warm up the laser system.

In the actual study, 30mm x 15mm petri dishes covered with aluminum foils were prepared ahead of time. Also, 1.5mL centrifuge tubes were labeled for each sample. Samples in the 96-well plates were checked under a bright field microscope to ensure spheroids were healthy. Then, they were removed from the 96-well plates to their respective tubes. Next, each were washed with PBS about five times. The washing step had to be done slowly and cautiously because spheroid pellets were very small. Finally, 10 $\mu\text{L}$  of samples in PBS were transferred to the aluminum foil in petri dishes and proceeded to Raman measurements. PBS prevents the spheroids from drying during acquisition.

### **3.2.8 Procedures for Raman spectral acquisition**

Once the system was ready, a quick calibration was performed to ensure the x-axis was calibrated properly. Initially, the samples were magnified under the 5X objective of the microscope to locate the sample. Then, the 5X objective of the microscope was switched to the 50X long working distance objective (LWD objective) to increase the magnification of the sample. Long working distance objective was used to avoid touching samples. A confocal microscopy image of the sample was taken at 50X LWD objective before measurement.

For preliminary study, 2 different point locations were selected on each sample and each point location generates one spectrum. Each spectrum was acquired at selected point location, using point-by-point mapping method, at 300mW with a spectrum window of 0 to 3000  $\text{cm}^{-1}$  over a period of 10 seconds per spectrum, with a repeated measurement, thus generating four spectrums in total for each sample.

In the actual study, spheroids were first identified under 5X objective. Then, a surface montage was performed to take an image of the whole spheroid under 50X objective. In the image, a circular area on the spheroid was selected to ensure the entire region of the spheroid was covered. For consistency, 69-point locations were selected within the circular area of the spheroid using the step size feature, to ensure points are evenly spaced and distributed over the area of interest. Each spectrum was acquired at selected point locations, using point-by-point mapping, raster scan mode, at 300mW with a spectrum window of 610 to 1726  $\text{cm}^{-1}$  over a period of 10 seconds per spectrum, without repeated measurements, thus generating 69 spectrums in total for each spheroid. For optimization study, laser irradiation powers were adjusted from 300mW (maximum power of 785 nm laser) to 150mW (50% maximum power of 785 nm laser), 30mW (10% maximum power of 785 nm laser), and 3mW (1% maximum power of 785 nm laser).

### **3.2.9 Data processing for Raman spectra**

All the raw data acquired on confocal Raman microscope (Renishaw inVia) were saved in the wintech drawing file (wdf) format. Therefore, these files need to be converted to text (txt) format using Renishaw BatchWire, before processing them in Python. Once the conversion was done, these files were imported in Python and all data were processed using ORPL (Open Raman Processing Library) Python script written by data science group at LUMED. Since spectra were measured with Renishaw inVia, only three steps are required for the processing. First, the cosmic rays from the spectra were removed. Cosmic rays are high energy particles that move through space and produce showers of particles when they hit the atmosphere. These random particles generate spurious spikes on Raman spectra. Next, the baseline or background which consists of fluorescence were removed from the raw spectra to obtain only Raman spectra. Finally, raw spectra were normalized using Standard Normal Variate (SNV) method to obtain processed spectra. A workflow for Raman signal processing is shown in Figure 3.3.

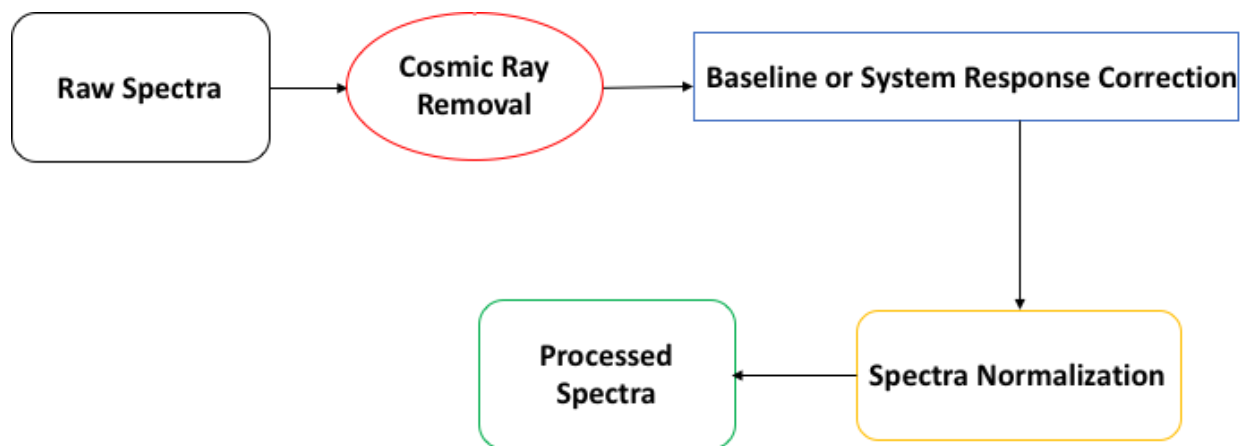


Figure 3.3 Raman signal processing workflow.

### 3.2.10 Generation of figures for results

#### 3.2.10.1 Preliminary study: characterize effect of functionalization on MWCNT

Figure 3.4 shows four Raman spectra representing different MWCNT samples, plotted on the same graph. Each Raman spectra represents a mean spectrum, by averaging spectrums over four different point locations. First, four raw spectrums of each sample were processed using ORPL Python script to remove cosmic rays and baseline. Next, the Raman shift (in wavenumbers) and Raman intensity of the four processed spectrums were averaged. Average Raman intensity was further normalized using Standard Normal Variate (SNV) method, to produce normalized Raman intensity, so each spectrum has a mean of 0 and standard deviation of 1. A y-axis offset was added to prevent spectra from overlapping and ensure that each spectrum can be viewed clearly. For each spectrum, the normalized Raman intensity was plotted against wavenumber as shown in Figure 3.4. Only CNT-COOH diluted at concentration 0.025mg/mL is plotted in this graph to compare to non-functionalized multi-walled carbon nanotubes with the same concentration of 0.025mg/mL.

The bar charts of D/G band ratio of functionalized and non-functionalized MWCNT shown in Figure 3.5 were plotted using Microsoft Excel and the calculations were computed in an Excel sheet. A sample calculation of MWCNT-GNR is shown below in Table 3.1, to demonstrate how the bar chart was plotted. Here, spectrums of four-point locations were not averaged, so that the values of Raman intensity of D band and G-band of, functionalized and non-functionalized MWCNT, can be obtained. In Table 3.1, “1” and “2”, each referred to point locations on the sample. In each row, the Raman intensity of D-band is divided over the Raman intensity of G-band to

calculate the D/G ratio. An average was computed at each column. Standard deviation was also calculated at each column. In Figure 3.5, the bar chart of MWCNT-GNR was plotted for D/G band ratio and standard deviation was plotted as an error bar on top of the bar chart.

Table 3.1 Sample calculation for MWCNT-GNR shown in Figure 3.5.

| MWCNT-GNR                               |                       | D band                          |                        | G band                          |                        | D/G Ratio |
|-----------------------------------------|-----------------------|---------------------------------|------------------------|---------------------------------|------------------------|-----------|
| Label                                   | Spectrum (Location #) | Raman shift (cm <sup>-1</sup> ) | Raman Intensity (a.u.) | Raman shift (cm <sup>-1</sup> ) | Raman Intensity (a.u.) |           |
| a                                       | 1                     | 1310.501                        | 69102.61               | 1604.829                        | 38494.43               | 1.795133  |
| b                                       | 2                     | 1311.568                        | 68294.23               | 1605.835                        | 37520.92               | 1.820164  |
| c                                       | 1                     | 1310.501                        | 70384.46               | 1604.829                        | 38864.52               | 1.811021  |
| d                                       | 2                     | 1310.501                        | 68507.38               | 1604.829                        | 38454.69               | 1.781509  |
| Average = (a+b+c+d)/4                   |                       | 1310.768                        | 69072.17               | 1605.081                        | 38333.64               | 1.801957  |
| Standard Deviation = STDEV (a, b, c, d) |                       | 0.5335                          | 939.3633               | 0.503                           | 572.3792               | 0.017111  |

### 3.2.10.2 Characterize the molecular fingerprint of prostate cancer cells using LNCaP spheroids as a model

Figure 3.6a shows a confocal image of spheroid before irradiation and the image was taken under 50X LWD objective. Figure 3.6b shows three Raman spectra representing different laser powers used for irradiating spheroids, all plotted on the same graph. Each Raman spectra represents a mean spectrum, computed by averaging spectrums over 69 different point locations on the spheroid. First, 69 spectrums of each sample were processed using ORPL Python script to remove cosmic rays and baseline. Next, the Raman shift (in wavenumbers) and Raman intensity of the 69 processed spectrums were averaged. Average Raman intensity was further normalized using Standard Normal Variate (SNV) method, to produce normalized Raman intensity, so each spectrum has a mean of 0 and standard deviation of 1. A y-axis offset was added to prevent spectra from overlapping and ensure that each spectrum can be viewed clearly. For each spectrum, the normalized Raman intensity was plotted against wavenumber as shown in Figure 3.6. Arrow labels were added on the graph to indicate prominent molecules of LNCaP spheroid.

Figure 3.7a shows confocal images of different sizes of spheroid before irradiation and images were taken under 50X LWD objective. Figure 3.7b shows three Raman spectra representing three different sizes of spheroids seeded at different cell densities, all plotted on the same graph. Each Raman spectra represents a mean spectrum, computed by averaging spectrums over 69 different point locations on the spheroid. First, 69 spectrums of each sample were processed using ORPL Python script to remove cosmic rays and baseline. Next, the Raman shift (in wavenumbers) and Raman intensity of the 69 processed spectrums were averaged. Average Raman intensity was further normalized using Standard Normal Variate (SNV) method, to produce normalized Raman intensity, so each spectrum has a mean of 0 and standard deviation of 1. A y-axis offset was added to prevent spectra from overlapping and ensure that each spectrum can be viewed clearly. For each spectrum, the normalized Raman intensity was plotted against wavenumber as shown in Figure 3.6. Arrow labels were added on the graph to indicate prominent molecules of LNCaP spheroid.

### **3.2.10.3 Characterization of CNT in LNCaP spheroids: probe interactions of multi-walled carbon nanotubes and cells**

Figure 3.9a shows a confocal image of spheroid with CNT before irradiation and the image was taken under 50X LWD objective. Two areas of interest were circled in red color. In Figure 3.9a, “With CNT” refers to the area where there are CNT. “Without CNT” refers to the area where there is no CNT. Areas of interest were identified based on their x and y coordinates on the confocal microscopy image. Figure 3.9b shows two spectra, LNCaP without carbon nanotubes and LNCaP with carbon nanotubes, both are plotted on the same graph. Each Raman spectra represents a mean spectrum, computed by averaging spectrums over 69 different point locations on the spheroid. First, 69 spectrums of each sample were processed using ORPL Python script to remove cosmic rays and baseline. Before normalization, the x and y coordinates of two areas were used to locate the two spectrums. Once the spectrums were identified, each of their Raman intensity was further normalized using Standard Normal Variate (SNV) method, to produce normalized Raman intensity, so each spectrum has a mean of 0 and standard deviation of 1. For each spectrum, the normalized Raman intensity was plotted against wavenumber as shown in Figure 3.9b. Arrow labels were added on the graph to indicate prominent molecules of LNCaP spheroid and multi-walled carbon nanotubes.

Figure 3.10 shows four Raman spectra representing different concentrations of CNT and a control with just LNCaP spheroid, all plotted on the same graph. Each Raman spectra represents a mean spectrum, computed by averaging spectrums over 69 different point locations on the spheroid. First, 69 spectrums of each sample were processed using ORPL Python script to remove cosmic rays and baseline. Next, the Raman shift (in wavenumbers) and Raman intensity of the 69 processed spectrums were averaged. Average Raman intensity was further normalized using Standard Normal Variate (SNV) method, to produce normalized Raman intensity, so each spectrum has a mean of 0 and standard deviation of 1. A y-axis offset was added to prevent spectra from overlapping and ensure that each spectrum can be viewed clearly. For each spectrum, the normalized Raman intensity was plotted against wavenumber as shown in Figure 3.10. Arrow labels were added on the graph to indicate prominent molecules of CNT-COOH in LNCaP spheroid.

Figure 3.11 shows four Raman spectra representing different concentrations of CNT-PEP and a control with just LNCaP spheroid, all plotted on the same graph. Each Raman spectra represents a mean spectrum, computed by averaging spectrums over 69 different point locations on the spheroid. First, 69 spectrums of each sample were processed using ORPL Python script to remove cosmic rays and baseline. Next, the Raman shift (in wavenumbers) and Raman intensity of the 69 processed spectrums were averaged. Average Raman intensity was further normalized using Standard Normal Variate (SNV) method, to produce normalized Raman intensity, so each spectrum has a mean of 0 and standard deviation of 1. A y-axis offset was added to prevent spectra from overlapping and ensure that each spectrum can be viewed clearly. For each spectrum, the normalized Raman intensity was plotted against wavenumber as shown in Figure 3.11. Arrow labels were added on the graph to indicate prominent molecules of CNT-PEP in LNCaP spheroid.

Figure 3.12 shows four Raman spectra representing different concentrations of CNT-PEG and a control with just LNCaP spheroid, all plotted on the same graph. Each Raman spectra represents a mean spectrum, computed by averaging spectrums over 69 different point locations on the spheroid. First, 69 spectrums of each sample were processed using ORPL Python script to remove cosmic rays and baseline. Next, the Raman shift (in wavenumbers) and Raman intensity of the 69 processed spectrums were averaged. Average Raman intensity was further normalized using Standard Normal Variate (SNV) method, to produce normalized Raman intensity, so each spectrum has a mean of 0 and standard deviation of 1. A y-axis offset was added to prevent spectra from overlapping and ensure that each spectrum can be viewed clearly. For each spectrum, the

normalized Raman intensity was plotted against wavenumber as shown in Figure 3.11. Arrow labels were added on the graph to indicate prominent molecules of CNT-PEG in LNCaP spheroid.

The graphs of Figure 3.13 were plotted using Microsoft Excel and the calculations were computed in an Excel sheet. The Raman intensity at D-band Raman shift ( $1360\text{cm}^{-1}$ ) and G-band Raman shift ( $1610\text{cm}^{-1}$ ) corresponding to different concentrations of CNT-PEG and PEG, were obtained from Figure 3.11 and Figure 3.12, all values are shown in Table 3.2. All values shown in Table 3.2 are computed from the mean of two repeated measurements. Based on these values, the Raman intensity of D-band ( $1360\text{cm}^{-1}$ ) were plotted against concentrations, for CNT-PEG and CNT-PEG, as shown in Figure 3.13a. The Raman intensity of G-band ( $1610\text{cm}^{-1}$ ) were plotted against concentrations, for CNT-PEG and CNT-PEG, as shown in Figure 3.13b. In each graph, a regression line has the form of  $y = mx + c$ , and was added to fit points of CNT-PEG and CNT-PEG, to model the relationship between Raman intensity and concentrations of CNTs. The  $r^2$  were computed from the regression line. The error bars shown in each curve were computed from standard error using the error bar feature in Excel.

Table 3.2 Raman intensity of D-band and G-band of different concentrations of CNT-PEG and CNT-PEG shown in Figure 3.11 and Figure 3.12.

| <b>CNT-PEG</b> | <b>Concentration<br/>(mg/mL)</b> | <b>Raman Intensity<br/>(D-band at <math>1360\text{cm}^{-1}</math>)</b> | <b>Raman Intensity<br/>(G-band at <math>1610\text{cm}^{-1}</math>)</b> |
|----------------|----------------------------------|------------------------------------------------------------------------|------------------------------------------------------------------------|
|                | 0                                | 0                                                                      | 0                                                                      |
|                | 0.01                             | 0.33945527                                                             | 0.27736132                                                             |
|                | 0.03                             | 0.64492754                                                             | 0.52561219                                                             |
|                | 0.04                             | 0.42566217                                                             | 0.36356822                                                             |
|                | 0.05                             | 0.64655172                                                             | 0.17206397                                                             |
|                |                                  |                                                                        |                                                                        |
| <b>CNT-PEG</b> | <b>Concentration<br/>(mg/mL)</b> | <b>Raman Intensity<br/>(D-band at <math>1360\text{cm}^{-1}</math>)</b> | <b>Raman Intensity<br/>(G-band at <math>1610\text{cm}^{-1}</math>)</b> |
|                | 0                                | 0                                                                      | 0                                                                      |
|                | 0.01                             | 0.44760101                                                             | 0.45833333                                                             |
|                | 0.03                             | 0.67108586                                                             | 0.68055556                                                             |
|                | 0.04                             | 0.90277778                                                             | 0.9                                                                    |
|                | 0.05                             | 0.89772727                                                             | 0.91666667                                                             |

### 3.3 Results

#### 3.3.1 Preliminary study: characterize effect of functionalization on MWCNT

MWCNT have unique molecular fingerprints in Raman spectra. The D and G-band are often described as the prominent bands of MWCNT. However, MWCNT tend to suffer from agglomeration due to their van der Waal forces. This can alter the shape or intensity of D and G-band, causing defects in MWCNT. Chemical functionalization showed a promising solution for reducing agglomeration of MWCNT. Functionalization involves attaching an organic functional group such as carboxylic acids to the surface of MWCNT. For preliminary study, the aim is to show how to characterize the effect of functionalization on MWCNT based on spectral information. For functionalized MWCNT, MWCNT were conjugated with carboxylic acids and referred to as COOH. Non-functionalized MWCNT are basically MWCNT without functional groups. Instead, they were conjugated directly with antibodies or gold nanorods. Antibodies used are related to prostate such thyroid stimulating hormone receptor (TSHR ab) and immunoglobulin G (IgG ab). First, the molecular fingerprints of functionalized MWCNT (COOH) and non-functionalized MWCNT (TSHR ab, IgG ab, GNR) were compared. Then, D/G ratio were calculated based on the intensity of D and G-band, to evaluate the defects present on the MWCNT, to determine if chemical functionalization could minimize the defects. This section presents two sub studies, i) Compare the molecular fingerprint of functionalized and non-functionalized MWCNT and ii) Analyze the effect of functionalization on MWCNT.

##### 3.3.1.1 Compare the Molecular Fingerprint of Functionalized and Non-Functionalized MWCNT

Both functionalized and non-functionalized MWCNT, with the same concentration of 0.025mg/mL were plotted in Figure 3.4. All the prominent Raman bands identified from Figure 3.4 were summarized in Table 3.3. Two distinct Raman peaks,  $1400\text{ cm}^{-1}$  and  $1600\text{ cm}^{-1}$  correspond to the D and G-band of carbon nanotubes, were observed consistently in all spectra. However, the D-band of TSHR ab and IgG ab, showed multiple spiky peaks around the D-band. The C-C radial breathing of carbon nanotubes were observed in COOH and GNR, at  $400\text{ cm}^{-1}$ . The peak at around  $2700\text{ cm}^{-1}$  that was observed in all spectra, was contributed by the C-H bond stretch of CNTs. There was no peak that corresponds to carboxylic acids in GNR. In contrast, the antibodies

introduced additional bands to CNTs. For instance, TSHR ab and IgG ab, showed bands corresponding to adenine, thymine, O-P-O symmetric stretching bands from  $0\text{ cm}^{-1}$  and  $800\text{ cm}^{-1}$ , phenylalanine at  $900\text{ cm}^{-1}$ , and adenine at  $1500\text{ cm}^{-1}$ .

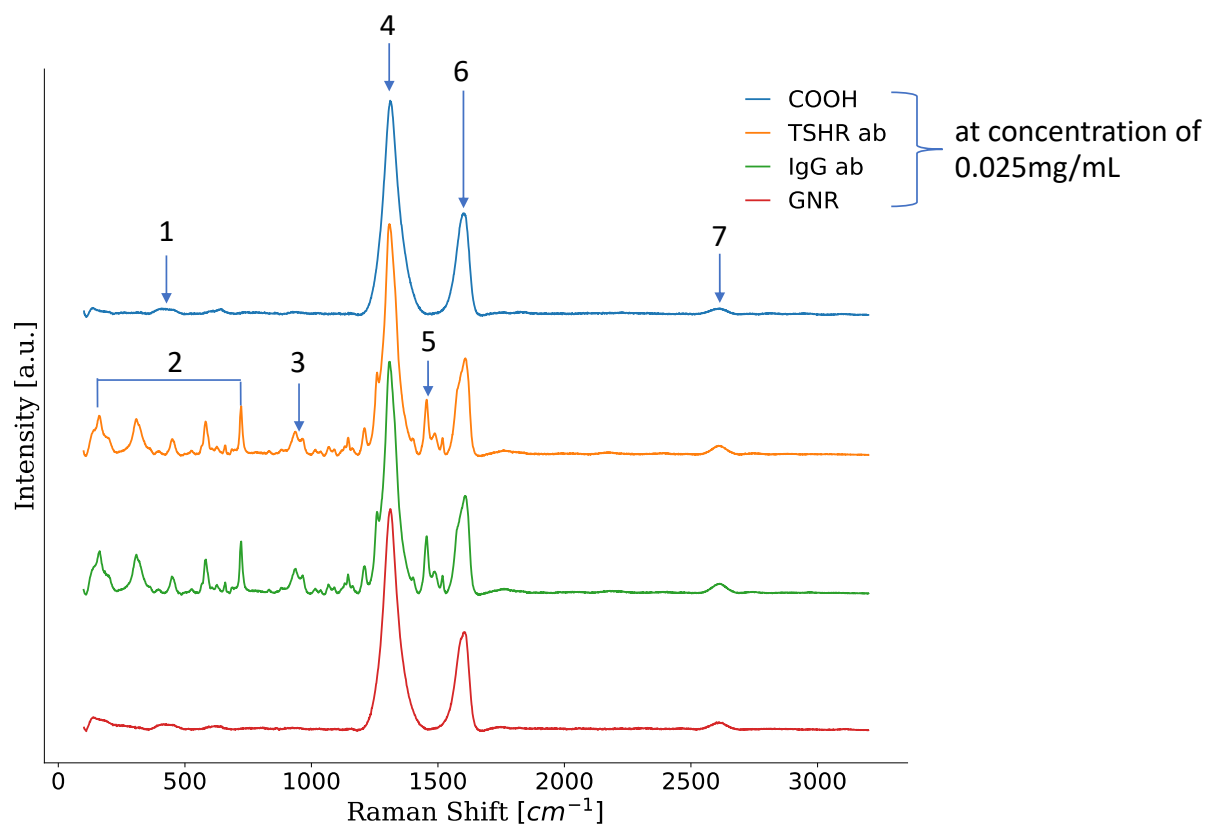


Figure 3.4 Raman spectra of functionalized MWCNT (COOH) and non-functionalized MWCNT (TSHR ab, IgG ab, GNR). All MWCNT have the same concentration of 0.025mg/mL. Prominent Raman peaks were identified as shown in Table 3.3.

Table 3.3 Prominent Raman bands identified from Figure 3.4.

| Band Label | Wavenumber ( $cm^{-1}$ ) | Molecular Bond Assignment                    | Molecular Species        |
|------------|--------------------------|----------------------------------------------|--------------------------|
| 1          | 400                      | C-C radial breathing (RBM)                   | carbon nanotubes         |
| 2          | 0 - 800                  | Adenine, Thymine, O-P-O symmetric stretching | nucleic acids (DNA, RNA) |
| 3          | 900                      | Ring breathing of phenylalanine (C-C)        | protein                  |
| 4          | 1400                     | D-band                                       | carbon nanotubes         |
| 5          | 1500                     | Adenine                                      | nucleic acids (DNA, RNA) |
| 6          | 1600                     | G-band                                       | carbon nanotubes         |
| 7          | 2700                     | C-H stretch                                  | carbon nanotubes         |

### 3.3.1.2 Analyze the Effect of Functionalization on MWCNT

The D/G band ratio was computed for each MWCNT based on their intensity of D and G-band. These values were then plotted as shown in Figure 3.5. On the left of the graph, those bar charts labeled with black and gray colors are referring to the functionalized MWCNT (COOH), diluted at different concentrations. Whereas, those bar charts on the right of the graph are non-functionalized MWCNT (TSHR ab, IgG ab, GNR). In Figure 3.5, the concentrations of COOH did not seem to affect its D/G band ratio. The COOH at 2-fold dilution (0.05mg/mL) and 4-fold dilution (0.025mg/mL) had the same D/G ratio of 1.7. Overall, non-functionalized MWCNT showed a higher D/G ratio in comparison to functionalized MWCNT. For instance, TSHR ab and IgG ab showed a D/G band ratio of 1.9, the D/G band ratio of GNR was about 1.8, while the average D/G band ratio of COOH was 1.7. The error bars of non-functionalized MWCNT were relatively larger than functionalized MWCNT.

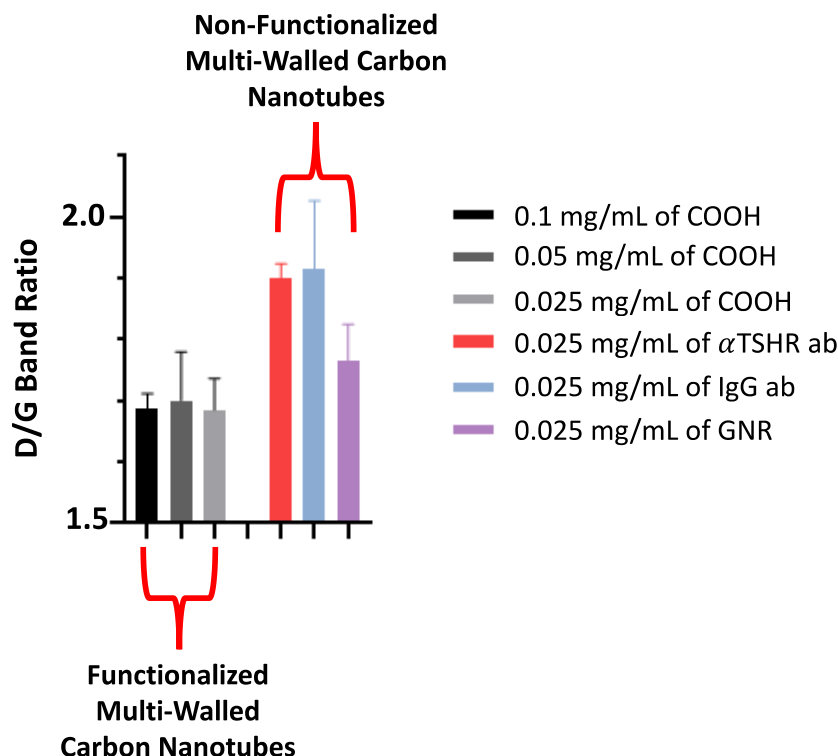


Figure 3.5 The effect of functionalization on the D/G ratio of MWCNT.

### 3.3.2 Characterize the molecular fingerprint of prostate cancer cells using LNCaP spheroids as a model

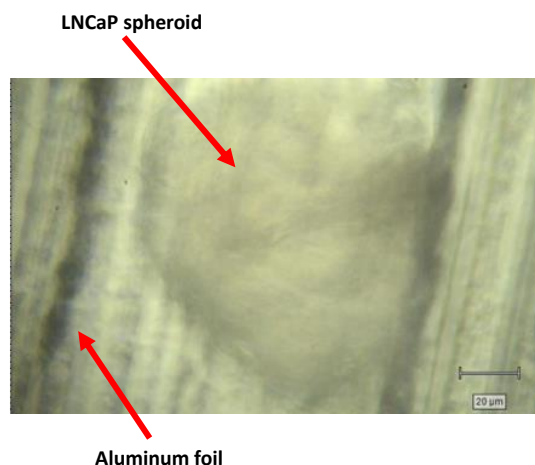
In this project, LNCaP spheroid were used as the model for prostate cancer because the spheroid exhibits remarkable resemblance to native cell structure and tumor tissue. It was essential to characterize the molecular fingerprint of LNCaP spheroid to identify prominent biomolecules that are associated with LNCaP cells. This section presents two sub studies, i) Optimize laser irradiation power for detecting prominent biomolecules associated with LNCaP cells and ii) Optimize the size of spheroid towards the detection of molecular fingerprint of LNCaP cells.

#### 3.3.2.1 Optimize Laser Irradiation Power for Detecting Prominent Biomolecules Associated with LNCaP Cells

A confocal microscopy image of LNCaP spheroid on an aluminum foil was shown in Figure 3.6a. Raman spectra of LNCaP spheroids acquired under irradiation laser power of 150mW, 30mW, and 3mW, as illustrated in Figure 3.6b. All the prominent Raman bands of LNCaP spheroid observed

from Figure 3.6b were summarized in Table 3.4. The intensity of Raman signals increased with laser irradiation power. Adenine at  $721\text{ cm}^{-1}$  and  $1317\text{ cm}^{-1}$ , were observed in all three spectrums as shown in Figure 3.6b. O-P-O and ( $PO_2^-$ ) symmetric stretching of nucleic acids were also observed at  $783\text{ cm}^{-1}$  and  $1096\text{ cm}^{-1}$  respectively for all spectrums, but these bands were more distinct when acquired at 150mW compared to 30mW and 3mW. 150mW produced a robust and sharp lipids band at  $1050\text{ cm}^{-1}$ . In comparison, the signal of lipids was significantly lower when acquired at 30mW and 3mW. The ring breathing of phenylalanine (C-C) at  $1001\text{ cm}^{-1}$  was observed in all spectrums, but its intensity was more robust at 150mW. Amide III at  $1263\text{ cm}^{-1}$ , Amide I at  $1657\text{ cm}^{-1}$  and nucleic acids band at  $1317\text{ cm}^{-1}$ , were spotted in all three spectrums. The valley between the Amide III and nucleic acids band disappeared at 150mW. At 3mW, the spectra showed a large noise background underneath, indicating the lowest signal-to-noise ratio among all the laser irradiation powers. Overall, the irradiation power of 150mW increased the Raman intensity and signal-noise-ratio, allowing prominent molecules of LNCaP be detected.

a)



b)

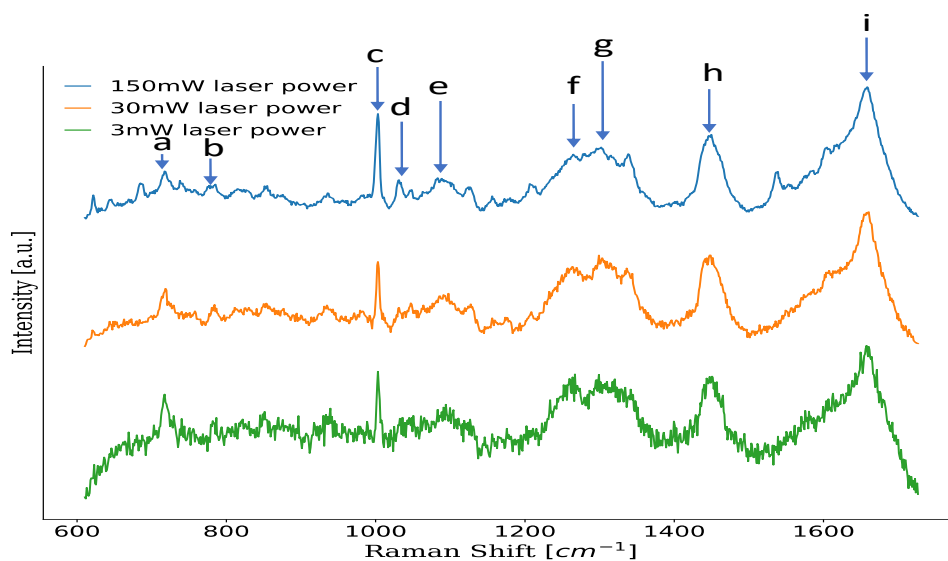


Figure 3.6 a) Confocal microscopy image of LNCaP spheroid; b) Raman spectra of LNCaP spheroids acquired at laser power of 150mW, 30mW, and 3mW were plotted on the same graph.

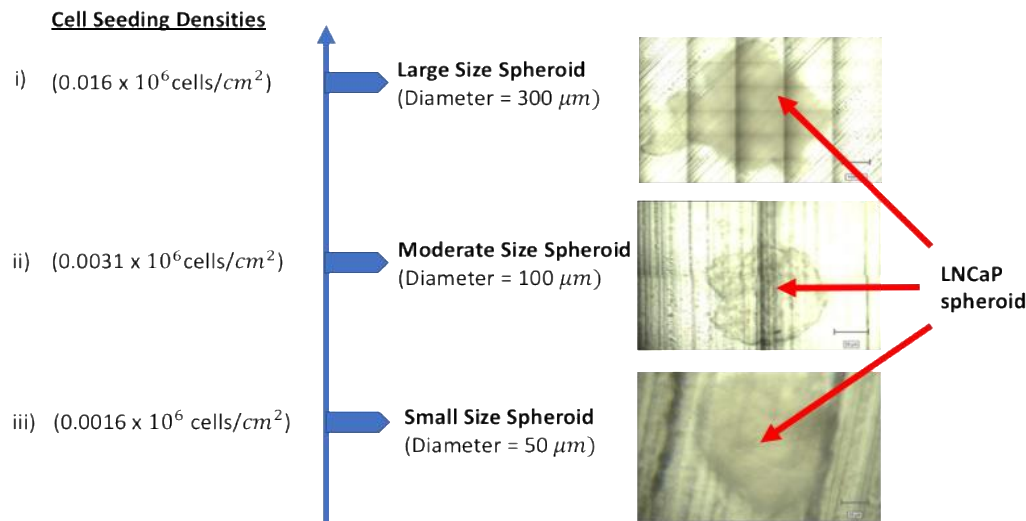
Table 3.4 Prominent Raman bands identified from Figure 3.6b and 3.7b.

| Band Label | Wavenumber ( $cm^{-1}$ ) | Molecular Bond Assignment             | Molecular Species        |
|------------|--------------------------|---------------------------------------|--------------------------|
| a          | 721                      | C-N head group of adenine             | nucleic acids (DNA, RNA) |
| b          | 783                      | O-P-O symmetric stretching            | nucleic acids (DNA, RNA) |
| c          | 1001                     | Ring breathing of phenylalanine (C-C) | protein                  |
| d          | 1050                     | Skeletal C-C stretch                  | lipids                   |
| e          | 1096                     | $(PO_2^-)$ symmetric stretching       | nucleic acids (DNA, RNA) |
| f          | 1263                     | Amide III (N-H)                       | $\alpha$ -helix protein  |
| g          | 1317                     | Adenine, Guanine                      | nucleic acids (DNA, RNA) |
| h          | 1450                     | $CH_2$ scissors                       | lipids                   |
| i          | 1657                     | Amide I (C=O, N-H)                    | $\alpha$ -helix protein  |

### 3.3.3.2 Optimize the Size of Spheroid Towards the Detection of The Molecular Fingerprint of LNCaP cells

Figure 3.7a shows the confocal microscopy images of spheroids of different sizes. The image brightness appears to be different for all images because of the intensity of illumination. Also, the image i) was taken with surface montage by accident, as a result, there are square grids around the image. All the labels from Figure 3.7b can be found in Table 3.4. In Figure 3.7a, there are three spheroids, each seeded at different cell densities, resulting in different sizes of spheroids. All cells were proliferated for three days to form spheroid. Spheroid size increased with the cell densities. At low seeding density, the spheroid has a diameter of 50  $\mu\text{m}$  which is relatively small. As the cell seeding density increases, the diameter of the spheroid doubled (100 $\mu\text{m}$ ). The resolution of its morphology also increased, revealing the structure of the spheroid. However, the structure resolution of spheroid decreases when cells were seeded at high density. At high density, spheroid diameter increased to 300  $\mu\text{m}$  and cells began to clump together instead of forming into cell aggregates. Figure 3.7b shows three Raman spectra acquired for different sizes of spheroid. The intensity of Raman signals increases with size. In Figure 3.7b, adenine at 721  $\text{cm}^{-1}$  and 1317  $\text{cm}^{-1}$  is observed in all spectrum, but the adenine band of moderate size spheroid at 721  $\text{cm}^{-1}$  has a sharp projecting point. The O-P-O and ( $\text{PO}_2^-$ ) symmetric stretching at 783  $\text{cm}^{-1}$  and 1096  $\text{cm}^{-1}$  respectively, are also observed in all three spectrums. The C-C stretch of lipids at 1050  $\text{cm}^{-1}$  has the same shape in large and moderate size spheroid. A high intensity of phenylalanine (C-C) band at 1001  $\text{cm}^{-1}$  is observed in moderate size spheroid. All sizes of spheroid have the same Amide III at 1263  $\text{cm}^{-1}$ , Amide I at 1657  $\text{cm}^{-1}$ , and nucleic acids at 1317  $\text{cm}^{-1}$ . Overall, the molecular fingerprint of LNCaP spheroid does not change significantly with size, but the signal-to-noise ratio does increase with size. Also, the size of the spheroid increased time for acquiring data. Among all three sizes of spheroid, a spheroid with a size of 100  $\mu\text{m}$  is suitable for mapping and extracting robust signals of prominent molecules of spheroid.

a)



b)

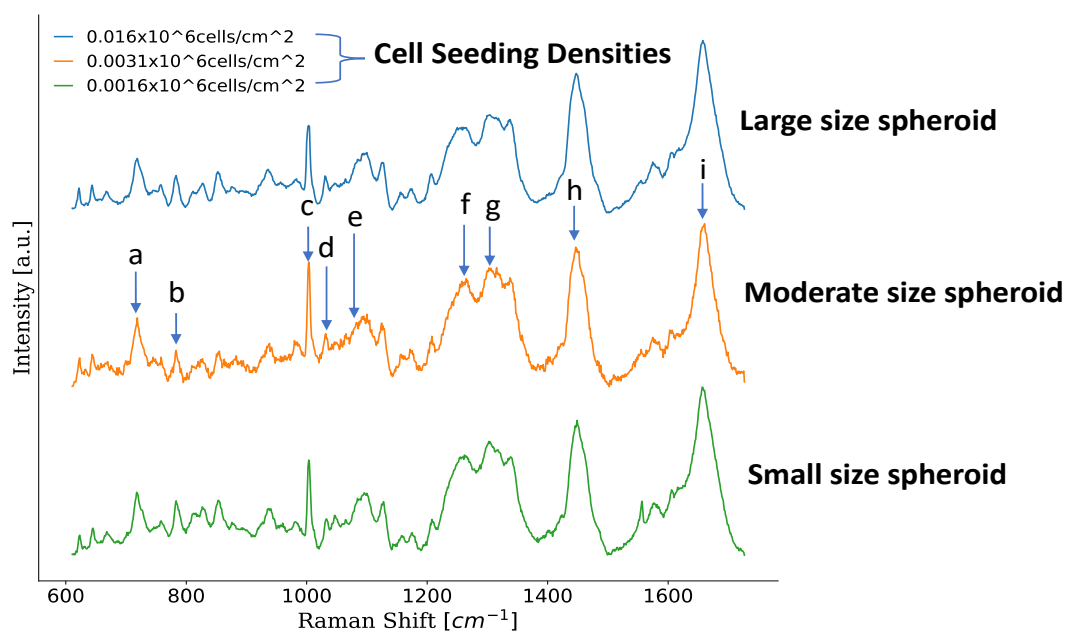


Figure 3.7 a) Confocal microscopy images of different sizes of LNCaP spheroid i) Seeding density =  $0.016 \times 10^6 \text{ cells/cm}^2$  and spheroid diameter of  $300 \mu\text{m}$ , ii) Seeding Density =  $0.0031 \times 10^6 \text{ cells/cm}^2$  and spheroid diameter of  $100 \mu\text{m}$ , and iii)  $0.0016 \times 10^6 \text{ cells/cm}^2$  and spheroid diameter of  $50 \mu\text{m}$ ; (b) Raman spectra of LNCaP spheroids of different sizes were all plotted on the same graph.

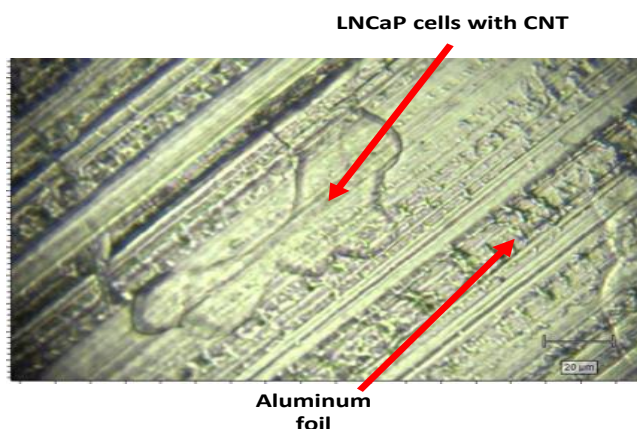
### 3.3.3 Characterization of CNT in LNCaP spheroids: probe interactions of multi-walled carbon nanotubes and cells

Based on preliminary study, results showed that functionalization minimizes defects or agglomeration of MWCNT. From this section onwards, all multi-walled carbon nanotubes that will be discussed in this section onwards were all functionalized with carboxylic acids (COOH) referred to as CNT in the following text. It was necessary to obtain the molecular fingerprint of CNT in spheroids. For photothermal therapy applications, CNT are normally conjugated with targeting molecules like peptidomimetics or antibodies for selective binding to antigens or receptors. Dr. Miltiadis Paliouras and his group have been testing CNT with peptidomimetic for targeting protein-specific membrane antigens (PSMA). In this study, CNT-PEP was characterized and compared to a control CNT-PEG. This section presents four sub studies, i) Optimize laser irradiation power for characterizing CNT in LNCaP spheroids, ii) Distinguish prominent Raman bands of CNT associated within LNCaP spheroids, iii) Obtain the molecular fingerprint of CNT in LNCaP spheroids, and iv) Compare the molecular fingerprint of CNT conjugated with peptidomimetic (CNT-PEP) and polyethylene glycol (CNT-PEG) in LNCaP spheroids.

#### 3.3.3.1 Optimize Laser Irradiation Power for Characterizing CNT in LNCaP Spheroid

The microscopy images shown in Figure 3.8 were taken with 50X objective of the confocal Raman microscopy. Before laser irradiation, the image of LNCaP cells with CNT was taken as shown in Figure 3.8a. After the laser irradiation, another image was taken to document the rupturing of cell membrane and CNT (dark color) releasing from the cells, as shown in Figure 3.8b.

a)



b)

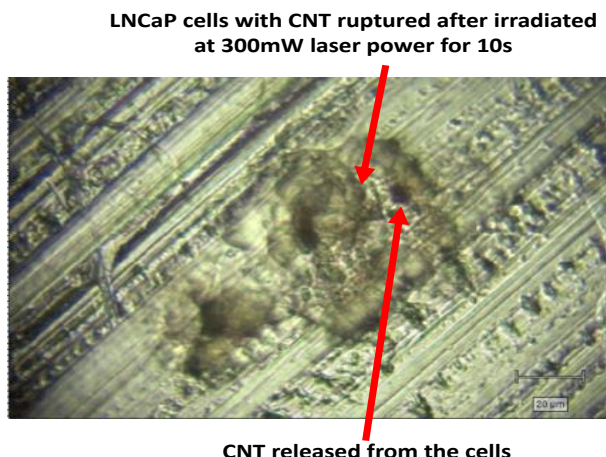


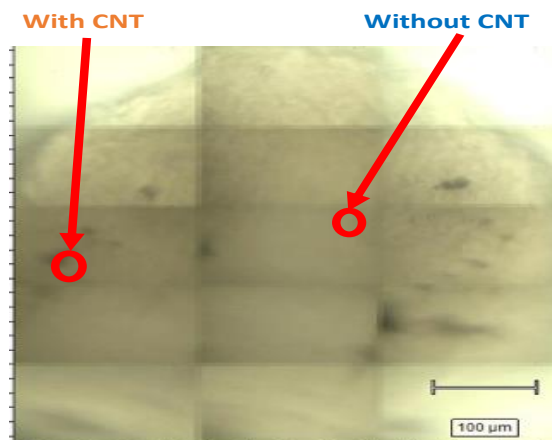
Figure 3.8 Confocal microscopy image of LNCaP cells with 0.1mg/mL of CNT a) before laser irradiation; b) after laser irradiation.

### 3.3.3.2 Distinguish Prominent Bands of CNT within LNCaP Spheroid

CNT that were present in spheroid can easily be spotted at the dark area, labeled as “with CNT”, as shown in Figure 3.9a. The area of spheroid where no CNT were present, was labeled as “no CNT”. Based on these areas, two Raman spectra were plotted on the same graph as shown in Figure 3.9b. Table 3.5 summarized all the prominent peaks identified from Figure 3.9b. At the lower region of molecular fingerprint ( $600 - 900 \text{ cm}^{-1}$ ), the two nucleic acids bands, adenine at  $721 \text{ cm}^{-1}$  and O-P-O symmetric stretching at  $783 \text{ cm}^{-1}$ , both were observed in both LNCaP spheroid and LNCaP spheroid with CNT, suggesting that nucleic acids of LNCaP spheroid were still detectable even in the presence of CNT. In the spectra of LNCaP without carbon nanotubes, the unique phenylalanine band was observed at  $1001 \text{ cm}^{-1}$  and the  $PO_2^-$  stretching is observed at  $1096 \text{ cm}^{-1}$  and a very small band of C-C stretch of lipids observed at  $1050 \text{ cm}^{-1}$ . In comparison, these prominent bands of spheroid were suppressed in the presence of CNT as shown in the spectra of LNCaP with CNT. The molecular features of CNT became visible starting from  $1200 \text{ cm}^{-1}$ . A sharp D-band of CNT was observed at  $1360 \text{ cm}^{-1}$ . When CNT were in the spheroid, D-band of CNT shifted from  $1400 \text{ cm}^{-1}$  down to  $1360 \text{ cm}^{-1}$ . In contrast, a broader sharp peak, G-band of CNT was observed at  $1610 \text{ cm}^{-1}$  shifted from  $1600 \text{ cm}^{-1}$ , when CNT were in the spheroid. Other prominent peaks of the LNCaP spheroid were observed in the region from  $1200 \text{ cm}^{-1}$  to  $1660 \text{ cm}^{-1}$ . For instance, the Raman band of  $\alpha$ -helix protein at  $1263 \text{ cm}^{-1}$  and  $1657 \text{ cm}^{-1}$ , nucleic

acids at  $1317\text{ cm}^{-1}$ , and lipids at  $1450\text{ cm}^{-1}$ . Overall, all prominent bands of CNT were detectable within the LNCaP spheroid, in the region ( $1000 - 1660\text{ cm}^{-1}$ ).

a)



b)

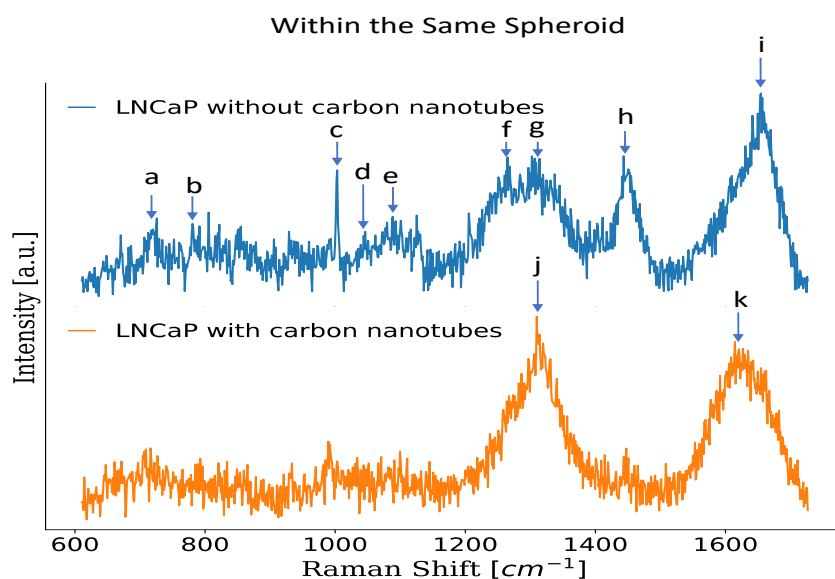


Figure 3.9(a) Confocal microscopy image showing that CNT can be detected by locating dark areas on the spheroid; (b) Raman spectra of LNCaP with carbon nanotubes and LNCaP without carbon nanotubes were plotted on the same graph. Each spectrum corresponds to selected areas shown in Figure 3.9a. Refer to Table 3.5 for the wavenumber and molecular bond assignment of prominent peaks (a, b, c, d, e, f, g, h, i, j, k).

Table 3.5 Prominent Raman bands identified from Figure 3.9b, 3.10, 3.11, and 3.12.

| Band Label | Wavenumber ( $\text{cm}^{-1}$ ) | Molecular Bond Assignment                | Molecular Species        |
|------------|---------------------------------|------------------------------------------|--------------------------|
| a          | 721                             | C-N head group of adenine                | nucleic acids (DNA, RNA) |
| b          | 783                             | O-P-O symmetric stretching               | nucleic acids (DNA, RNA) |
| c          | 1001                            | Ring breathing of phenylalanine (C-C)    | protein                  |
| d          | 1050                            | Skeletal C-C stretch                     | lipids                   |
| e          | 1096                            | ( $\text{PO}_2^-$ ) symmetric stretching | nucleic acids (DNA, RNA) |
| f          | 1263                            | Amide III (N-H)                          | $\alpha$ -helix protein  |
| g          | 1317                            | Adenine, Guanine                         | nucleic acids (DNA, RNA) |
| h          | 1450                            | $\text{CH}_2$ scissors                   | lipids                   |
| i          | 1657                            | Amide I (C=O, N-H)                       | $\alpha$ -helix protein  |
| j          | 1360                            | D-Band                                   | carbon nanotubes         |
| k          | 1610                            | G-Band                                   | carbon nanotubes         |

### Obtain the Molecular Fingerprint of CNT in LNCaP Spheroid

The molecular fingerprint of CNT at different concentrations (2.5-fold dilutions) and LNCaP spheroid is included here as a control, as illustrated in Figure 3.10. Table 3.5 summarized all the prominent peaks identified from Figure 3.10. The intensity of Raman signals increased with the concentration of CNT as shown in Figure 3.10. In Figure 3.10, the D and G-band of CNT had the same wavenumbers as those observed in Figure 3.9b. For instance, the D-band of CNT at  $1360\text{ cm}^{-1}$  and the G-band at  $1610\text{ cm}^{-1}$ . The signature of D-band was observed even at the concentration of  $0.0016\text{mg/mL}$  CNT. At the concentration of  $0.0016\text{mg/mL}$  CNT, G-band splitted into “two valleys”, and merged into a single peak at the concentration of  $0.025\text{mg/mL}$  CNT. At the concentration of  $0.0016\text{mg/mL}$  CNT, the prominent bands of LNCaP spheroid did not change

significantly. For instance, the phenylalanine band at  $1001\text{ cm}^{-1}$  along with nucleic acids bands of LNCaP spheroid at  $721\text{ cm}^{-1}$  and  $1096\text{ cm}^{-1}$  were still noticeable. But as the concentration increases, most of these nucleic acid bands disappeared. The phenylalanine band was still observed at the concentration of  $0.025\text{ mg/mL}$  CNT, the only difference was a small reduction in its intensity. The intensity of the phenylalanine band was significantly lower at  $0.01\text{ mg/mL}$ . The  $PO_2^-$  stretching observed at  $1096\text{ cm}^{-1}$  disappeared from the spectra and only C-O stretching of the DNA was spotted here. Overall, it was possible to distinguish CNT from LNCaP spheroid if the concentration of CNT is at least  $0.025\text{ mg/mL}$  or more. The molecular fingerprint of CNT in LNCaP spheroid can be defined by the prominent bands of CNT (D and G-band) and small phenylalanine band of LNCaP spheroid.

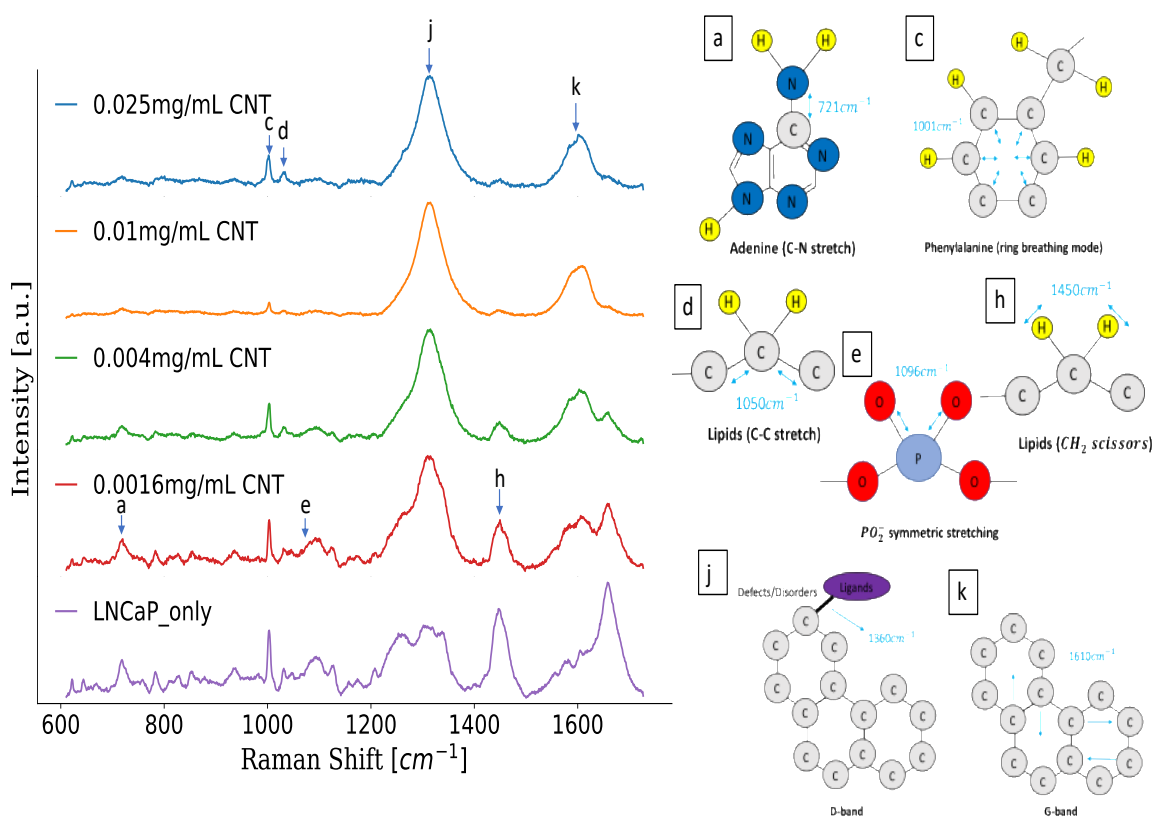


Figure 3.10 Molecular fingerprint of CNT in LNCaP spheroids. Raman spectra of different concentrations of CNT are plotted on the same graph and LNCaP is included here as a control. The prominent bands (a, c, d, e, i, g, j) vary from low to high concentration of CNT.

## Compare the Molecular Fingerprint of CNT-PEP and CNT-PEG in LNCaP Spheroid

The molecular fingerprint of CNT-PEP at different concentrations (1.25-fold dilutions) and LNCaP spheroid is included here as a control, as illustrated in Figure 3.11. The intensity of Raman signals increased with the concentration of CNT-PEP and CNT-PEG. The prominent D-band was observed at  $1360\text{ cm}^{-1}$  for both CNT-PEP and CNT-PEG. G-band was also observed at  $1610\text{ cm}^{-1}$  for CNT-PEP and CNT-PEG. Although the G-band of CNT-PEP and CNT-PEG had the same wavenumbers as CNT, but, the shape of the G-band changed dramatically for both CNT-PEP and CNT-PEG. In Figure 3.11, the tip of G-band formed into a small dent. The phenylalanine band of LNCaP spheroid at  $1001\text{ cm}^{-1}$  disappeared from  $0.03\text{mg/mL}$  onwards. Similarly, the lipids band at  $1450\text{ cm}^{-1}$  also disappeared from  $0.03\text{mg/mL}$  onwards. In comparison, the phenylalanine band of LNCaP spheroids at around  $1001\text{ cm}^{-1}$  was observed at  $0.05\text{mg/mL}$  of CNT-PEG, as shown in Figure 3.12. It is important to note that  $0.02\text{mg/mL}$  was dropped from the experiment due to instrumentation error during measurements. Fortunately, this did not affect the qualitative interpretation as long as there are sufficient concentrations for comparisons.

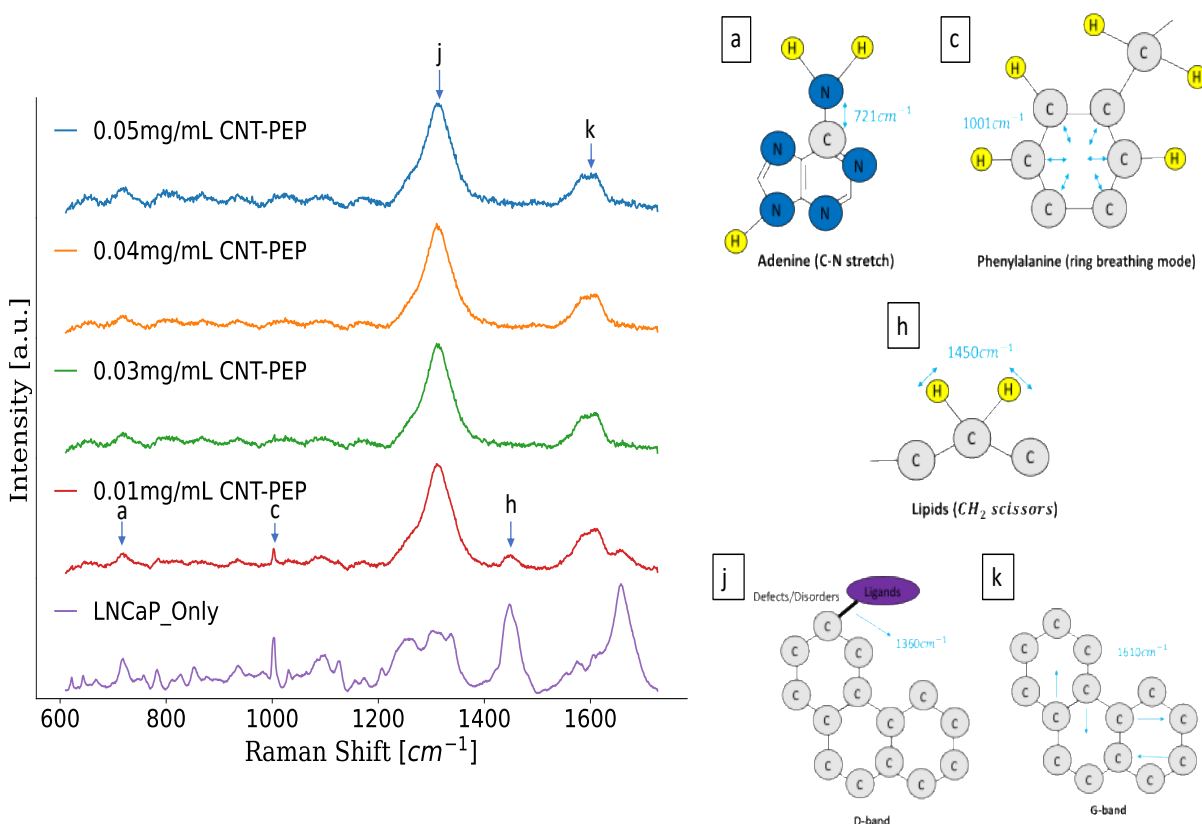


Figure 3.11 Molecular fingerprint of CNT-PEG (targeting PSMA) in LNCaP spheroid. Raman spectra of different concentrations of CNT-PEG are plotted on the same graph and LNCaP is included here as a control. The prominent bands (a, c, i, g, j) vary from low to high concentration of CNT-PEG. 0.02mg/mL was dropped from the experiment due to instrumentation error during measurements.

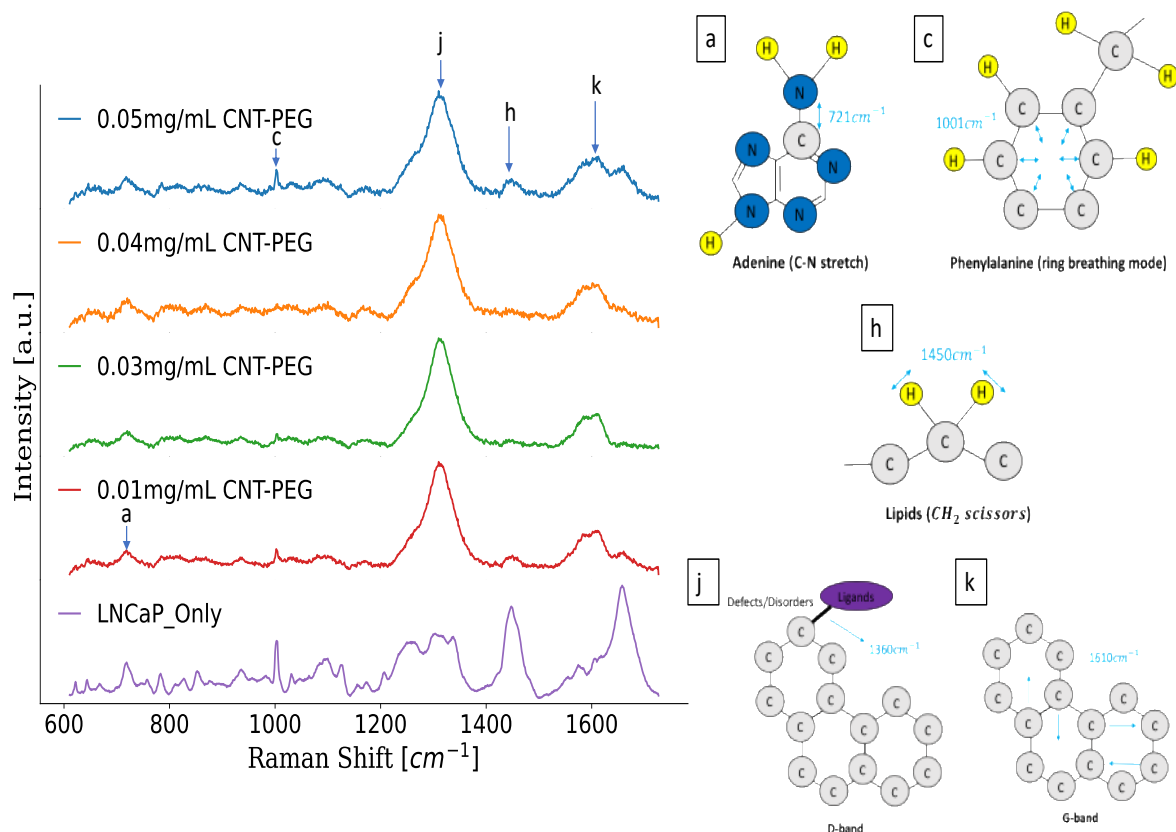
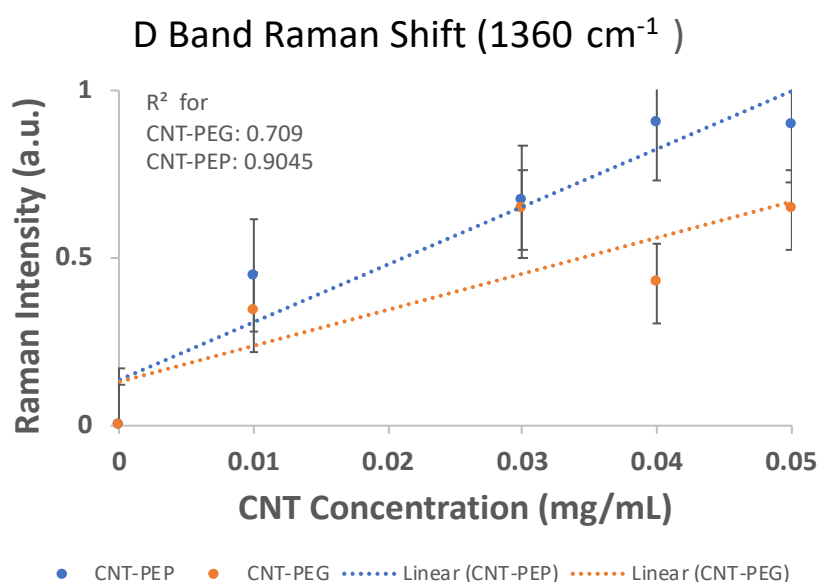


Figure 3.12 Molecular fingerprint of CNT-PEG (non-specific to PSMA) in LNCaP spheroid. Raman spectra of different concentrations of CNT-PEG are plotted on the same graph and LNCaP is included here as a control. The prominent bands (a, c, i, g, j) vary from low to high concentration of CNT-PEG. 0.02mg/mL was dropped from the experiment due to instrumentation error during measurements.

The Raman intensities of D-band were plotted against concentrations for CNT-PEG and CNT-PEG, as shown in Figure 3.13a. Figure 3.13b shows the plot of Raman intensities of G-band against the

concentrations for CNT-PEP and CNT-PEG. The linear regression analysis was applied to both plots to model the relationship between concentration and the intensity of D or G-band. The  $r^2$  were also included in both plots. Because 0.02mg/mL was dropped from the experiment, it was also excluded in linear regression analysis. In Figure 3.13a, most of D-band intensities of CNT-PEP falls along the fitted line and  $r^2$  values, were close to 0.90, suggesting that there is a correlation between the intensity of CNT-PEP and its concentration. There was no correlation between intensity for CNT-PEG and its concentration. This was also reflected in significant difference in  $r^2$  values of CNT-PEG (0.709 and 0.1759). The standard error bars of CNT-PEP and CNT-PEG in both graphs, showed there are small variations between the two repeated measurements.

a)



b)

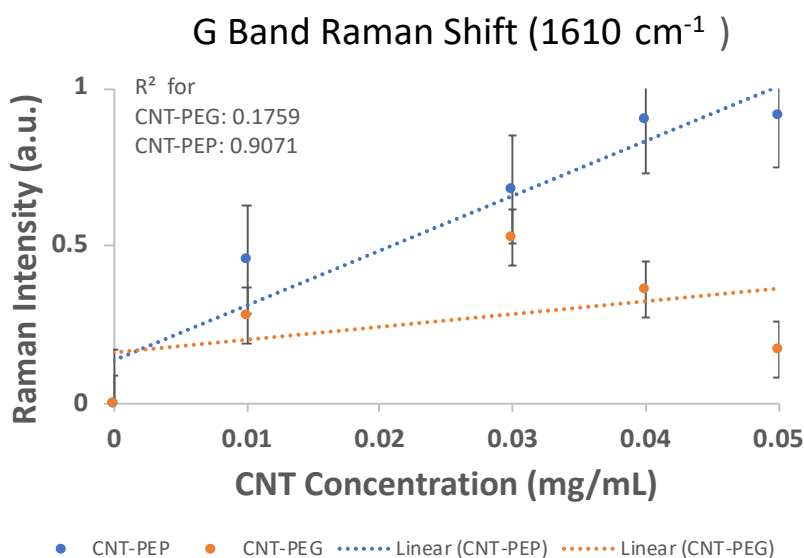


Figure 3.13 Linear Regression a) Comparing the D bands of CNT-PEP and CNT-PEG at  $1360\text{ cm}^{-1}$  ; (b) Comparing the G bands of CNT-PEP and CNT-PEG at  $1610\text{ cm}^{-1}$ . Note that  $0.02\text{mg/mL}$  was excluded from the linear regression analysis due to instrumentation error during measurements.

### 3.4 Discussions

#### 3.4.1 Preliminary study: characterize effect of functionalization on MWCNT

Dr. Miltiadis Paliouras and his group have been developing MWCNT as photothermal agents. They showed that MWCNT conjugated with prostate-specific membrane antigens antibodies enhance the nearfield cell ablation effect in LNCaP cells and reduce the potential damage around healthy tissue [22]. However, there are inherent challenges with the current design such as agglomeration of MWCNT. MWCNT are inert and tend to agglomerate due to van der Waals forces, reducing their dispersion and reinforcing effect [46]. They tend to agglomerate due to their bending stiffness and high aspect ratio [68]. This could affect the efficacy of photothermal agents at tumor sites. Recent study showed that functionalization reduces the agglomeration of carbon nanotubes and increases its dispersibility [46]. Furthermore, organic functional groups open up possibilities to conjugate with antibodies or drug molecules. This can allow tailoring properties MWCNT and

extending their application areas. Currently, Dr. Miltiadis Paliouras and his group are exploring chemical functionalization as a way to reduce agglomeration of MWCNT. By chemical functionalization, organic functional groups such as carboxylic acids are attached to the side-wall or the tip of multi-walled carbon nanotubes. Functionalized MWCNT provide possibility of further chemical derivatization which could reduce the agglomeration [46]. Confocal Raman microscopy was used in this study to determine if chemical functionalization can help to minimize agglomeration of MWCNT that hinder its application as a photothermal agent.

Multiple tiny spiky bands were observed around the D-band of the non-functionalized MWCNT (TSHR ab and IgG ab). The D/G band ratio of those two carbon nanotubes is relatively higher than COOH-functionalized MWCNT, suggesting the antibodies could be attaching to the open end of carbon nanotubes rather the side-wall of carbon nanotubes, thus disrupting the planar sheet configurations causing defects [54]. The defects lead to inter-channel scattering observed as spikey bands around D-band [69]. The molecular fingerprints of GNR are identical to the COOH. Gold nanorods are made of a capping agent, citrate, which contains three carboxylic acids. In principle, there should be a Raman band at around  $1610 - 1740\text{ cm}^{-1}$  that corresponds to these carboxylic acids. However, there is no sign of this band in the spectrum of GNR. There is a possibility that the gold nanorods (GNR) did not attach well to the surface of MWCNT, thus leading to weak Raman scattering. In contrast, the antibodies introduced additional amino acids and nucleotide base bands to MWCNT. Antibodies are made up of polypeptide chains and these chains are made of amino acids linked together to form the building blocks of proteins. The functionalized MWCNT at different concentrations have about the same D/G ratio of 1.7, which suggests that higher concentrations are needed to observe a change in D/G ratio. Based on the intensity of spectra, COOH are more detectable than non-functionalized MWCNT. It also has a lower D/G ratio, indicating less defects, thus less chances of agglomeration of carbon nanotubes. Therefore, functionalization helps to reduce defects or agglomeration of MWCNT, ensuring its function as a photothermal agent.

### **3.4.2 Characterize the molecular fingerprint of prostate cancer cells using LNCaP spheroids as a model**

Dr. Miltiadis Paliouras and his group have tested MWCNT in LNCaP cells. They will be transplanting the same cell line in mice to test the efficacy of MWCNT for photothermal ablation.

Therefore, the same prostate cell line was used in this study to generate spheroid. Spheroid exhibits greater physiological relevance than conventional 2D cell cultures and can mimic the interactions of MWCNT and cells in the target tumor tissue. The second objective of this project is to detect the prominent biomolecules associated with LNCaP cells. Laser irradiation power needs to be optimized so that biomolecules of LNCaP cells are detectable. Three laser irradiation powers, at 150mW, 30mW, and 3mW, were selected to test if prominent molecules of LNCaP cells could be detected. 150mW has been used in our lab to obtain Raman molecular fingerprints of prostate cancer tissue from patients. 30mW and 3mW are used to test if it is still possible to detect prominent molecules of LNCaP spheroids at low laser powers. The intensity of prominent molecules of LNCaP cells increases with laser irradiation power. For instance, ( $PO_2^-$ ) symmetric band of nucleic acids was distinct when acquired at 150mW compared to 30mW and 3mW. 150mW also produces a robust and sharp lipids band at  $1050\text{ cm}^{-1}$  compared to 30mW and 3mW. Nucleic acids are generally found in the cell nucleus or cytoplasm. While lipids are found in the membrane of cells. Since LNCaP spheroids were not bounded by CNTs, so the cells were basically exposed upon light irradiation. Thus, the photons generated by 150mW laser power is able to penetrate into cell without hindrance, to nucleic acids in the cytoplasm or mitochondria as well as the phenylalanine (C-C) at transport membrane proteins. At 3mW laser power, the spectra have the lowest signal-to-noise ratio, suggesting that it is not suitable for detecting prominent molecules of LNCaP cells. The irradiation power of 150mW increases the Raman intensity and signal-noise-ratio, allowing prominent molecules of LNCaP be detected. Thus, 150mW is sufficient to detect prominent biomolecules associated with LNCaP cells. For Raman characterization, the size of a spheroid needs to be optimized to obtain the molecular fingerprint of LNCaP cells. The size of a spheroid is determined by the cell seeding densities. The higher the seeding density, the larger the spheroid. Furthermore, cells expand in size as they proliferate, resulting in changes of surface area over time that could affect molecular fingerprint of LNCaP cells. In this study, three different sizes of spheroids were prepared at different cell seeding densities. The surface area of 50  $\mu\text{m}$  spheroid is too small to obtain the molecular fingerprint of LNCaP cells. On the other hand, 300 $\mu\text{m}$  spheroid loses its structure resolution because overgrown cells clumped together. Spheroids at this size are also prone to cell death due to the accumulation of toxic waste and lack of nutrients and oxygen supply to the inner core of the spheroid. Spheroid with a size of 100 $\mu\text{m}$  diameter has the best structure resolution among all sizes of spheroid. The molecular fingerprint of LNCaP spheroid did

not change significantly with size. The intensity of signals and signal-to-noise ratio do increase with size. Time acquisition also increase with size. Therefore, a spheroid with a size of 100  $\mu\text{m}$  is the optimum size for obtaining the molecular fingerprint of LNCaP cells. The size of a prostate tumor typically falls between the range of 0.3 – 2.5cm [70], which is clearly much larger than 100  $\mu\text{m}$ . Since tumors are formed from lumps of cells, the prominent bands of LNCaP cells should not shift significantly in frequency as long as the excitation wavelength (785nm) remain the same.

### **3.4.3 Characterization of CNT in LNCaP spheroids: probe interactions of multi-walled carbon nanotubes and cells**

Up till now, the molecular fingerprints of CNT and LNCaP cells have been obtained in separate studies. In this study, the aim is to be able to characterize CNT when they are bound on or buried inside LNCaP cells. Cell seeding density are optimized at  $0.0031 \times 10^6 \text{ cells}/\text{cm}^2$  to achieve a desirable spheroid size of 100  $\mu\text{m}$  for detecting the molecular fingerprint of LNCaP cells. Two different laser irradiation powers were used for previous studies. LNCaP spheroids irradiated at 150mW (50% of maximum power of 785 nm laser) for 10 seconds were able to detect signals of prominent biomolecules associated with LNCaP cells. Cells survived at 150mW laser irradiation ( $8475 \frac{\text{mW}}{\text{cm}^2}$ ). CNT that were irradiated at 300mW ( $16949 \frac{\text{mW}}{\text{cm}^2}$ ) for 10 seconds also showed a robust Raman signal. In theory, the higher the laser power, the more intense the signal. However, high laser power can directly kill the cells by destroying the cell envelope [71]. The two highest laser powers that are available on the confocal Raman microscope (Renishaw inVia, 785 nm laser) are 300mW and 150mW. This raises the question of whether 300mW or 150mW, should be used when characterizing CNT on spheroids without triggering cell death in the LNCaP cells. Since the spheroids have been tested with 150mW, an optimization study was established to test if spheroids could survive at 300mW. For optimization study, LNCaP cells were used instead of spheroids because it is easier to observe the shape and condition of cells when they are grown on a flat surface in comparison to agglomeration of cells. Functionalization MWCNT (CNT) have shown significant reduction in agglomeration of MWCNT. LNCaP cells were incubated with CNT at a concentration of 0.1mg/mL to ensure that cells are covered with CNT. Cells were then irradiated at 300mW laser power for 10 seconds. LNCaP cells have an irregular shape, viable, and intact in the aluminum foil before laser irradiation [72]. After 10 seconds of 300mW laser irradiation, the cell envelopes begin to rupture and cell walls can no longer stay intact. Eventually, cells collapsed

leading to cell death, releasing CNT from cells into its external environment. This observation concludes that 150mW ( $8475 \frac{mW}{cm^2}$ ) is the best irradiation laser power for characterizing CNT in spheroids without disrupting the cell membranes. It should be noted that this study tested 300mW and 150mW because of the limitations of instrumentation. If the laser power is not constrained, it is recommended to test CNT in LNCaP spheroid at a range between 150mW to 280mW to determine the maximum laser power to extract the best possible Raman signals.

When CNT are added to spheroids, they could either be internalized by spheroids, bind onto the surface of spheroid, or even absorb or penetrate into the interior of spheroids [73]. If 150mW is sufficient to induce strong scattering from this CNT, the molecular fingerprint of CNT should be detectable and differentiable even within the LNCaP spheroid. To test this hypothesis, LNCaP spheroids were injected with CNT and irradiated at 150mW. D-band and G-band, are only detectable in the spectra labeled with “LNCaP with carbon nanotubes”, suggesting the D and G-band are specific to carbon nanotubes or CNTs. The positions of prominent bands do however shift when CNT are in spheroids. For instance, the D-band shifted from  $1400 \text{ cm}^{-1}$  down to  $1360 \text{ cm}^{-1}$ . In contrast, the G-band of CNT shifted from  $1600 \text{ cm}^{-1}$  up to  $1610 \text{ cm}^{-1}$ . In Raman spectra, the Raman shift is basically the frequency shift from the original light beam and the Raman scattered light. The shifting of bands, whether to lower or higher wavenumbers, is related to the length of chemical bonds of the molecules. A shorter bond length causes higher Raman shift. For instance, O-H bond with length of 96 picometer has observed at a Raman shift of  $3250 \text{ cm}^{-1}$ , whereas a C=C bond of 134 picometer observed a lower Raman shift, at  $1655 \text{ cm}^{-1}$ . In this case, the shift of D-band is due to the change in the bond length, contributed by the addition of COOH at the surface of carbon nanotubes. On the other hand, the small shift of the G-band is due to hybridization at  $sp_2$  carbon. The nucleic acid and lipids bands of LNCaP spheroid, at  $721 \text{ cm}^{-1}$ ,  $783 \text{ cm}^{-1}$ ,  $1450 \text{ cm}^{-1}$  could still be detectable in the presence of CNT. Indeed, 150mW laser power is sufficient for obtaining strong prominent bands of CNT at fingerprint region ( $1000 - 1660 \text{ cm}^{-1}$ ) even when CNT are bound on spheroids. The molecular fingerprint of CNT is distinguishable even within the same spheroid. It is important to note that the spectra shown is only from the selected area for simplicity. As a result, the signal to noise ratio is low.

D or G-band of CNT do change when they are uptake by spheroids. At the lowest CNT concentration (0.0016mg/mL), the distinct D and G-band could still be detected. But the spectra are dominated mostly by the prominent bands of LNCaP cells. Since there are less CNT uptake by spheroids, most of the areas of spheroids do not have CNT, so the higher the chances of molecules in those areas are prone to stretching upon light irradiation, distorting the electron cloud that leads to scattering. As the CNT concentration increases, prominent bands of LNCaP cells are gradually suppressed due to presence of more CNT hindering the vibrational modes of molecules, which explains why the D and G-band becomes more evident. CNT without target ligands tend to bind onto cells via integrin receptor, facilitated by the adsorption of serum and cell-secreted adhesive extracellular matrix (ECM) proteins [74]. At the highest CNT concentration (0.025mg/mL), the intensity of D and G-band also increases. The phenylalanine (C-C) band and lipids are still noticeable in the spectra. Large neutral amino acids like phenylalanine transport through cell membranes into cytoplasm via membrane transport proteins [75]. There is a possibility that CNT uptake by spheroids are not blocking all membrane transport proteins of cells, thus scattering from phenylalanine could still be detected. At 0.01mg/mL, the intensity of the phenylalanine band is significantly lower than the phenylalanine band at 0.025mg/mL, possibly because there is less phenylalanine detected in this area. Subcellular localization of carbon nanotubes depends on the size of CNT. A study on the intracellular uptake of CNT have shown that multiwalled-carbon nanotubes (0.5-2 $\mu$ m in length, 10-30 nm in diameter) are excluded from the interior of the cell and only single-walled nanotubes of length 50-100 nm can partly reside in the cell nucleus [76]. Lipids resided on the cell membrane are detected in the spectra. As the concentration of CNT increases, the intensity of prominent bands also increases. In summary, the concentration of CNT does affect the molecular fingerprint. The minimal detectable concentration of CNT is 0.025mg/mL. The intensity of phenylalanine and lipids band is expected to drop gradually when the concentration of CNT increases. The molecular fingerprint of CNT in LNCaP spheroid can be defined by the prominent bands D and G-band, phenylalanine band of LNCaP spheroid, and skeletal C-C stretch of lipids. These prominent bands can be used as “markers” to probe any molecular changes that happen when CNT are bound on the spheroids.

Photothermal therapy relies on the activation of photothermal agents (carbon nanotubes) upon laser irradiation. Its effectiveness depends strongly on binding of ligands of carbon nanotubes to specific

sites of tumor. Also, the concentration of carbon nanotubes is important to ensure there are sufficient to induce localized heating around tumor sites [77]. Dr. Miltiadis Paliouras and his group have been developing MWCNT as photothermal agents and testing MWCNT with different ligands for targeting prostate-specific membrane antigens expressed by prostate cancer cells. Third objective of this project is to be able to probe interactions of carbon nanotubes and cells. CNT were detectable at concentration of 0.025mg/mL or higher. In this study, CNT were conjugated with peptidomimetics (CNT-PEP) that were modified to bind PSMA and also with polyethylene glycol (CNT-PEG) as a control. Peptidomimetic, is a small molecule with modification of peptide sequences that improve biological properties and the replacement of the peptide backbone with a molecular scaffold [78]. They are designed to mimic peptides, known to be stable, non-toxic, and easily absorbed by prostate cancer cells [79]. They have a strong affinity and selectivity for PSMA that are expressed on the surface of LNCaP spheroid and in contrast, PEG do not bind specifically to PSMA. To probe for interactions between CNT-PEP and LNCaP cells, it is crucial to identify molecular changes between CNT-PEP and CNT-PEG. The prominent D and G-bands of CNT are observed in the Raman spectra of CNT-PEP and CNT-PEG. However, there is a small dent in the tip of G-band CNT-PEP. Peptides of peptidomimetics can be trapped by CNT through van der Waals interactions [80]. The trapping of peptide at CNT could affect the in-plane vibration of the  $sp^2$  bonded carbon, creating a small dent in the tip of G-band. For CNT-PEP, the phenylalanine band of LNCaP spheroid disappears starting from 0.03mg/mL. CNT-PEP binds favorably to the prostate-specific membrane antigens expressed on the cell surface [81], so there is a possibility that, at 0.03mg/mL, there is sufficient PEP binding to PSMA at the membrane of LNCaP, hindering the ring-breathing of phenylalanine. In comparison, CNT, which bind non-specifically to any molecules, still have the phenylalanine band at 0.025mg/mL. Similarly, CNT-PEG also has the phenylalanine band even at 0.05mg/mL. Polyethylene glycol (PEG) has a molecular weight of 2 kDa and 0.35nm [82]. The light weight of PEG does not constrain the  $CH_2$  scissors of lipids, this it is still detectable in CNT-PEG at 0.05mg/mL. In summary, these molecular features specific to each type of CNT (PEP, PEG), can be described as “markers”, define the unique molecular fingerprint of CNT-PEP and CNT-PEG. For instance, the unique shape of G-band and the absence of phenylalanine band is only observed in CNT-PEP. The minimal detectable concentration of CNT-PEP is 0.03mg/mL.

The intensity of D-band at  $1360\text{ cm}^{-1}$  and G-band at  $1610\text{ cm}^{-1}$  increase with the concentration of CNT-PEP. Further linear regression analysis reveals the correlation between the concentration of CNT-PEP and Raman intensity. Most of the points of CNT-PEP fall on the regression line and  $r^2$  values are relatively higher, suggesting that CNT-PEP bind specifically to LNCaP cells. In contrast, there is no linear correlation between the Raman intensity and the concentration of CNT-PEG, suggesting the non-specific binding of CNT-PEG to LNCaP cells were removed during the washing. PEG behaves like weak organic solvent and tend to interact unfavorably with polar and nonpolar charged groups such as phospholipids on cell surface [83]. Their unfavorable interactions with phospholipids constrain their binding on the surface of spheroids. Linear regression analysis also showed that the intensity of D and G band of CNT-PEP at  $0.04\text{ mg/mL}$  is approaching a plateau, indicating that it is close to the steady state. The maximal detectable concentration of CNT-PEP is expected at higher concentration than  $0.04\text{ mg/mL}$ . Linear regression analysis does not explain if PEP binds specifically to PSMA, so kinetic binding assays of CNT-PEP are required to evaluate the binding affinity of PEP to PSMA.

## CHAPTER 4 CONCLUSION AND FUTURE RESEARCH

Since the last decade, rapid development of MWCNT has promoted a significant increase of interests in MWCNT as photothermal agents. Recent study showed that MWCNT combined with 1064nm laser can elevate the local temperature of the target tumor cells to induce apoptosis, without the need to increase the laser irradiation power, minimizing thermal damage around healthy tissue. Despite of its growth, MWCNT are still facing a significant challenge. Particularly, the lack of characterization of MWCNT in cells, is hindering its development for in vivo photothermal therapy. The characterization of MWCNT in cells is important because it reveals the binding interactions of MWCNT with tumor cells. A poor binding interaction of MWCNT and cells can lessen the efficacy of MWCNT as a photothermal agent. To assess the binding interactions of MWCNT and cells, a confocal Raman microscopy was proposed as the method to characterize MWCNT in cells. The aim of this thesis was to obtain the molecular profiles of MWCNT in prostate cancer cells (LNCaP) as well as identifying its minimal detectable concentration, using confocal Raman microscopy.

All the three main objectives of the project have been achieved and two hypotheses have been answered. The preliminary study was included here to test if functionalization could help to solve the issue of agglomeration of carbon nanotubes. The changes in vibrational features (band frequencies) and intensity enhancement of D and G-bands revealed that functionalization increases the detectability of MWCNT. D/G band ratio computed from the intensity of D and G-bands disclosed the structural defects of non-functionalized MWCNT. These results confirmed that functionalization of MWCNT is necessary to minimize the agglomeration of MWCNT before conjugating with ligands, which in turn, improves its detectability in Raman. LNCaP cells will be transplanted into healthy mice to form tumors. Hence, the same cell line (LNCaP cells) was used in this study to enable us to understand how MWCNT actually works in vivo. Confocal Raman microscopy was able to capture the full molecular fingerprint of MWCNT in cells and distinguish its molecular fingerprint of MWCNT from cells. The changes in band frequencies and intensity of prominent MWCNT bands along with bands of nucleic acids and proteins, offers insights into the binding interactions of MWCNT and cells. By probing the interactions of CNT-PEP/PEG and cells, it revealed unique fingerprint pertaining to CNT-PEP (specific binding to PSMA) and CNT-PEG (control). These unique fingerprints allowed us to verify the binding between MWCNT and cells

in the target tumor tissue in vivo. As mentioned earlier, this project is the preliminary phase of a grand project as described in Figure 4.1. The proposed grand project consists of four phases (preliminary, Phase I, Phase II, Phase III), utilize the dual functionality of MWCNT (CNT-PEP) combined with the sensitivity of Raman spectroscopy to carbon nanotubes and versatility of fluorescence, to enhance the efficacy of photothermal ablation. The minimal detectable concentration of CNT-PEP identified from the preliminary phase will allow us to set the range of concentrations to obtain the maximum detectable concentration of CNT-PEP. Both concentrations are necessary for plotting the calibration curve that will be used to quantify the actual concentration of MWCNT present on the target tumor in vivo, as part of Phase I and II. In parallel, we will coordinate with Dr. Miltiadis Paliouras's lab to transfer the mice over to CRCHUM so we can transplant LNCaP cells into the mice, incubate mice with CNT-PEP, and harvest the prostate tissues. Currently, our group is still working to set up a portable imaging system that contains an image box equipped with two lasers (785nm and 1064nm) and an InGaAs camera for detecting near-infrared wavelengths (800 – 2500nm). In the meantime, a well-established portable single-point system (785nm) is available for characterizing the fresh prostate tissue harvested from mice. The goal here is to compare the prominent peaks of CNT-PEP in these tumor tissue with the preliminary data to ensure their frequencies do not shift significantly. With this system and portable single-point system, we envision to integrate both systems under the same platform for Phase II, as illustrated in Figure 4.2. Since MWCNT and clinically approved indocyanine green fluorescence probe (ICG) both can emit at different wavelengths (ICG: 814nm and MWCNT: 800 – 1064nm), this sparked the idea of developing CNT- PEP-ICG as a contrast agent for photothermal therapy. First, mice will be anesthetized with isoflurane before the injection. Once the mice are anesthetized, they will be injected with a solution containing CNT-PEP and a fluorescence probe (ICG) at different concentrations via intravenous administration (tail vein). CNT-PEP and ICG will travel along the blood vessels before they cross the barrier between blood vessels and cells, and bound onto PSMA expressed on the LNCaP cells in the target tumor tissue. 785nm will be used to excite the fluorescence emission of ICG to visualize the target tumor. Once the tumor location is identified, 785nm Raman probe will be used to confirm CNT-PEP binding at the target tumor and to determine the actual amount of CNT-PEP that present at the target tumor. Finally, the laser ablation will be performed (under anesthesia) using 1064nm with different laser irradiations and timepoints, to determine the best parameter to induce heat at the target tumor to trigger apoptosis

for destroying tumor cells. The tumor volumes will then be analyzed as four independent groups (CNT-PEP + laser, laser only, CNT-PEP only, no treatment). Mice will be monitored with fluorescence imaging for the next following days to assess the efficacy of PTT treatment and any adverse effects. Cellular toxicity and immunological effects of CNT-PEP will be assessed in a separate study to support the Phase II data. All the results from Phase II will help us to assess the efficacy of photothermal agent in vivo and improve our imaging system. Phase III is no doubt the most challenging phases among all due to hurdles of clinical trials such as patient's recruitment and data collection. In terms of technical aspects, we would need to optimize: 1) the imaging system to adapt to surgical robotics (require surgical incision to perform ablation of prostate tumor accurately), 2) the laser irradiation power, and 3) the concentration of CNT-PEP. Figure 4.2 depicts an illustration of Phase III we visualize. An imaging system that equipped with both NIR and visible laser, instead of dual laser system. Also, the photothermal agent (CNT-PEP and ICG) solution will be injected through IV pumps.

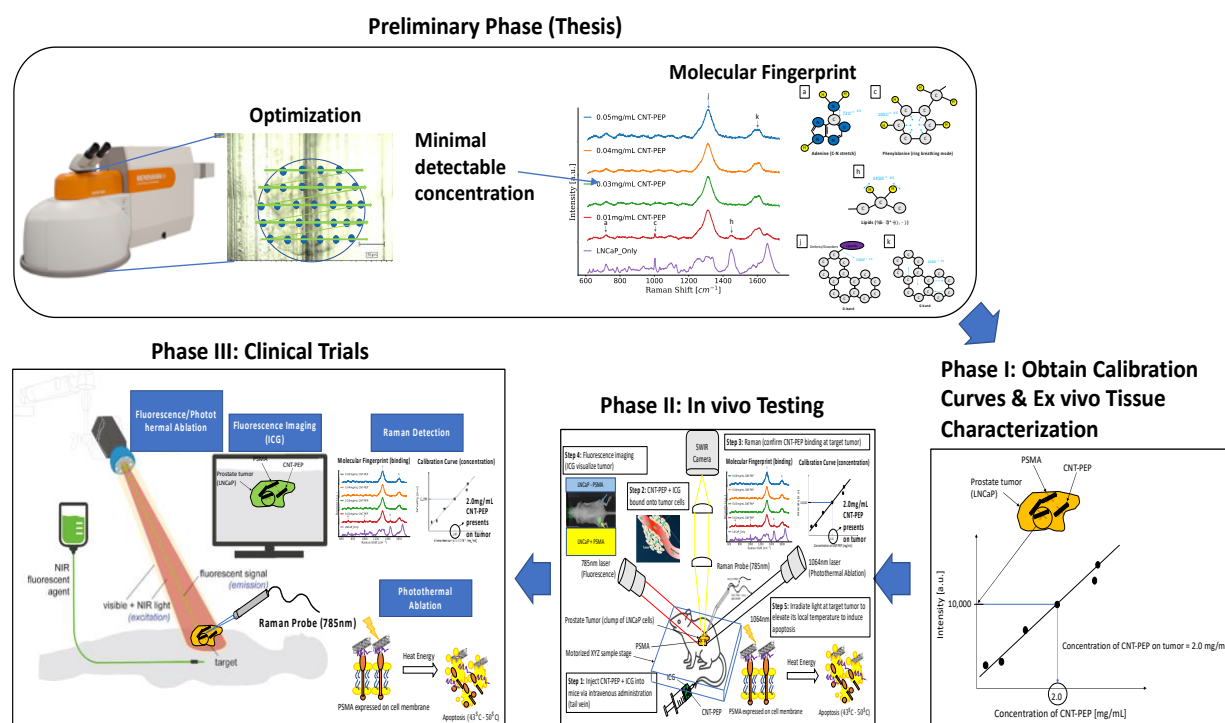


Figure 4.1 Illustration of Proposed Grand Project Phases (Preliminary, Phase I, Phase II, Phase III) (Adapted from [1], [2], [3]).

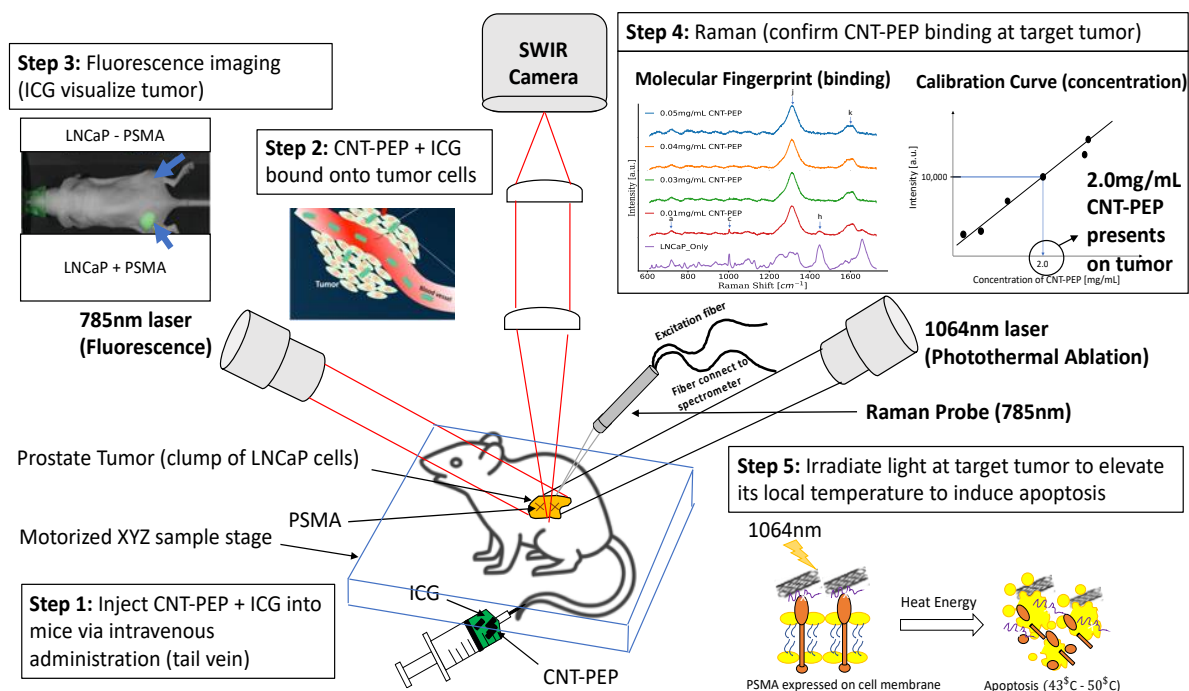


Figure 4.2 Illustration of Proposed Integration System (Raman/Fluorescence Imaging and Photothermal Ablation) for Phase II (Adapted from [1] and [2]).

## BIBLIOGRAPHY

- [1] T. T. V. Phan *et al.*, "Photoacoustic Imaging-Guided Photothermal Therapy with Tumor-Targeting HA-FeOOH@PPy Nanorods," *Sci Rep*, vol. 8, no. 1, p. 8809, Jun 11 2018, doi: 10.1038/s41598-018-27204-8.
- [2] T. Nakajima, M. Mitsunaga, N. H. Bander, W. D. Heston, P. L. Choyke, and H. Kobayashi, "Targeted, activatable, in vivo fluorescence imaging of prostate-specific membrane antigen (PSMA) positive tumors using the quenched humanized J591 antibody-indocyanine green (ICG) conjugate," *Bioconjug Chem*, vol. 22, no. 8, pp. 1700-5, Aug 17 2011, doi: 10.1021/bc2002715.
- [3] H. A. Galema *et al.*, "Fluorescence-guided surgery in colorectal cancer; A review on clinical results and future perspectives," *Eur J Surg Oncol*, vol. 48, no. 4, pp. 810-821, Apr 2022, doi: 10.1016/j.ejso.2021.10.005.
- [4] C. C. Society. "Prostate cancer statistics." <https://cancer.ca/en/cancer-information/cancer-types/prostate/statistics> (accessed 2023).
- [5] R. J. Rebello *et al.*, "Prostate cancer," *Nat Rev Dis Primers*, vol. 7, no. 1, p. 9, Feb 4 2021, doi: 10.1038/s41572-020-00243-0.
- [6] C. A. Heinlein and C. Chang, "Androgen receptor in prostate cancer," *Endocr Rev*, vol. 25, no. 2, pp. 276-308, Apr 2004, doi: 10.1210/er.2002-0032.
- [7] M. Eiber *et al.*, "Prostate-Specific Membrane Antigen Ligands for Imaging and Therapy," *J Nucl Med*, vol. 58, no. Suppl 2, pp. 67S-76S, Sep 2017, doi: 10.2967/jnumed.116.186767.
- [8] M. Bath, "The Prostate Gland," ed. TeachMe Anatomy, 2019.
- [9] J. M. Stern, V. V. Kibanov Solomonov, E. Sazykina, J. A. Schwartz, S. C. Gad, and G. P. Goodrich, "Initial Evaluation of the Safety of Nanoshell-Directed Photothermal Therapy in the Treatment of Prostate Disease," *Int J Toxicol*, vol. 35, no. 1, pp. 38-46, Jan-Feb 2016, doi: 10.1177/1091581815600170.
- [10] D. F. Penson *et al.*, "5-year urinary and sexual outcomes after radical prostatectomy: results from the prostate cancer outcomes study," *J Urol*, vol. 173, no. 5, pp. 1701-5, May 2005, doi: 10.1097/01.ju.0000154637.38262.3a.
- [11] T. J. Wilt, R. MacDonald, I. Rutks, T. A. Shamliyan, B. C. Taylor, and R. L. Kane, "Systematic review: comparative effectiveness and harms of treatments for clinically localized prostate cancer," *Ann Intern Med*, vol. 148, no. 6, pp. 435-48, Mar 18 2008, doi: 10.7326/0003-4819-148-6-200803180-00209.
- [12] E. A. o. Urology, "Radiation Therapy," F. 1, Ed., ed. European Association of Urology Patient Information, 2023.
- [13] P. Colin *et al.*, "Focal laser ablation of prostate cancer: definition, needs, and future," *Adv Urol*, vol. 2012, p. 589160, 2012, doi: 10.1155/2012/589160.
- [14] A. Rakauskas *et al.*, "Focal Therapy for Prostate Cancer: Complications and Their Treatment," *Front Surg*, vol. 8, p. 696242, 2021, doi: 10.3389/fsurg.2021.696242.

- [15] S. Nomura *et al.*, "Highly reliable, targeted photothermal cancer therapy combined with thermal dosimetry using a near-infrared absorbent," *Sci Rep*, vol. 10, no. 1, p. 9765, Jun 17 2020, doi: 10.1038/s41598-020-66646-x.
- [16] J. R. Melamed, R. S. Edelstein, and E. S. Day, "Elucidating the fundamental mechanisms of cell death triggered by photothermal therapy," *ACS Nano*, vol. 9, no. 1, pp. 6-11, Jan 27 2015, doi: 10.1021/acsnano.5b00021.
- [17] M. Kim, G. Kim, D. Kim, J. Yoo, D. K. Kim, and H. Kim, "Numerical Study on Effective Conditions for the Induction of Apoptotic Temperatures for Various Tumor Aspect Ratios Using a Single Continuous-Wave Laser in Photothermal Therapy Using Gold Nanorods," *Cancers (Basel)*, vol. 11, no. 6, May 31 2019, doi: 10.3390/cancers11060764.
- [18] J. Kim *et al.*, "Gold nanoparticle clusters for the investigation of therapeutic efficiency against prostate cancer under near-infrared irradiation," *Nano Converge*, vol. 7, no. 1, p. 5, Feb 17 2020, doi: 10.1186/s40580-019-0216-z.
- [19] S. Cui *et al.*, "Ultra-homogeneous NIR-II fluorescent self-assembled nanoprobe with AIE properties for photothermal therapy of prostate cancer," *Nanoscale*, vol. 13, no. 37, pp. 15569-15575, Oct 1 2021, doi: 10.1039/d1nr04227k.
- [20] A. R. Rastinehad *et al.*, "Gold nanoshell-localized photothermal ablation of prostate tumors in a clinical pilot device study," *Proc Natl Acad Sci U S A*, vol. 116, no. 37, pp. 18590-18596, Sep 10 2019, doi: 10.1073/pnas.1906929116.
- [21] J. W. Fisher *et al.*, "Photothermal response of human and murine cancer cells to multiwalled carbon nanotubes after laser irradiation," *Cancer Res*, vol. 70, no. 23, pp. 9855-64, Dec 1 2010, doi: 10.1158/0008-5472.CAN-10-0250.
- [22] P. J. R. Seung S Lee, Paresa N Giannopoulos, Elliot J Mitmaker, Michael Tamilia, Miltiadis Paliouras, and Mark A Trifiro, "Prostate-specific membrane antigen– directed nanoparticle targeting for extreme nearfield ablation of prostate cancer cells," *Tumor Biology*, pp. 1-12, 2017, doi: 10.177/101042831769543.
- [23] W. Jin, C. Dong, D. Yang, R. Zhang, T. Jiang, and D. Wu, "Nano-Carriers of Combination Tumor Physical Stimuli-Responsive Therapies," *Curr Drug Deliv*, vol. 17, no. 7, pp. 577-587, 2020, doi: 10.2174/1567201817666200525004225.
- [24] D. Cai *et al.*, "Interaction between carbon nanotubes and mammalian cells: characterization by flow cytometry and application," *Nanotechnology*, vol. 19, no. 34, pp. 1-10, Aug 27 2008, doi: 10.1088/0957-4484/19/34/345102.
- [25] A. I. Lopez-Lorente, B. M. Simonet, and M. Valcarcel, "Raman spectroscopic characterization of single walled carbon nanotubes: influence of the sample aggregation state," *Analyst*, vol. 139, no. 1, pp. 290-8, Jan 7 2014, doi: 10.1039/c3an00642e.
- [26] D. O. Grajales Lopera *et al.*, "Image-guided Raman spectroscopy navigation system to improve transperineal prostate cancer detection. Part 2: in-vivo tumor-targeting using a classification model combining spectral and MRI-radiomics features," *J Biomed Opt*, vol. 27, no. 9, Sep 2022, doi: 10.1117/1.JBO.27.9.095004.

- [27] M. Pinto *et al.*, "Integration of a Raman spectroscopy system to a robotic-assisted surgical system for real-time tissue characterization during radical prostatectomy procedures," *J Biomed Opt*, vol. 24, no. 2, pp. 1-10, Feb 2019, doi: 10.1117/1.JBO.24.2.025001.
- [28] K. Aubertin *et al.*, "Mesoscopic characterization of prostate cancer using Raman spectroscopy: potential for diagnostics and therapeutics," *BJU Int*, vol. 122, no. 2, pp. 326-336, Aug 2018, doi: 10.1111/bju.14199.
- [29] A. A. Grosset *et al.*, "Identification of intraductal carcinoma of the prostate on tissue specimens using Raman micro-spectroscopy: A diagnostic accuracy case-control study with multicohort validation," *PLoS Med*, vol. 17, no. 8, p. e1003281, Aug 2020, doi: 10.1371/journal.pmed.1003281.
- [30] J. Toporski, T. Dieing, and C. Heim, "Raman Microscopy (Confocal)," in *Encyclopedia of Geobiology*, (Encyclopedia of Earth Sciences Series, 2011, ch. Chapter 173, pp. 754-761.
- [31] S. Gomes da Costa, A. Richter, U. Schmidt, S. Breuninger, and O. Hollricher, "Confocal Raman microscopy in life sciences," *Morphologie*, vol. 103, no. 341, pp. 11-16, Mar 2019, doi: 10.1016/j.morpho.2018.12.003.
- [32] M. S. Bergholt, A. Serio, and M. B. Albro, "Raman Spectroscopy: Guiding Light for the Extracellular Matrix," *Front Bioeng Biotechnol*, vol. 7, p. 303, 2019, doi: 10.3389/fbioe.2019.00303.
- [33] T. Dieing, O. Hollricher, and J. Toporski, <Confocal Raman Microscopy Book.pdf> (Springer Series in Optical Sciences). Springer, 2010.
- [34] E. Instruments. "How to Reduce Fluorescence in Raman Spectroscopy." <https://www.edinst.com/how-to-reduce-fluorescence-in-raman-spectroscopy/> (accessed 2023).
- [35] Renishaw, "StreamLine™: generate chemical images rapidly," ed. Renishaw.
- [36] S. Dunst and P. Tomancak, "Imaging Flies by Fluorescence Microscopy: Principles, Technologies, and Applications," *Genetics*, vol. 211, no. 1, pp. 15-34, Jan 2019, doi: 10.1534/genetics.118.300227.
- [37] M. Abramowitz and M. W. Davidson, "Image Formation," D. P. o. N. a. W. Slits, Ed., ed. Evident.
- [38] TUBALL. "Single-walled carbon nanotubes: structure, properties, applications, and health & safety." [https://tuball.com/pages/single-walled-carbon-nanotubes?gclid=CjwKCAjw2bmLBhBREiwAZ6ugo4ZATWdOvvE2MHAM3A-NXAnHVftjStuQBE5m6CBXYaERhnaYphMxphoCZuUQAvD\\_BwE](https://tuball.com/pages/single-walled-carbon-nanotubes?gclid=CjwKCAjw2bmLBhBREiwAZ6ugo4ZATWdOvvE2MHAM3A-NXAnHVftjStuQBE5m6CBXYaERhnaYphMxphoCZuUQAvD_BwE) (accessed.
- [39] B. Abdallah, A. M. A. Elhissi, W. Ahmed, and M. Najlah, "Carbon nanotubes drug delivery system for cancer treatment," in *Advances in Medical and Surgical Engineering*, 2020, pp. 313-332.
- [40] I. S. Nefedov, M. V. Davidovich, O. E. Glukhova, M. M. Slepchenkov, and J. M. Rubi, "Radiative heat transfer between two carbon nanotubes," *Sci Rep*, vol. 12, no. 1, p. 17930, Oct 26 2022, doi: 10.1038/s41598-022-22138-8.

- [41] Z. Han and A. Fina, "Thermal conductivity of carbon nanotubes and their polymer nanocomposites: A review," *Progress in Polymer Science*, vol. 36, no. 7, pp. 914-944, 2011, doi: 10.1016/j.progpolymsci.2010.11.004.
- [42] Y. K. Koh, "Thermal Conductivity and Phonon Transport," in *Encyclopedia of Nanotechnology*, B. Bhushan Ed. Dordrecht: Springer Netherlands, 2012, pp. 2704-2711.
- [43] W. Zhang, Z. Zhu, F. Wang, T. Wang, L. Sun, and Z. Wang, "Chirality dependence of the thermal conductivity of carbon nanotubes," *Nanotechnology*, vol. 15, no. 8, pp. 936-939, 2004, doi: 10.1088/0957-4484/15/8/010.
- [44] S. Y. Yue, T. Ouyang, and M. Hu, "Diameter Dependence of Lattice Thermal Conductivity of Single-Walled Carbon Nanotubes: Study from Ab Initio," *Sci Rep*, vol. 5, p. 15440, Oct 22 2015, doi: 10.1038/srep15440.
- [45] M. Khalkhali, F. Khoeini, and A. Rajabpour, "Thermal transport in silicene nanotubes: Effects of length, grain boundary and strain," *International Journal of Heat and Mass Transfer*, vol. 134, pp. 503-510, 2019, doi: 10.1016/j.ijheatmasstransfer.2019.01.074.
- [46] R. Dubey, D. Dutta, A. Sarkar, and P. Chattopadhyay, "Functionalized carbon nanotubes: synthesis, properties and applications in water purification, drug delivery, and material and biomedical sciences," *Nanoscale Adv*, vol. 3, no. 20, pp. 5722-5744, Oct 12 2021, doi: 10.1039/d1na00293g.
- [47] G. Bounos, K. S. Andrikopoulos, T. K. Karachalios, and G. A. Voyiatzis, "Evaluation of multi-walled carbon nanotube concentrations in polymer nanocomposites by Raman spectroscopy," *Carbon*, vol. 76, pp. 301-309, 2014, doi: 10.1016/j.carbon.2014.04.081.
- [48] M. S. Dresselhaus and P. C. Eklund, "Phonons in carbon nanotubes," *Advances in Physics*, vol. 49, no. 6, pp. 705-814, 2000, doi: 10.1080/000187300413184.
- [49] X. Zhao, Y. Ando, L. Qin, H. Kataura, Y. Maniwa, and R. Saito, "Radial breathing modes of multiwalled carbon nanotubes," *Chemical Physics Letters*, vol. 361, pp. 169 - 174, 2002.
- [50] S. Botti, A. Rufoloni, T. Rindzevicius, and M. S. Schmidt, "Surface-Enhanced Raman Spectroscopy Characterization of Pristine and Functionalized Carbon Nanotubes and Graphene," in *Raman Spectroscopy*, 2018, ch. Chapter 10.
- [51] X. Zhao, Y. Ando, L.-C. Qin, H. Kataura, Y. Maniwa, and R. Saito, "Multiple splitting of G-band modes from individual multiwalled carbon nanotubes," *Applied Physics Letters*, vol. 81, no. 14, pp. 2550-2552, 2002, doi: 10.1063/1.1502196.
- [52] J. H. Lehman, M. Terrones, E. Mansfield, K. E. Hurst, and V. Meunier, "Evaluating the characteristics of multiwall carbon nanotubes," *Carbon*, vol. 49, no. 8, pp. 2581-2602, 2011, doi: 10.1016/j.carbon.2011.03.028.
- [53] R. N. Gontijo, G. C. Resende, C. Fantini, and B. R. Carvalho, "Double resonance Raman scattering process in 2D materials," *Journal of Materials Research*, vol. 34, no. 12, pp. 1976-1992, 2019, doi: 10.1557/jmr.2019.167.
- [54] J. Hodkiewicz. "Rapid Quality Screening of Carbon Nanotubes with Raman Spectroscopy." <https://assets.thermofisher.com/TFS-Assets/CAD/Application-Notes/D19506~.pdf> (accessed 2023).

- [55] C. C. Kao and R. J. Young, "A Raman spectroscopic investigation of heating effects and the deformation behaviour of epoxy/SWNT composites," *Composites Science and Technology*, vol. 64, no. 15, pp. 2291-2295, 2004, doi: 10.1016/j.compscitech.2004.01.019.
- [56] T. L. d. A. Montanheiro, F. H. Cristóvan, J. P. B. Machado, D. B. Tada, N. Durán, and A. P. Lemes, "Effect of MWCNT functionalization on thermal and electrical properties of PHBV/MWCNT nanocomposites," *Journal of Materials Research*, vol. 30, no. 1, pp. 55-65, 2014, doi: 10.1557/jmr.2014.303.
- [57] G. Pezzotti, "Raman spectroscopy in cell biology and microbiology," *Journal of Raman Spectroscopy*, vol. 52, no. 12, pp. 2348-2443, 2021, doi: 10.1002/jrs.6204.
- [58] D. W. Shipp, F. Sinjab, and I. Notingher, "Raman spectroscopy: techniques and applications in the life sciences," *Advances in Optics and Photonics*, vol. 9, pp. 315-428, 2017.
- [59] A. A. Bhirde *et al.*, "Combining portable Raman probes with nanotubes for theranostic applications," *Theranostics*, vol. 1, pp. 310-21, 2011, doi: 10.7150/thno/v01p0310.
- [60] W. Bian *et al.*, "Review of Functionalized Nanomaterials for Photothermal Therapy of Cancers," *ACS Applied Nano Materials*, vol. 4, no. 11, pp. 11353-11385, 2021, doi: 10.1021/acsanm.1c01903.
- [61] W. Cai, T. Gao, H. Hong, and J. Sun, "Applications of gold nanoparticles in cancer nanotechnology," *Nanotechnol Sci Appl*, vol. 1, pp. 17-32, Sep 19 2008, doi: 10.2147/nsa.s3788.
- [62] R. inVia, "inVia™ confocal Raman microscope," ed.
- [63] E. W. Young, E. Berthier, and D. J. Beebe, "Assessment of enhanced autofluorescence and impact on cell microscopy for microfabricated thermoplastic devices," *Anal Chem*, vol. 85, no. 1, pp. 44-9, Jan 2 2013, doi: 10.1021/ac3034773.
- [64] M. Mazilu, A. C. De Luca, A. Riches, C. S. Herrington, and K. Dholakia, "Optimal algorithm for fluorescence suppression of modulated Raman spectroscopy," *Opt Express*, vol. 18, no. 11, pp. 11382-95, May 24 2010, doi: 10.1364/OE.18.011382.
- [65] L. Cui, H. J. Butler, P. Martin-Hirsch, and F. L. Martin, "Aluminium foil as a potential substrate for ATR-FTIR, transfection FTIR or Raman spectrochemical analysis of biological specimens " *Analytical Methods*, vol. 8, no. 3, 2016, doi: <https://doi.org/10.1039/C5AY02638E>
- [66] E. C. Santos, H. S. Martinho, K. Annes, R. F. Leite, and M. P. Milazzotto, "Rapid and noninvasive technique to assess the metabolomics profile of bovine embryos produced in vitro by Raman spectroscopy," *Biomed Opt Express*, vol. 6, no. 8, pp. 2830-9, Aug 1 2015, doi: 10.1364/BOE.6.002830.
- [67] P. Crow *et al.*, "The use of Raman spectroscopy to differentiate between different prostatic adenocarcinoma cell lines," *Br J Cancer*, vol. 92, no. 12, pp. 2166-70, Jun 20 2005, doi: 10.1038/sj.bjc.6602638.
- [68] M. Soleymani, A. R. Ghasemi, and B. Moslemi-Abyaneh, "Agglomerated carbon nanotubes effects on buckling behavior of fiber metal laminated nanocomposite shells," *Polymers and Polymer Composites*, vol. 30, 2022, doi: 10.1177/09673911221108743.

- [69] P. G. Collins, *DEFECTS AND DISORDER IN CARBON NANOTUBES*. University of California at Irvine: Oxford University Press, 2009.
- [70] A. A. Renshaw, J. P. Richie, K. R. Loughlin, M. Jiroutek, A. Chung, and A. V. D'Amico, "The greatest dimension of prostate carcinoma is a simple, inexpensive predictor of prostate specific antigen failure in radical prostatectomy specimens," *Cancer*, vol. 83, no. 4, pp. 748-52, Aug 15 1998. [Online]. Available: <https://www.ncbi.nlm.nih.gov/pubmed/9708940>.
- [71] X. Yuan *et al.*, "Effect of Laser Irradiation on Cell Function and Its Implications in Raman Spectroscopy," *Appl Environ Microbiol*, vol. 84, no. 8, Apr 15 2018, doi: 10.1128/AEM.02508-17.
- [72] A. I. Baba and C. Catoi, "Tumor Cell Morphology," in *Comparative Oncology*. Bucharest (RO), 2007.
- [73] R. A. Anisimov, D. A. Gorin, and A. A. Abalymov, "3D Cell Spheroids as a Tool for Evaluating the Effectiveness of Carbon Nanotubes as a Drug Delivery and Photothermal Therapy Agents," *C*, vol. 8, no. 4, 2022, doi: 10.3390/c8040056.
- [74] M. Imaninezhad, J. Schober, D. Griggs, P. Ruminski, I. Kuljanishvili, and S. P. Zustiak, "Cell Attachment and Spreading on Carbon Nanotubes Is Facilitated by Integrin Binding," *Front Bioeng Biotechnol*, vol. 6, p. 129, 2018, doi: 10.3389/fbioe.2018.00129.
- [75] Y. Sanayama, A. Matsumoto, N. Shimojo, Y. Kohno, and H. Nakaya, "Phenylalanine sensitive K562-D cells for the analysis of the biochemical impact of excess amino acid," *Sci Rep*, vol. 4, p. 6941, Nov 6 2014, doi: 10.1038/srep06941.
- [76] S. Kang, M. Pinault, L. D. Pfefferle, and M. Elimelech, "Single-walled carbon nanotubes exhibit strong antimicrobial activity," *Langmuir*, vol. 23, no. 17, pp. 8670-3, Aug 14 2007, doi: 10.1021/la701067r.
- [77] L. Zhao, X. Zhang, X. Wang, X. Guan, W. Zhang, and J. Ma, "Recent advances in selective photothermal therapy of tumor," *J Nanobiotechnology*, vol. 19, no. 1, p. 335, Oct 24 2021, doi: 10.1186/s12951-021-01080-3.
- [78] R. Kuppusamy, M. Willcox, D. S. Black, and N. Kumar, "Short Cationic Peptidomimetic Antimicrobials," *Antibiotics (Basel)*, vol. 8, no. 2, Apr 18 2019, doi: 10.3390/antibiotics8020044.
- [79] P. Ravindranathan *et al.*, "Peptidomimetic targeting of critical androgen receptor-coregulator interactions in prostate cancer," *Nat Commun*, vol. 4, p. 1923, 2013, doi: 10.1038/ncomms2912.
- [80] Y. Chen *et al.*, "Synthesis and biological evaluation of indole-based peptidomimetics as antibacterial agents against Gram-positive bacteria," *Eur J Med Chem*, vol. 226, p. 113813, Dec 15 2021, doi: 10.1016/j.ejmech.2021.113813.
- [81] M. M. Gomari *et al.*, "Peptidomimetics in cancer targeting," *Mol Med*, vol. 28, no. 1, p. 146, Dec 7 2022, doi: 10.1186/s10020-022-00577-3.
- [82] D. B. Chithrani, "Polyethylene Glycol Density and Length Affects Nanoparticle Uptake by Cancer Cells," *Journal of Nanomedicine Research*, vol. 1, no. 1, 2014, doi: 10.15406/jnmr.2014.01.00006.

- [83] A. Hirano, K. Shiraki, and T. Arakawa, "Polyethylene glycol behaves like weak organic solvent," *Biopolymers*, vol. 97, no. 2, pp. 117-22, Feb 2012, doi: 10.1002/bip.21708.
- [84] E. H. Hasdeo, A. R. T. Nugraha, K. Sato, M. S. Dresselhaus, and R. Saito, "Electronic Raman scattering and the Fano resonance in metallic carbon nanotubes," *Physical Review B*, vol. 88, no. 11, 2013, doi: 10.1103/PhysRevB.88.115107