CHAPTER III
EXPERIMENTAL

3.1 Chitosan synthesis

3.1.1 Chemicals and Materials

Chitin extracted from shrimp and used in the synthesis of chitosan was obtained from A.N. (aquatic nutrition lab) Ltd., Thailand. Concentrated sodium hydroxide (NaOH) 50 % w/w was purchased from Vittayasom Co., Ltd., Thailand. Commercial grade ethanol was purchased from Italmar Co. Ltd., Thailand.

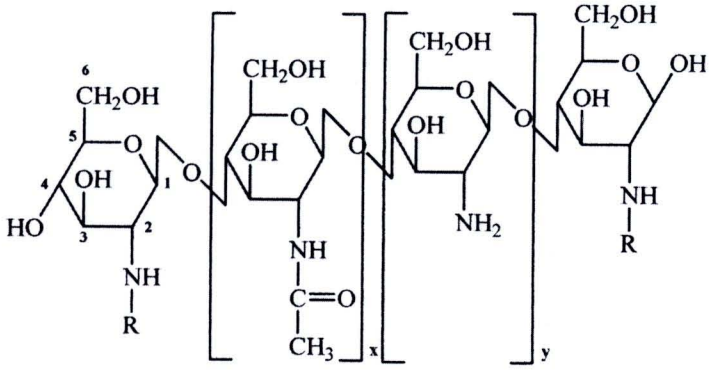


Figure 3.1 Chemical structure of Chitosan: x = N-acetyl-D-glucosamine unit, y = D-glucosamine unit: $x > 50\%$ = Chitin, $y > 50\%$ = Chitosan.

3.1.2 Methodology of chitosan synthesis with various degree of deacetylation

Chitosan with various degree of deacetylation (%*DD*) were prepared by reacting 50 g of chitin extracted from shrimp with 750 ml of concentrated sodium hydroxide (50%w/w) under constant shaking (ratio 1 g : 15 ml). Different chitosan batches of increasing %*DD* were obtained by varying the reaction time from 2 to 7 days at ambient temperature. The resulting chitosan powder was then filtered and rinsed with water until

obtaining neutral *pH* in the rinsed water. Thereafter, chitosan batches were rinsed with ethanol 50%, 70%, and 95% and finally air-dried. The chitosan, which was deacetylated for 7 days, was secondly deacetylated with concentrated sodium hydroxide for 3 days. The secondary deacetylated chitosan was deacetylated again with concentrated sodium hydroxide for 3 days to obtain high degree of deacetylation of chitosan. The *%DD* of each chitosan batches was measured by first derivative technique using a UV-Vis spectrophotometer (SPECORD S 100, Analytikjena). In addition, the average molecular weights of the chitosan were investigated using gel permeation chromatography.

3.1.3 Determination of degree of deacetylation of chitosan using first derivative UV-Vis Spectroscopy technique

First derivative UV-Vis spectroscopy technique was used to determine degree of deacetylation of resulting chitosan [61]. Chitosan powder (0.01 g) were dissolved in 0.01M diluted acetic acid 100 ml, then UV-Vis absorption spectras in term first derivative were obtained at fixed wavelength 201.4 nm by using UV-Vis spectrophotometer. *%DD* can be calculated from the equation [1] as follow,

$$\%DD = \{1 - \{\frac{A}{\frac{((10\times W) - 204A)}{161} + A}\}\}\times 100 \quad \ldots\ldots\ldots\ldots [1]$$

$$A = \frac{\text{Concentration of N - acetyl - D - glucosamine (g/lit)}}{204}$$

W = Weight of chitosan in 0.01M acetic acid

161 = Molecular weight of D-glucosamine

204 = Molecular weight of N-acetyl-D-glucosamine

To identify the N-acetyl-D-glucosamine in chitosan structure, the calibration curve of N-acetyl-D-glucosamine in various concentrations were prepared by plotting from first derivative absorbance spectra (Figure 3.2) at wavelength 201.4 nm as a function of N-acetyl-D-glucosamine concentration as shown in Figure 3.3.

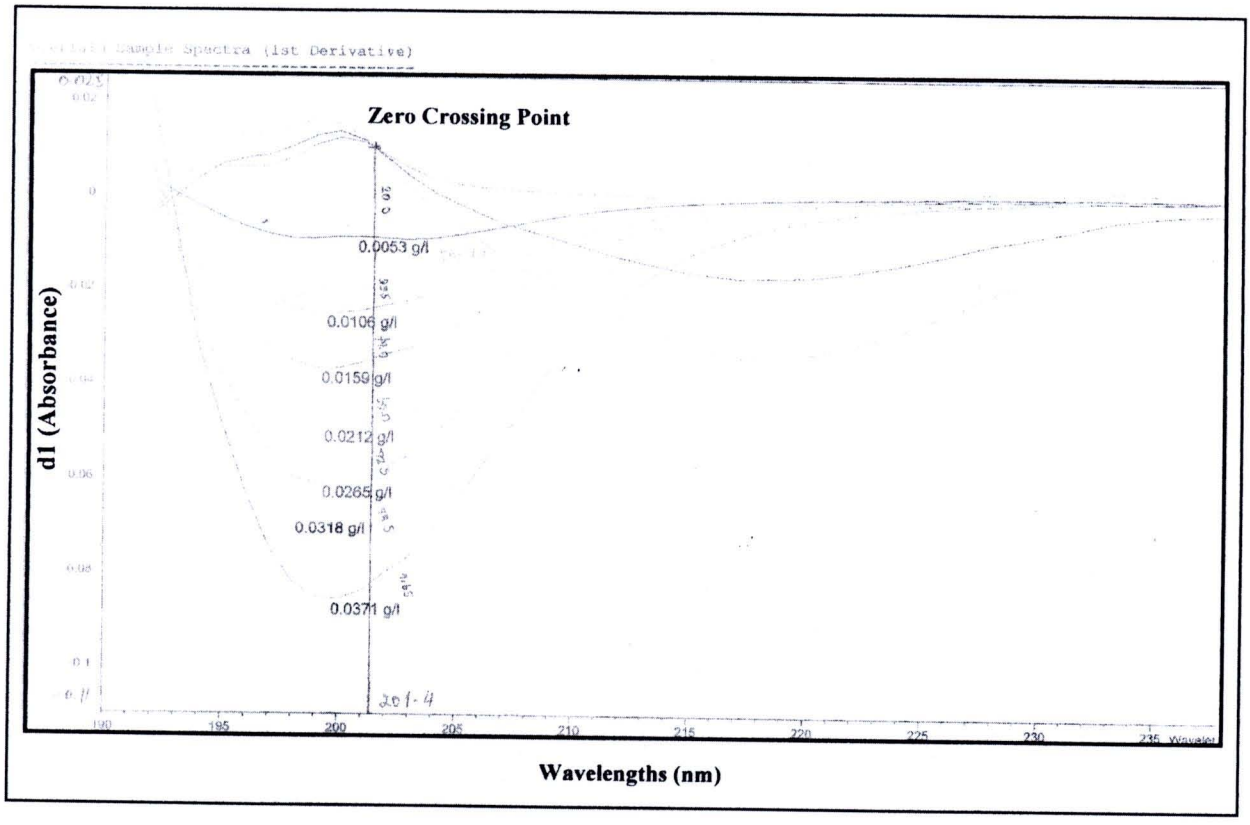


Figure 3.2 First derivative absorbance of N-acetyl-D-glucosamine with different N-acetyl-D-glucosamine concentrations (g/l).

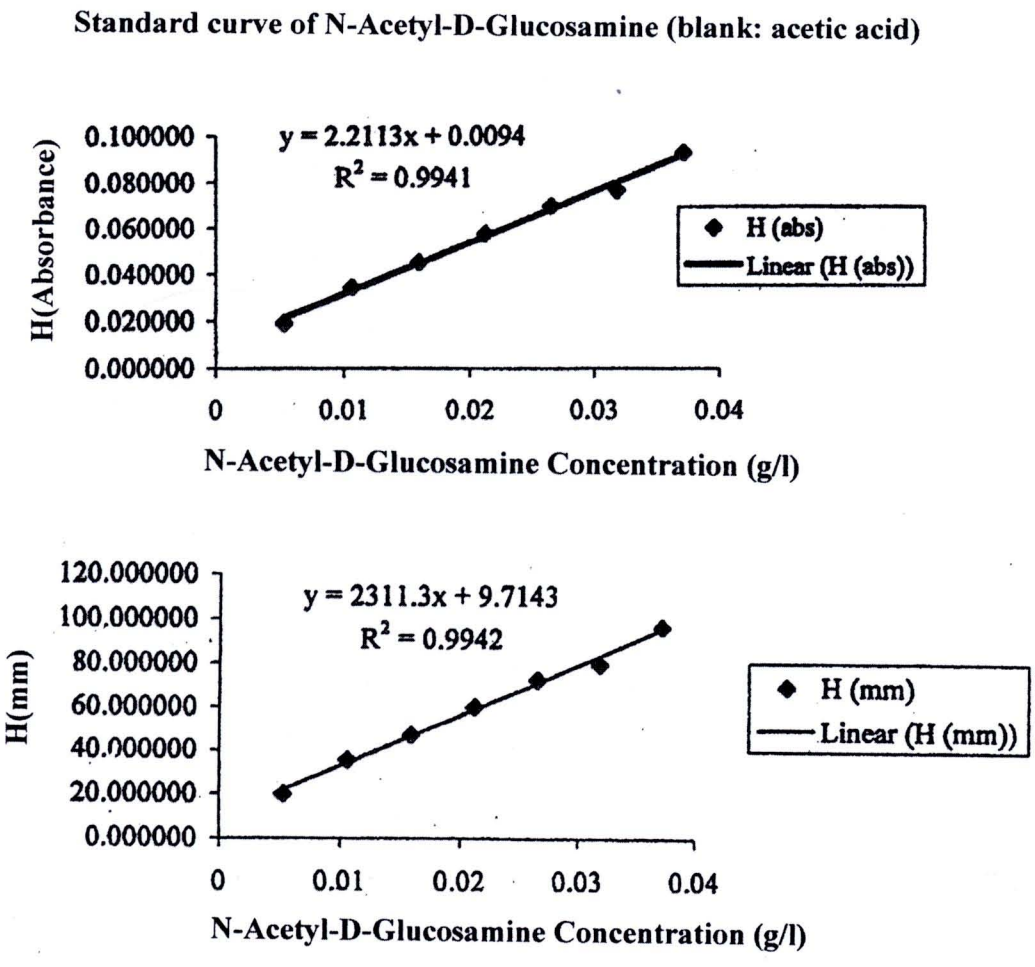


Figure 3.3 Calibration curve of N-acetyl-D-glucosamine in acetic acid 0.1 M with (a) Height (H) of derivative absorbance and (b) Height (H) in mm from zero crossing point as a function of the concentration of N-acetyl-D-glucosamine.

3.1.4 Determination of average molecular weight of chitosan using gel permeation chromatography (GPC Waters 600E)

Table 3.1 Gel Permeation Chromatography test condition.

Types	Condition
Eluent	Acetate buffer pH~4
Flow rate	0.6 ml/min
Injection volume	20 µl
Temperature	30 °C
Column set	Ultrahydrogel linear 1 column (MW resolving range 1,000-20,000,000) 1 column + guard column Standard pollulans (MW 5,900-788,000)
Calibration method	Polysaccharide standard calibration
Detector	Refractive Index Detector

3.1.4.1 Sample preparation

Chitosan with different degree of deacetylation (61, 70, 78, 84, 90, and 93%*DD*) 2 mg/ml were dissolved in eluent and filtered using nylon 66 membrane (pore size 0.45 µm) before injection.

3.2 Effect of the degree of deacetylation of chitosan on its dispersion of carbon nanotubes

3.2.1 Chemicals and Materials

Multiwall carbon nanotubes with a diameter of 110-170 nm and length of 5-9 micrometer were purchased from Aldrich, Thailand. Chitosan with various degree of deacetylation (61, 71, 78, 84, 90, and 93%DD) and their molecular weight in a rage of 630-530 kDa. Analytical grade glacial acetic acid was purchased from Labscan Asia Co.,

Ltd., Thailand. All chemicals and solvents were used as received without any further purification. Double distilled water was used in all experiments.

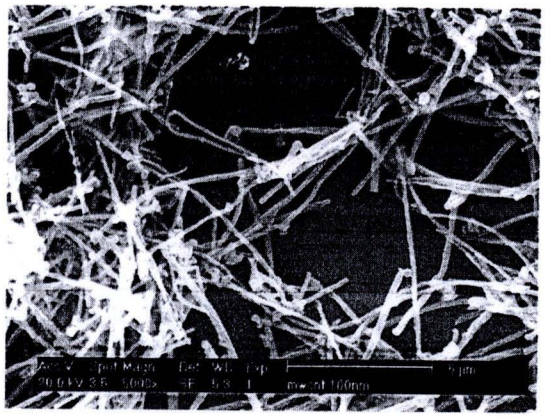


Figure 3.4 Morphology image of pristine CNT characterized by scanning electron microscope (Phillips XL30CP), a diameter in a range of 110-170 nm and length in a range of 5-9 μm.

3.2.2 Effect of chitosan concentration on the dispersion of MWCNTs

UV-Vis spectroscopy was used to determine the efficiency of the dispersion of the carbon nanotubes by turbidity measurements at 550 nm. 5 mg of MWCNT were mixed in 100 ml of a 0.01 mM chitosan solution (%*DD* = 61). The pH of the solution was adjusted to pH 4 with 20 mM of acetic acid. The absorbance of the solution was measured after each adjunction of chitosan until the final concentration of 10 mM chitosan was reached. In each step, the mixture was stirred and sonicated for 10 minutes using an ultrasonic bath (CREST Model 275D, USA).

3.2.3 Effect of sonication times on MWCNTs dispersion with chitosan

5 mg of MWCNT were mixed in 100 ml of a 5 mM chitosan solution (%*DD* = 61, 78 and 93). The mixture was stirred and sonicated using an ultrasonic bath with varying sonication time (15 to 150 minutes). In each period with different sonication time, the

turbidity of carbon nanotubes dispersion were determined by UV-Vis spectroscopy at wavelength 550 nm.

3.2.4 Effect of %DD of chitosan on the dispersion of MWCNTs

To evaluate the effect of the *%DD* on the dispersion efficiency of MWCNT by chitosan, 2.5 mg of MWCNTs were added to different solutions of chitosan having a fixed volume of 50 ml and a fixed concentration of 5 mM but increasing *%DD* (61, 71, 78, 84, 90, and 93%). After mixing, the MWCNT and the chitosan solutions were stirred and sonicated for 30 minutes. The absorbance at 550 nm of the pitch-black solution was then measured by using UV-Vis spectroscopy and recorded.

3.2.5 Surface charge of the modified MWCNTs

To evaluate the surface charge of each MWCNT modified with chitosan of various %DD, samples of the prepared solutions were measured with a Zetasizer (NanoZS4700 nanoseries, Malvern Instruments, UK). The samples were taken from the solutions of MWCNTs modified with 5 mM chitosan having various degree of deacetylation (61, 71, 78, 84, 93%*DD*) and sonicated for 10 minutes. The modified MWCNT with chitosan solution were centrifuged at 4,000 rpm for 15 minutes in order to remove the excess of chitosan. While the supernatant was removed, 25 ml acetic acid, 20 mM, was added to the remaining MWCNT and re-dispersed by vortex and sonication. The precipitation and re-dispersion step was repeated 3 times in order to remove the excess chitosan. Zeta potential of modified MWCNTs with chitosan were obtained as the average of three measurements at 25 °C.

3.3 Molecular Dynamics Simulation: Dispersion and seperation of chitosan wrapping on SWCNTs by noncovalently modification.

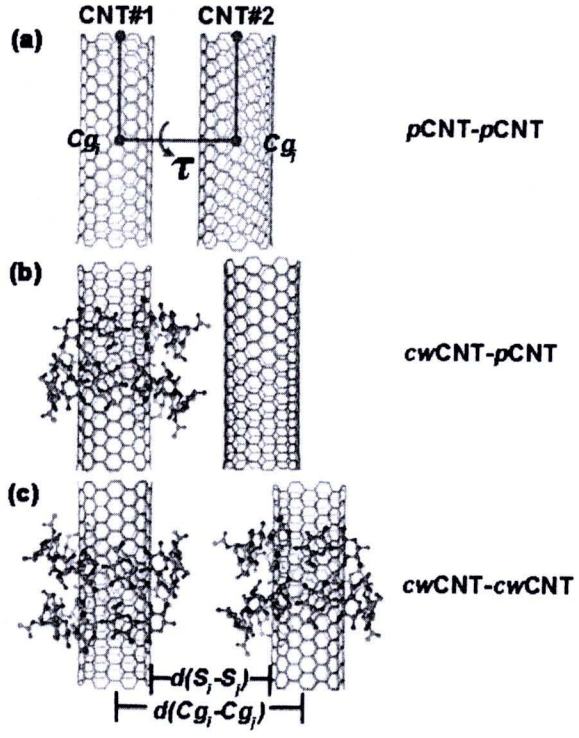
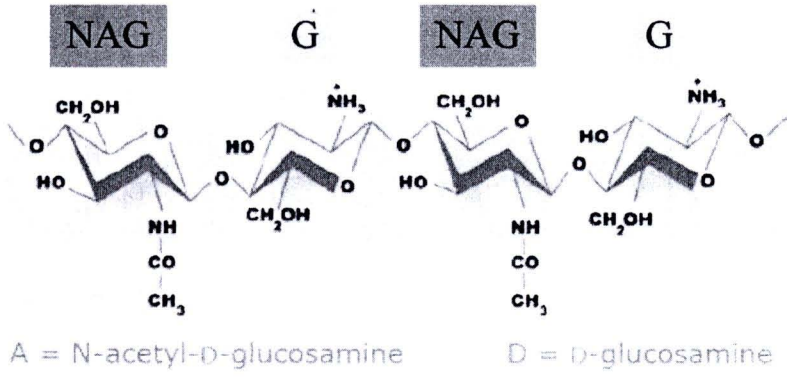


Figure 3.5 Schematic views of (a) two pristine CNTs (*p*CNT-*p*CNT), (b) a pristine CNT - a wrapped CNT with chitosan (*p*CNT-*cw*CNT), and (c) two chitosan-wrapped CNTs (*cw*CNT-*cw*CNT) where the SWCNT and the polymer used are the (8,8) armchair and 60%*DD* chitosan, respectively. The distances ($d(Cgi\text{-}Cgj)$) and ($d(Sgi\text{-}Sgj)$) and torsion angle (τ) between the two SWCNTs were defined through the center of gravity (*Cg*) and the surface of each tube in which $\tau = 0°$ and the two tubes are parallel.



60%*DD* = G-G-N-G-N- G-N-G-N-G-G-N-G-N-G- N-G-N-G-G

NAG = N-Acetyl-D-Glucosamine G = D-Glucosamine

Figure 3.6 Chemical structure of chitosan 60%*DD* were alternate the repeating unit between N-Acetyl-D-Glucosamine and D-Glucosamine.

3.3.1 Materials and methods

The 20 repeating units of 60%*DD* chitosan and the (8,8) armchair of SWCNTs with diameter of 11 Å, chiral vectors n=8 and m=8, and 12 repeating units were constructed using Material Studio 4.3 package. The three models, as shown in Figure 3.5, are (a) two pristine CNTs (*p*CNT-*p*CNT), (b) a pristine CNT – a chitosan wrapped CNT (*p*CNT-*cw*CNT), and (c) two chitosan wrapped CNTs (*cw*CNT-*cw*CNT). Each model was solvated in aqueous solution. The MD simulations were set up and carried out according to our previous works [62, 63]. The CNT and chitosan were parameterized by AMBER03 [64] and GLYCAM06 [65] force fields, respectively. The SPC/E water model with octagonal box over 12 Å from the system surface was applied to all systems. The simulations were calculated using the AMBER10 programme package [66] with the NPT ensemble at 1 atm and a time step of 2 fs. The SHAKE algorithm was applied to all bonds involving hydrogen atoms to constraint their motions. The periodic boundary conditions were applied and the cutoff function was set at 12 Å for nonbonded interactions and particle mesh Ewald method. The whole system was heated from 10 K to 300 K for 200 ps and equilibrated at 300 K for 5 ns. Finally, the production stage was performed until 20 ns and the structural coordinates were saved every 1 ps for analysis.

3.4 Covalent surface modification of multiwall carbon nanotubes with acid oxidation (H_2SO_4 and HNO_3)

3.4.1 Chemicals and materials

Multiwall carbon nanotubes: MWCNT (baytubes® C 150 P, outer diameter distribution 5-20 nm and length 1 - >10 μm, (Figure 3.7)) were kindly donated from Bayer Co., Ltd., Thailand. Concentrated sulfuric acid 98% and nitric acid 90% A.R. grade were purchased Labscan Asia Co., Ltd., Thailand. Sodium hydroxide was purchased from Aldrich, Thailand. All chemicals and solvents were used as received without any further purification. Double distilled water was used in all experiments.

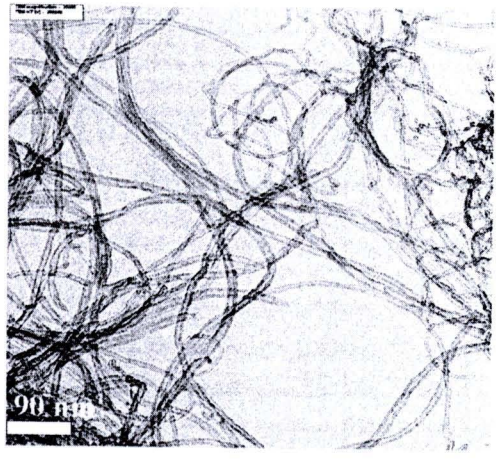


Figure 3.7 Transmission electron microscopic image of pristine multiwall carbon nanotubes (baytubes® C 150 P).

3.4.2 Acid treatment of carbon nanotubes

To prepare multiwall carbon nanotubes surface prior to coating with multilayer thin film, MWCNTs were properly treated with strong acid to provide a negative charge on their surface. 0.5 g of MWCNTs was mixed with H_2SO_4: HNO_3 = 3:1 (v/v) in 80 ml, and sonicated for 2 hr at temperature 50 °C. The treated MWCNTs mixture was then neutralized with NaOH until the pH reached a value of 7. Treated MWCNT were dialyzed in distilled water for 4 days in order to remove salt ions from the acid and base used. Finally the treated carbon nanotubes were centrifuged at 4,000 rpm for 30 minutes and collected as in powder form.

The treated multiwalled carbon nanotubes were characterized on their chemical structure using Raman spectroscopy (Perkin Elmer Spectrum-GX, USA) and FTIR spectroscopy (Perkin Elmer Spectrum One, USA).

3.5 Layer-by-layer deposition on treated carbon nanotubes with polyelectrolyte; PDADMAC and PSS without centrifugation process

3.5.1 Chemicals and materials

Treated multiwall carbon nanotubes were obtained from acid treatement. Poly(diallyldimethylammonium chloride): PDADMAC (medium molecular weight, 20 wt% in water, typical Mw 200,000 – 350,000) and poly(sodium 4-styrene sulfonate): PSS (typical Mw 70,000) were purchased from Aldrich, Thailand. Sodium hydroxide was purchased from Aldrich, Thailand. All chemicals and solvents were used as received without any further purification. Double distilled water was used in all experiments.

3.5.1.1 Poly(diallyldimethylammonium chloride)

PDADMAC (medium molecular weight, 20 wt% in water, typical Mw 200,000 – 350,000), was a strong cationic polyelectrolyte with positive charges along the backbone chain. The chemical structure of PDADMAC is shown in Figure 3.8.

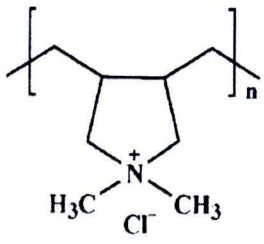


Figure 3.8 Chemical structure of poly(diallyldimethylammonium chloride): PDADMAC.

3.5.1.2. Poly(sodium 4-styrene sulfonate)

PSS (typical M_w 70,000), was a strong anionic polyelectrolyte with negative charges along the backbone chain. The chemical structure of PSS is shown in Figure 3.9.

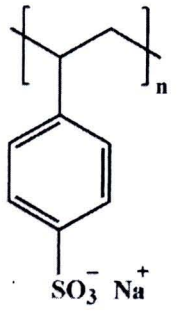


Figure 3.9 Chemical structure of poly(sodium 4-styrene sulfonate): PSS.

3.5.2 Deposition of polyelectrolyte multilayers on multiwall carbon nanotubes via layer-by-layer technique

For the primary coating of the MWCNT, various amounts of carbon nanotubes (5, 10 and 20 mg) were dispersed by sonication in 400 ml of 1 mM NaOH in order to deprotonate carboxylic acid become to carboxylate group which provided negatively charged MWCNT. In different vials, 20 ml of PDADMAC with concentrations ranging

from 0.0005 to 0.25 mM were mixed with 20 ml of carbon nanotubes solutions. For the deposition of the secondary layer of polyanionic PSS on the positively charged primary coated MWCNT, aliquots of 20 ml taken from a 200 ml of the CNT mixed with 0.15 mM PDADMAC were mixed 20 ml of PSS having concentration ranging from 0.001-0.3 mM.

Finally for the deposition of the tertiary layer of PDADMAC on the negatively charged carbon nanotubes, aliquots of 20 ml were taken from a 200 ml solution containing of 0.09 mM of PSS as secondary layers were added to 20 ml of PDADMAC having concentrations ranging from 0.001 to 0.1mM. All of mixture solutions were investigated on their stability by measuring the turbidity of carbon nanotubes solution using UV-Vis spectroscopy and their surface charge density using zeta potential measurement as shown in Figure 3.10.

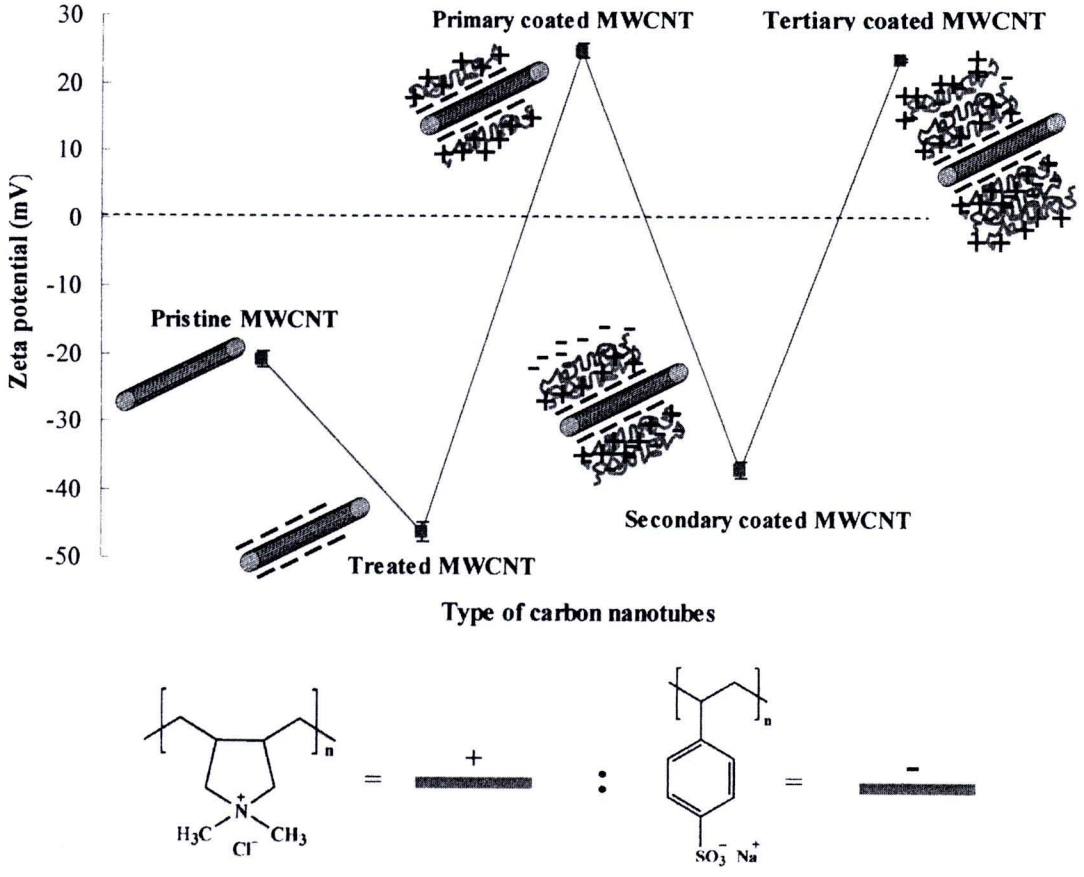


Figure 3.10 Diagram of modified carbon nanotubes with PDADMAC and PSS as polyelectrolyte multilayers.

3.5.3 Stability of modified MWCNT (treated MWCNT and primary coating MWCNT with PDADMAC)

3.5.3.1 Effect of salt concentration on stability of modified MWCNT

25 μg/ml of treated MWCNT and primary coating MWCNT with PDADMAC 20 ml were mixed with 20 ml of different concentrations of NaCl (0-1.5 M). The mixture solution was stored at the room temperature for 1 week. The turbidity of the mixture solutions were measured by UV-Vis spectroscopy at a wavelength of 550 nm.

3.5.3.2 Effect of pH on stability of modified MWCNT

25 μg/ml of treated MWCNT and primary coating MWCNT with PDADMAC 20 ml were mixed with 20 ml of different *pH* buffer (*pH* 2 to11). The mixture solution was stored at the room temperature for 24 hour. The turbidity of the mixture solutions were measured by UV-Vis spectroscopy at a wavelength of 550 nm.

3.5.4 Characterization Technique

UV-Vis spectrophotometer (SPECORD S 100, Analytikjena, Germany) was used to investigate the turbidity of modified carbon nanotubes in aqueous solution in order to identify the stability of modified CNT. To confirm the successful coating on treated carbon nanotubes, the surface charge of modified carbon nanotubes with polycation and polyanion were measured by Zetasizer (NanoZS4700 nanoseries, Malvern Instruments, UK). Transmission electron microscope (TEM model JEM-2100) was used to investigate the morphology of pristine MWCNT and modified MWCNT with polyelectrolyte.

3.6 Hydrophilic model drugs: gentian violet and diclofenac loading and recovery on modified multiwalled carbon nanotubes

3.6.1 Chemicals and materials

Treated multiwall carbon nanotubes were obtained from acid treatement. Poly(diallyldimethylammonium chloride): PDADMAC (medium molecular weight, 20 wt% in water, typical Mw 200,000 – 350,000) and poly(sodium 4-styrene sulfonate): PSS (typical Mw 70,000) were purchased from Aldrich, Thailand. Gentian violet was purchased from Vittayasom Co., Ltd., Thailand. Diclofenac Sodium was purchased from Aldrich, Thailand. Sodium chloride, Disodium hydrogen phosphate, potassium chloride and Potassium dihydrogen phosphate were purchased from Carlo Erba, Thailand.

3.6.1.1 Gentian violet

Gentian violet (crystal violet, Methyl Violet 10B, hexamethyl pararosaniline chloride) is a bactericide and an antifungal agent, the primary agent used in the Gram stain test, perhaps the single most important bacterial identification test in use today, and it is also used by hospitals for the treatment of serious heat burns and other injuries to the skin and gums. The chemical structure of gentian violet was shown in Figure 3.11.

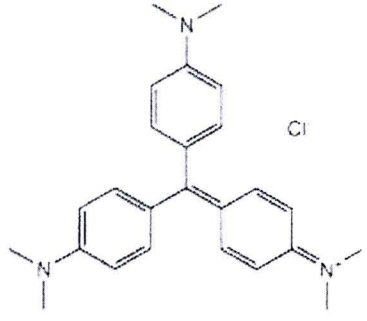


Figure 3.11 Chemical structure of Gentian violet (crystal violet, Methyl Violet 10B, hexamethyl pararosaniline chloride).

3.6.1.2 Diclofenac

Diclofenac (marketed as Voltaren and under a number of other trade names, see Figure 3.12) is a non-steroidal anti-inflammatory drug (NSAID) taken to reduce inflammation and as an analgesic reducing pain in conditions such as arthritis or acute injury. It can also be used to reduce menstrual pain, dysmenorrhea. The name is derived from its chemical name: 2-(2,6-dichloranilino)phenylacetic acid.

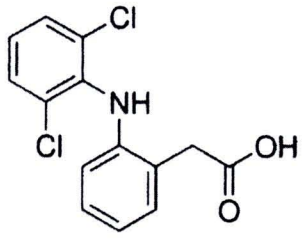


Figure 3.12 Chemical structure of Diclofenac (2-(2,6-dichloranilino) phenylacetic acid).

3.6.2 Preparation of phosphate buffer saline

Phosphate buffer saline *pH* 7.4 was prepared by dissolving 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, 0.25 g of KH_2PO_4 in 800 ml of distilled H_2O. Sodium chloride, Potassium chloride, Potassium dihydrogen phosphate and Disodium hydrogen phosphate were purchased from Carlo Erba, Thailand. The *pH* of aqueous solution was adjusted to 7.4 with HCl or NaOH then added distilled water was added to 1 liter.

3.6.3 Layer-by-layer deposition on treated multiwall carbon nanotubes with polyelectrolyte in 0.1xPBS buffer

For the primary coating of the MWCNT, various amounts of MWCNT 20 mg were dispersed in 400 ml of 0.1xPBS by sonicator. In different vials, 20 ml of PDADMAC with concentrations ranging from 0.02 to 0.12 mM were mixed with 20 ml of carbon nanotubes solutions. For the deposition of the secondary layer of polyanionic

PSS on the positively charged primary coated MWCNT, aliquots of 20 ml from a 200 ml of the MWCNT mixed with 0.09 mM PDADMAC, were mixed with 20 ml of PSS having concentration ranging from 0.02 to 0.12 mM. Finally, for the deposition of the tertiary layer of PDADMAC on the negatively charged MWCNT, aliquots of 20 ml were taken from a 200 ml solution containing 0.04 mM of PSS as secondary layers were added to 20 ml of PDADMAC having concentrations ranging from 0.005 to 0.1 mM. All of mixture solutions were investigated on their stability by measuring the turbidity of carbon nanotubes solution using UV-Vis spectroscopy and their surface charge density using zeta potential measurement.

3.6.4 Preparation of primary and secondary layers coating on MWCNTs with polyelectrolyte

3.6.4.1 Deposition of primary layer on carbon nanotubes:

The amount of treated carbon nanotubes (10 mg) were sonicated in 400 ml of 0.1x PBS buffer *pH* 7.4. While 200 ml of 0.1mM of PDADMAC were stirred, 200 ml of carbon nanotubes solution were added into the PDADMAC solution.

3.6.4.2 Deposition of secondary layer on carbon nanotubes:

To deposit polyanion as poly(sodium 4-styrene sulfonate): PSS on positive charge of primary coated carbon nanotubes, 20 mg of treated carbon nanotubes were sonicated in 400 ml of 0.1xPBS buffer *pH* 7.4. While 200 ml of 0.15 mM PDADMAC concentration were stirred, 200 ml of treated carbon nanotubes solution were added into the PDADMAC solution. Thereafter, while 200 ml of 0.04 mM of PSS concentration were stirred, 200 ml of primary coating MWCNT with PDADMAC solution were added into the PSS solution.

In this work, we try to load hydrophilic model drug as Gentian violet as a cationic drug and Diclofenac as an anionic drug onto modified MWCNT.

3.6.5 Loading and recovery gentian violet from modified multiwall carbon nanotubes; Treated MWCNT, Primary coating MWCNT with PDADMAC and Secondary coating MWCNT with PDADMAC/PSS

Treated carbon nanotubes, primary coating MWCNT with PDADMAC and secondary coating MWCNT with PDADMAC/PSS were prepared in 0.1xPBS buffer, *pH* 7.4. Gentian violet was prepared at different concentrations (0.0003-0.003 μM) in 0.1xPBS buffer. 20 ml of modified MWCNT were mixed with 20 ml of gentian violet in different concentrations. The solution were kept for 24 hr, after that the mixture solution were centrifuged at 14,000 rpm for 15 minutes. The supernatant solutions were measured by UV-Vis spectroscopy to determine unbound gentian violet. The decant that consist of gentian violet adsorbed MWCNT were rinsed with 0.1xPBS buffer, thereafter dissolved in ethanol which was the best solvent to dissolve gentian violet. The mixtures were vortexed for 5 minutes then centrifuged again to measure the absorbance of supernatant solution using UV-Vis spectrophotometer.

3.6.6 Loading and recovery diclofenac sodium from modified multiwall carbon nanotubes; Treated MWCNT, Primary coating MWCNT with PDADMAC and Secondary coating MWCNT with PDADMAC/PSS

Treated MWCNT, primary coating MWCNT with PDADMAC and secondary coating MWCNT with PDADMAC/PSS were prepared in 0.1 x PBS buffer, *pH* 7.4. Diclofenac sodium was prepared different concentrations (0.00025-0.01%w/v) in 0.1 x PBS buffer. 20 ml of modified MWCNT were mixed with 20 ml of diclofenac in different concentrations. The solution were kept for 24 hr, after that the mixture solution were centrifuged at 14,000 rpm for 15 minutes. The supernatant solutions were measured by UV-Vis spectroscopy to determine unbound diclofenac concentration. In this case, surprising that modified MWCNT with any coating had no effect on loading efficiency of diclofenac.

3.7 Cytotoxicity of modified multiwall carbon nanotubes using MTT assay

3.7.1 Chemicals and Materials

Untreated multiwall carbon nanotubes (baytubes® C 150 P, outer diameter distribution 5-20 nm and length 1 - >10 μm (Figure 3.7)) were kindly donated from Bayer Co., Ltd., Thailand. Treated multiwall carbon nanotubes were received from covalent surface modification process in 3.4. L929 fibroblast mouse cell line were purchased from the American Type Culture Collection (A.T.C.C.) (Rockville, U.S.A). Dulbecco's Modified Eagle Medium (DMEM)-high glucose was purchased from Sigma-Aldrich, U.S.A. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from United States Bio- medical (USB) Corp. (Cleveland, Ohio). Dimethylsulfoxide (DMSO) was purchased from Riedel-de Haën (Seelze, Germany).

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) as shown in Figure 3.13, is reduced to purple formazan in living cells. A solubilization solution (usually either dimethyl sulfoxide, an acidified ethanol solution, or a solution of the detergent sodium dodecyl sulfate in diluted hydrochloric acid) is added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer. The absorption maximum is dependent on the solvent employed.

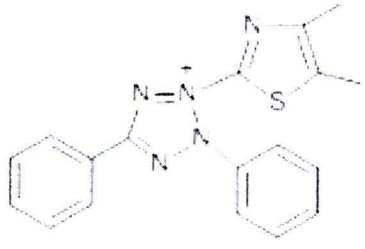


Figure 3.13 Chemical structure of MTT; (3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide, a yellow tetrazole)

L929 fibroblast cells, derived from an immortalized mouse fibroblast cell line, are internationally recognized cells that are routinely used in in-vitro cytotoxicity assessments.

3.7.2 L929 fibroblast cells preparation

L929 cells line were frozen with the mixture of serum and DMSO 5-10% in cryo-tube. The cells were defrosted into 37 °C and 5 ml of DMEM were added in the cell. The cell suspensions were centrifuged and the supernatant were removed. DMEM were added in the cells decant and re-suspended by mixing with micropipette. Cells suspended with DMEM were replaced into tissue and tune flash, then incubated in oven 37 °C.

Once, L929 cells were proliferated and adhered at the bottom, the media were removed. Trypsin enzyme was added in order to peel the cells adhesion and re-suspend in the trypsin solution. The trypsinized cells were incubated at 37 °C for 5 minutes. DMEM were added into the cell solution. The cells suspensions were centrifuged and the supernatant was removed. DMEM was added into cell and the cells were counted by hemo-cytometry. The number of L929 cells were prepared at 10,000 cells per well per 100 µl. The cells in 96 wells plate were incubated at 37 °C for 24 hr.

3.7.3 Interference of formazan adsorption on untreated and treated multiwall carbon nanotubes

L929 fibroblast cells were seed at 10,000 cells/well in 96 wells. L929 cells were incubated for 24 hr at 37 °C. Thereafter, the DMEM media were refreshed with the new DMEM media. The L929 cells were incubated for 12 hr. MTT solution was added to cells and incubated for 4 hr. Formazan crystal was dissolved with DMSO then untreated and treated MWCNT concentrations at 6.25, 12.5, 25, 50, 100µg/ml were exposed in formazan solutions for 1 hr. MWCNTs were removed from formazan solution by centrifugation at 4,000 rpm. The remaining formazan solution was measured using UV-Vis spectroscopy at a wavelength of 570 nm. MWCNT samples were sterilized by

exposing with UV lamp for 1-2 hr. 8 replications of samples were tested in each concentration.

3.7.4 Cytotoxicity test: MTT Assay

L929 fibroblast cells were seed at 10,000 cells/well in 96 wells. L929 cells were incubated for 24 hr at 37 °C. Thereafter, the DMEM were refreshed. The L929 cells were incubated for 12 hr. Pristine and treated multiwall carbon nanotubes concentrations 3.125, 6.25, 12.5, 25 and 50 μg/ml were exposed in L929 fibroblast cells and then incubated for 24 hr. Carbon nanotubes were removed after exposing by washing with PBS buffer pH 7.4 twice. MTT solutions was added and incubated for 4 hr at 37 °C. Formazan crystal were dissolved with DMSO and centrifugation at rotation speed 4000 rpm. OD of formazan solutions were measured by UV-Vis spectroscopy at a wavelength of 570 nm. CNT samples were sterilized by exposing on UV lamp for 1-2 hr. 8 replications of samples were tested in each concentration.

3.7.5 Effect of functional groups of modified multiwall carbon nanotubes on their cytotoxicity

3.7.5.1 Preparation of modified carbon nanotubes with poly(diallyldimethyl ammonium chloride) and poly(sodium 4-styrene sulfonate)

Treated carbon nanotubes 0.05 mg/ml in volume 100 ml were dispersed in 0.1x PBS buffer by sonicator. To prepare the primary coating carbon nanotubes with PDADMAC, treated carbon nanotubes solution (30 ml) were dropped in 0.15 mM poly(diallyldimethyl ammonium chloride)(30 ml) and stirred at the same time. To prepare the secondary coating carbon nanotubes with PSS, the primary coating carbon nanotubes with PDADMAC solution (30 ml) were dropped in 30 ml of 0.1 mM PSS solution. To prepare the tertiary coating carbon nanotubes with PDADMAC, the secondary coating on carbon nanotubes solution 25 ml were dropped in 0.04 mM poly(diallyldimethyl ammonium chloride)(25 ml) and stirred at the same time.

3.7.5.2 Preparation of modified multiwall carbon nanotubes for testing MTT assay

Sample tests; All of the samples were exposed with UV lamp for 3-4 hr.

Solution samples: The modified MWCNT samples were diluted in different concentrations with nutrient broth solution.

- **Primary coating CNT with PDADMAC**: 12.5 μg/ml, 6.25 μg/ml, 3.125 μg/ml, 1.5625 μg/ml and 0.78125 μg/ml.
- **Secondary coating CNT with PDADMAC/PSS**: 6.25 μg/ml, 3.125 μg/ml, 1.5625 μg/ml and 0.78125 μg/ml.
- **Tertiary coating CNT with PDADMAC/PSS/PDADMAC**: 3.125 μg/ml, 1.5625 μg/ml and 0.78125 μg/ml.

Powder samples: The modified MWCNT samples were centrifuged to remove the polyelectrolyte excess at 10,000 rpm for 10 minutes. The modified MWCNT powders were collected and re-dispersed in the nutrient broth by sonication.

- **Primary coating CNT with PDADMAC**: 50 μg/ml, 25 μg/ml, 12.5 μg/ml, 6.25 μg/ml, 3.125 μg/ml and 1.5625 μg/ml.
- **Secondary coating CNT with PDADMAC/PSS**: 50 μg/ml, 25 μg/ml, 12.5 μg/ml, 6.25 μg/ml, 3.125 μg/ml and 1.5625 μg/ml.
- **Tertiary coating CNT with PDADMAC/PSS/PDADMAC**: 50 μg/ml, 25 μg/ml, 12.5 μg/ml, 6.25 μg/ml, 3.125 μg/ml and 1.5625 μg/ml.

3.7.5.3 Cytotoxicity: MTT Assay

L929 fibroblast cells were seed 10,000 cells/well in 96 wells. L929 cells were incubated for 24 hr. at temperature 37 °C. Thereafter, the old DMEM media were refreshed with the new DMEM media. The L929 cells were incubated with new DMEM media for 12 hr. Untreated and treated MWCNT concentrations 3.125, 6.25, 12.5, 25 and 50 μg/ml and modified MWCNT with polyelectrolyte which were

Solution samples:

- **Primary coating CNT with PDADMAC**: 12.5 μg/ml, 6.25 μg/ml, 3.125 μg/ml, 1.5625 μg/ml and 0.78125 μg/ml.
- **Secondary coating CNT with PDADMAC/PSS**: 6.25 μg/ml, 3.125 μg/ml, 1.5625 μg/ml and 0.78125 μg/ml.
- **Tertiary coating CNT with PDADMAC/PSS/PDADMAC**: 3.125 μg/ml, 1.5625 μg/ml and 0.78125 μg/ml.

Powder samples:

- **Primary coating CNT with PDADMAC**: 50 μg/ml, 25 μg/ml,12.5 μg/ml, 6.25 μg/ml, 3.125 μg/ml and 1.5625 μg/ml.
- **Secondary coating CNT with PDADMAC/PSS**: 50 μg/ml, 25 μg/ml, 12.5 μg/ml, 6.25 μg/ml, 3.125 μg/ml and 1.5625 μg/ml.
- **Tertiary coating CNT with PDADMAC/PSS/PDADMAC**: 50 μg/ml, 25 μg/ml, 12.5 μg/ml, 6.25 μg/ml, 3.125 μg/ml and 1.5625 μg/ml.

, were exposed in L929 fibroblast cells and then incubated for 24 hr. MWCNTs were removed after exposing by washing with PBS buffer *pH* 7.4 twice times. MTT solutions was added into cells and incubated for 4 hr at temperature 37 °C. Living cells in each well can convert MTT to be formazan crystal. Formazan crystal were dissolved with DMSO and centrifuged at rotation speed of 4,000 rpm. OD of formazan solutions were measure by UV-Vis spectroscopy at wavelength 570 nm. MWCNT samples were sterilized by exposing on UV lamp for 3-4 hr. 8 replications of samples were tested in each concentration.

3.8 Characterization Technique

3.8.1 UV-Vis spectroscopy [67]

Ultraviolet-Visible spectroscopy (UV = 200-400 nm, Visible = 400-800 nm) corresponds to electronic excitations between the energy levels that correspond to the molecular orbitals of the systems. In particular, transitions involving p orbitals and lone pairs (n = non-bonding) are important and so UV-Vis spectroscopy is of most use for

identifying conjugated systems which tend to have stronger absorptions. This technique can be used for analyze the degree of deacetylation of chitosan, stability of carbon nanotubes in chitosan solution, the drug concentration after loading and release from the functionalized carbon nanotubes.

3.8.2 Zeta potential measurement [68]

Zeta potential is a scientific term for electrokinetic potential in colloidal systems. In the colloidal chemistry literature, it is usually denoted using the Greek letter zeta, hence ζ-potential. From a theoretical viewpoint, zeta potential is electric potential in the interfacial double layer (DL) at the location of the slipping plane versus a point in the bulk fluid away from the interface. In other words, zeta potential is the potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed particle. A value of 25 mV (positive or negative) can be taken as the arbitrary value that separates low-charged surfaces from highly-charged surfaces.

3.8.3 Transmission electron microscopy (TEM) [69]

Transmission electron microscopy (TEM) is an imaging technique where a beam of electrons is focused onto a specimen causing an enlarged version to appear on a fluorescent screen or layer of photographic film. Raw carbon nanotubes and functionalized carbon nanotubes can be imaged their morphology clearly in 2 dimensions. This technique will be used for CNTs with diameter less than 100 nm.

3.8.4 Scanning electron microscopy (SEM) [69]

Scanning electron microscopy (SEM) is an imaging technique to produce high resolution images of a sample surface. Due to the manner in SEM, the image is created, its images have a characteristic three-dimensional appearance and are useful to show the surface structure of the sample. This technique will be used for CNTs with diameter more than 100 nm.

3.8.5 Gel permeation chromatography (GPC) [70]

Gel Permeation Chromatography (GPC) is one of the most versatile and powerful analytical techniques available for understanding and predicting polymer performance. GPC is well established for determining the molar mass of polymers. It has the advantage that it determines complete distributions of molar masses as opposed to merely an average molecular weight. This technique indicated the molecular weight of Chitosan with varying degree of deacetylation.

3.8.6 Fourier Transform Infrared spectroscopy (FTIR) [71]

FT-IR is the preferred method of infrared spectroscopy. Infrared spectroscopy, IR radiation is passed through a sample. Some of the infrared radiation absorbed by the sample and some of it is passed through (transmitted). The resulting spectrum represents the molecular absorption and transmission, creating a molecular fingerprint of the sample. Like a fingerprint no two unique molecular structures produce the same infrared spectrum. This technique will be used to analyze functional groups of modified CNTs.

3.8.7 Raman spectroscopy [72]

Raman spectroscopy (named after C. V. Raman) is a spectroscopic technique used to study vibrational, rotational, and other low-frequency modes in a system. It relies on inelastic scattering, or Raman scattering, of monochromatic light, usually from a laser in the visible, near infrared, or near ultraviolet range. The laser light interacts with phonons or other excitations in the system, resulting in the energy of the laser photons being shifted up or down. The shift in energy gives information about the phonon modes in the system.