CHAPTER III

EXPERIMENTAL

3.1. Materials and Chemicals

All chemicals were analytical grade reagents. Natural flake graphite (NFG), size of 200-300 μm, was obtained from Thai Carbon and Graphite Co., Ltd (Bangkok, Thailand). Sulfuric acid (95-98%), Dimethyl sulfoxide (DMSO) and tetramethylrhodamine-5-carbonyl azide (TAMRA) were obtained from Sigma-Aldrich (St. Louis, USA). Dimethyl formamide (DMF) was purchased from Labscan (Bangkok, Thailand). Ethanol was purchased from Merck (Darmstadt,Germany). Potassium permanganate, sodium nitrate and hydrogen peroxide (18%) were obtained from Suksapan (Bangkok, Thailand). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI), N-hydroxy succunamide (NHS) and curcumin were purchased from Acros Organics (Geel, Belgium). Dialysis tubing cellulose membrane (MWCO = 12-14 kDa, avg flat width 76 mm (3.0 in.)), 17.9 mL cm-1 volume capacity, was purchased from Sigma Aldrich (St. Louis, USA).

Dulbecco's modified Eagle's medium (DMEM), Roswell park memorial institute medium 1640 (RPMI), fetal bovine serum (FBS), trypan blue dye, sodium pyruvate and N-2-hydroxyethylpiperazine-N'-2- ethanesulfonic acid (HEPES) were purchased from HyClone (Logan, USA). Penicillin was obtained from General Drugs House Co. Ltd (Bangkok, Thailand). Streptomycin was obtained from M&H Manufacturing Co. Ltd (Samut Prakan, Thailand). Lipolipid polysaccharide (LPS) from Salmonella was purchased from Sigma Aldrich (St. Louis, USA). Murine recombinant interferon gamma (rmIFN-γ) was obtained from R&D Systems (Minneapolis, USA). 4-Paraformadehyde was purchased from Sigma Aldrich (St. Louis, USA). TriZol reagent was purchased from Invitrogen (Paisley, UK). Random hexamer primer was purchased from Qiagen (Hilden, Germany). Reverse transcriptase was obtained from Fermentas (Glen Burnie, USA). iQ^{TM} SYBR Green Supermix was purchased from Biorad (Richmond, USA). 25 cm^2 vented culture flasks, treated petri dish (150x 90 mm) and untreated petri dish (150x 90 mm) were purchased from HyClone (Georgia, Germany).

3.2. The oxidation of graphite

The natural graphite flake (200-300 μm in size) was oxidized using follow method. One gram of graphite flake was mixed with 1.0 g of sodium nitrate and 50 mL 95-98% sulfuric acid. The mixture was sonicated at 40 KHz at room temperature for 1 h. Then the mixture was added with 6.0 g of potassium permanganate and stirred at room temperature for 90 min. Then 100 mL of distilled water was added and the mixture was heat up to 90°C and stirred for another 30 min. After that, 300 mL of distilled water was then added into the mixture and stirring was continued for another 10 min at room temperature. Subsequently, excess potassium permanganate was eliminated by adding 50 mL of 5% hydrogen peroxide and stirred at room temperature for 30 min. Finally, the obtained mixture was washed with distilled water using high speed centrifugation at 10,000 rpm (9,400 g) for 15 min, and then pellet was collected. The pellet was re-suspended in 100 mL distilled water then the suspension was poured into dialysis bag and dialyzed against distilled water until pH 5.5. The product of various morphologies was obtained at this stage. After dialysis, the suspension in the dialysis bag was centrifuged at 10,000 rpm (9,400 g) for 10 min, to remove various artifacts in the precipitate. The supernatant was then centrifuged at 12,000 rpm (11,300 g) for 15 min. The precipitate was re-dispersed with distilled water. This fraction is called fraction 1 (F1). The supernatant was then centrifuged at 15,000 rpm (21,100 g) for 15 min. The precipitate was re-dispersed with distilled water. This fraction is called fraction 2 (F2). The supernatant was centrifuged at 20,000 rpm (37,600 g) for 30 min. The precipitate was re-dispersed with distilled water. This fraction is called fraction 3 (F3). The supernatant is called fraction 4 (F4). The F4 was then filtrated by ultrafiltration with pore size cutoff 100 nm. The filtrated suspension is called fraction 5 (F5).

All fractions, F1-F5, were analyzed morphology by scanning electron microscopy, transmission electron microscopy and high-resolution transmission electron microscopy. The obtained F3 and F4 was further subjected to a range of analytical techniques including; UV-spectrometry, selected area electron diffraction analysis, attenuated total reflectance-fourier transform infrared spectroscopy, raman spectroscopy, elemental analysis, thermo-gravitational analysis, X-ray photoelectron

spectroscopy, dynamic light scattering analysis, confocal laser scanning fluorescence microscopy.

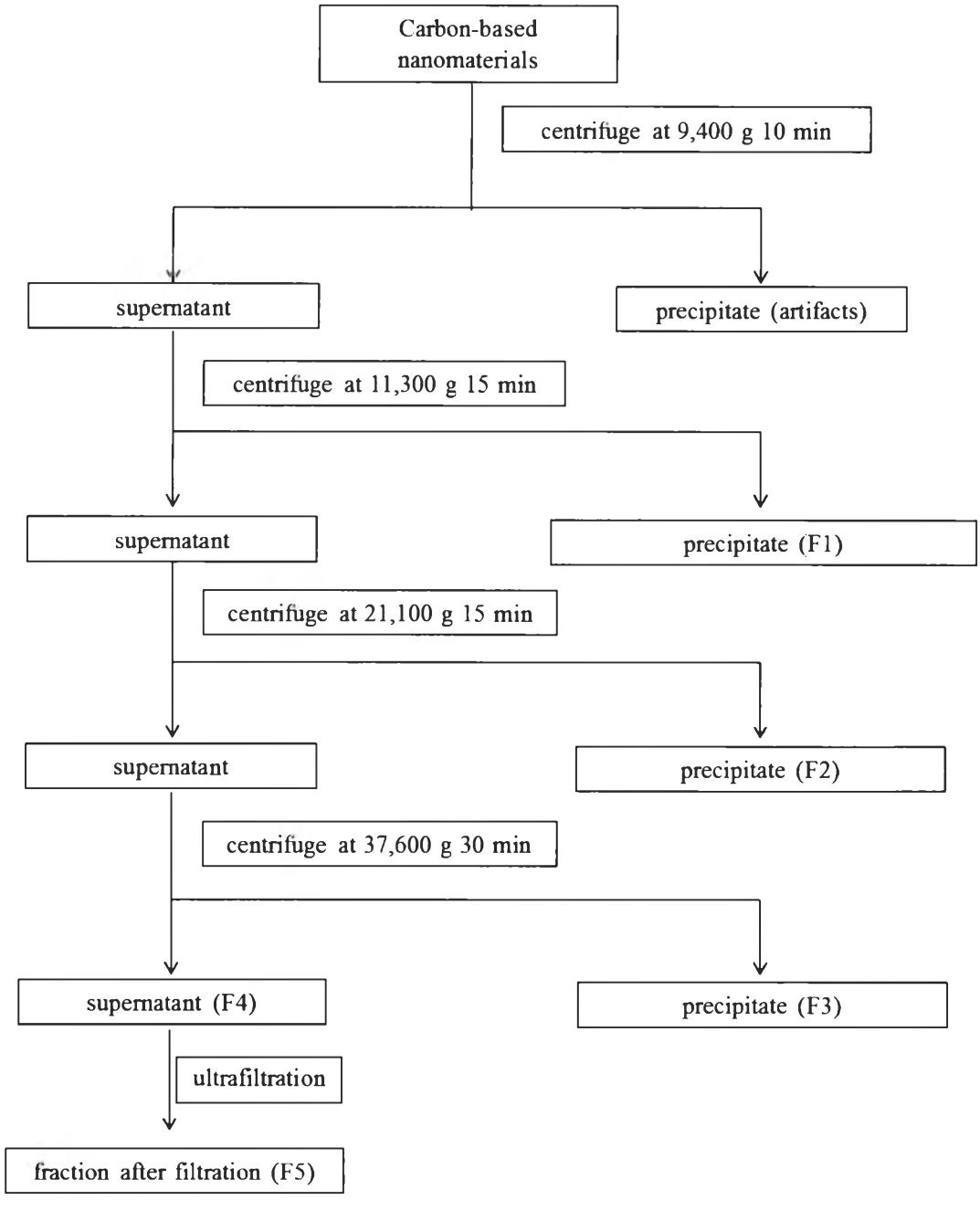

Figure 3. 1 Separation of carbon-based nanomaterials

3.3 Characterization

3.3.1 Scanning electron microscopy (SEM)

The F2 and F4 were diluted with distilled water to make final concentration of 10 ppm. A drop of the suspension was placed on a glass slide and dried in desiccators for 24 h. Subsequently, the samples were coated by gold under vacuum at 15 kV for 90 s. The morphology of samples was analyzed by a scanning electron microscope (JSM-6400 scanning electron microscope, JEOL, Tokyo, Japan and S400, Hitachi, Tokyo, Japan). Visualization proceeded at an accelerating voltage of 15 kV and 20 kV.

3.3.2 Transmission electron microscopy (TEM)

The F1, F3, F4 and F5 were diluted with distilled water to make final concentration of 10 ppm. A drop of the suspension was placed on a glass slide and dried in desiccators for 24 h. The morphology of the samples was analyzed by transmission electron microscopy (TEM, JEM-2100, JEOL, Tokyo, Japan and TECNAI 20 TWIN, Tokyo, Japan). Observation was done at 100-120 kV.

3.3.3 High-resolution transmission electron microscopy (HRTEM)

The F3 and F4 were diluted with distilled water to make final concentration of 10 ppm. A drop of the suspension was placed on a carbon film coated copper grid and dried in desiccators for 24 h. The nanoscale structure of samples was observed using high-resolution transmission electron microscopy (HRTEM, Hitachi H-9000NAR, Hitachi, Ltd., Tokyo, Japan) operating at 300 kV.

3.3.4 Dynamic light scattering (DLS)

The F3 and F4 were diluted with distilled water to make final concentration of 0.8 ppm. The average particle size (z-average size) and zeta potential of samples were measured by zetasizer nano series model (Zetasizer nanoseries model S4700, Malvern Instruments, Worcestershire, UK) equipped with a He-Ne laser beam at 632.8 nm (scattering angle of 173°) at 25 ± 2°C. Each measurement was repeated three times and an average value was reported.

3.3.5 UV-Vis spectroscopy

The F3 and F4 were diluted with distilled water to make final concentration of 8 ppm. UV absorption spectra were acquired with a UV 2500 UV/vis double beam spectrophotometer (Shimadzu Corporation, Kyoto, Japan) using a quartz cell with 1 cm path-length. The absorbance of sample was scanned from 200 to 700 nm.

3.3.6 Selected area electron diffraction analysis (SAED)

The graphite, graphene nanopellet (GNPs), F3 and F4 were diluted with distilled water to make final concentration of 10 ppm. A drop of the suspension was placed on a carbon film coated copper grid and dried in desiccators for 24 h. The crystallinity pattern of the samples was examined by means of selected-area electron diffraction (SAED, JEM-2100, JEOL, Japan) patterns. The camera length was correctly determined using graphite. The nanoscale pattern was observed at 100-120 kV.

3.3.7 Attenuated total reflectance-fourier transform infrared spectroscopy (ATR-FTIR)

The F4 was dried by freeze-drying. Small amount of F4 powder was mix with KBr and ground until physically homogeneous. The mixture was mounted into sample cell. The functional group of nanoparticles in F4 was determined by attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR; Nicolet 6700 FT-IR spectrometer, Thermo Electron Corporation, Madison, WI, USA). The spectra were recorded with 64 scans in the mid-infrared region (4000 – 650 cm^{-1}).

3.3.8 Raman spectroscopy

The F4 was diluted with distilled water to make final concentration of 1000 ppm. A drop of the suspension was placed on a glass slide and driec in desiccators for 24 h. Raman spectroscopy was performed using thermo electron raman spectrometer equipped with a triple monochromator. A 5 mW Ar^{+} laser (λ = 780 nm) was used as an excitation source. Charge coupled detector (CCD) was used as the detector and data were processed with the subtraction of laser-induced background luminescence using a personal computer.

3.3.9 X-ray photoelectron spectroscopy (XPS)

The F3 and F4 were dried by freeze-drying. The surface functional groups of graphite, F3 and F4 were determined by X-ray photoelectron spectroscopy (XPS, Kratos AXIS Ultra DLD instrument, Kratos, Manchester, England) using a monochromatic Al K_α X-ray source at 1486.6 eV and operated at 150 W, 15 kV and 10 mA with a base pressure in the XPS analysis chamber of 5×10^{-8} Torr. High resolution spectra (C1s and O1s) were acquired using a pass energy of 20 eV and 0.1 eV energy steps. All binding energies (BEs) were referenced to the hydrocarbon C1s peak at 285 eV.

3.3.9 Elemental analysis (EA)

The F3 and F4 were dried by freeze-drying. Quantitative analysis of the elemental composition of samples; graphite, GNPs, F3 and F4, was identified by combustion elemental analysis (EA, CHNS/O Analyzer, PE2400 Series II, Perkin Elmer).

3.3.10 X-ray diffraction analysis (XRD)

The F3 and F4 were dried by freeze-drying. The crystallinity of samples; graphite, F3 and F4, was examined by X-ray diffraction analysis (XRD, a Rigaku DMAX 2200/Ultima$^+$ diffractometer, Japan) using Cu K_α radiation source and operating at 40 kV and 30 mA.

3.3.11 Thermo-gravitational analysis (TGA)

The F3 and F4 were dried by freeze-drying. Five mg of the sample; graphite, F3 and F4, were measured. The thermal properties of the samples were characterized by thermo-gravitational analysis (TGA, SDTA 8515 METTLER-TOLEDO thermo-gravimetric analyzer). TGA was performed under a nitrogen flow (100 mL/min) and the mass was recorded as a function of temperature. The samples were heated from room temperature to 800 °C at the temperature ramp of 5 °C/min.

3.4. Synthesis of TAMRA-labeled the cluster of carbon nanoparticles (CCNsT)

To synthesized fluorescence labeled CCNs, five milligram of fluorescence dye, tetramethylrhodamine-5-carbonyl azide (TAMRA) was dissolved in 1 mL of dimethyl formamide (DMF) in round bottle at 0 °C. Then 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI) solution, 6 mg of EDCI in 0.1 mL DI water, was added and the mixture was stirred at 0 °C for 30 min. The N-hydroxy succunamide (NHS) solution (4 mg of NHS in 0.1 mL DI water) and 100 mL of CCN suspension (0.5 mg/mL) were added and the mixture was stirred on ice bath overnight. Finally, TAMRA-labeled the cluster of carbon oxide nanoparticles (CCNsT) suspension was dialyzed against distilled water under light-proof condition until TAMRA could not be detected in the dialysate. The detection of TAMRA in dialysate was analyzed by UV/vis spectroscopy.

3.4.1 Confocal laser scanning fluorescence microscopy (CLSFM)

The CCNsT solution was diluted with distilled water to make final concentration of 100 ppm. A drop of the suspension was placed on a glass slide and covered with glass cover slip. The CLSFM carried out using Nikon Digital Eclipse C1-Si (Tokyo, Japan) equipped with Plan Apochromat VC 100x, BDLaser (561 nm (Melles Griot, Carlsbad, CA, USA)), Nikon TE2000-U microscope, 32-channel-PMT-spectral-detector and Nikon-EZ-C1 Gold Version 3.80 software.

3.4.2 Dynamic light scattering (DLS)

The CCNsT solution was diluted with distilled water to make final concentration of 0.8 ppm. The average particle size (z-average size) and zeta potential of samples were measured by zetasizer nano series model (Zetasizer nanoseries model S4700, Malvern Instruments, Worcestershire, UK) equipped with a He-Ne laser beam at 632.8 nm (scattering angle of 173°) at 25 ± 2°C. Each measurement was repeated three times and an average value was reported.

3.5. Curcumin-loaded CCNs or CCNsT nanoparticles (CCNs-C or CCNsT-C)

To prepare curcumin-loaded CCNs or CCNsT, 4 mg of curcumin was dissolved in 0.5 mL of ethanol and then added to the aqueous suspension of CCNs (10 mL containing 8 mg CCNs) or CCNsT (10 mL containing 8 mg CCNsT). The mixture was incubated at room temperature for 3 h. The suspension was dialyzed against distilled water (3 x 1000 mL) under light-proof condition. The suspension in the dialysis bag was then collected. Finally, the sample was calculated encapsulation efficiency (%EE) and active loading (% active loading).

2.5.1 Confocal laser scanning fluorescence microscopy (CLSFM)

The CCNsT-C solution was diluted with distilled water to make final concentration of 100 ppm. A drop of the suspension was placed on a glass slide and covered with glass cover slip. The CLSFM carried out using Nikon Digital Eclipse C1-Si (Tokyo, Japan) equipped with Plan Apochromat VC 100x, BDLaser (405 nm and 561 nm (Melles Griot, Carlsbad, CA, USA)), Nikon TE2000-U microscope, 32-channel-PMT-spectral-detector and Nikon-EZ-C1 Gold Version 3.80 software.

2.5.2 Dynamic light scattering (DLS)

The CCNsT-C solution was diluted with distilled water to make final concentration of 0.8 ppm. The average particle size (z-average size) and zeta potential of samples were measured by zetasizer nano series model (Zetasizer nanoseries model S4700, Malvern Instruments, Worcestershire, UK) equipped with a He-Ne laser beam at 632.8 nm (scattering angle of 173°) at 25 ± 2°C. Each measurement was repeated three times and an average value was reported.

3.6. Encapsulation efficiency (%EE) and active loading (% active loading)

To calculate the encapsulation efficiency and loading capacity, one mg of the freeze-dried curcumin-loaded CCNs was dissolved in 5.0 mL of ethanol. The mixture was incubated in light-proof condition at room temperature for 1 h before being filtering centrifuged. The ethanol extract was collected and concentration of curcumin in the ethanol extract was determined by UV/vis spectroscopy with the aid of calibration curve. The encapsulation efficiency (%EE) and loading capacity were calculated using equation (1) and (2), respectively.

$$\%EE = \frac{\text{Weight of extracted active}}{\text{Weight of active initially used}} \times 100 \quad (1)$$

$$\%\text{Active loading} = \frac{\text{Weight of extracted active}}{\text{Weight of active loaded spheres}} \times 100 \quad (2)$$

3.7. Synthesis of (1S, 2S)-2-aminocyclopentanecarboxylic acid (acpcPNA)

To synthesize PNA, the (1S, 2S)-2-aminocyclopentanecarboxylic acid (acpcPNA) was synthesized by Vilaivan laboratory[137]. The full constructed PNAs, Flu-O-TGTCAACTGACT-LysNH_2 (random sequences, PNA_{flu}) and Flu-O-GGGATTTTCCCA-LysNH_2 (NF-κB specific sequences, NF-$κB_{flu}$ PNA) for intercellular trafficking and the full constructed Bz-TGTCAACTGACT-LysNH_2 (random sequences, PNA) and Bz-GGGATTTTCCCA-LysNH_2 (NF-κB specific sequences, NF-κB PNA) for cytotoxicity test and IL6 expression test, were purified by high-performance liquid chromatography (HPLC).

3.8 PNA-loaded CCNs or CCNsT nanoparticles (CCNs-PNA or CCNsT-PNA)

To prepare PNA-loaded CCNs or CCNsT, the fluorescein-labeled PNA (NF-$κB_{flu}$ PNA and PNA_{flu}) or unlabeled PNA (PNA and NF-κB) solution (10 µL of 100 µM in PBS pH 7.4) was added to the aqueous suspension of CCNs (90 µL containing 100 µg CCNs) or CCNsT (90 µL containing 100 µg CCNsT). The mixture was incubated at 4 °C for 24 h under light-proof condition. To confirm NF-$κB_{flu}$ PNA-loaded CCNs (CCNs-NF-$κB_{flu}$ PNA), the sample was confirmed by CLSFM.

3.9. PNA loading

To calculate the PNA loading, the PNA_{flu}-loaded CCNs (CCNs- PNA_{flu}) suspension was used to confirm PNA loading by fluorescence microscope. PNA_{flu} solution (10 µL of 100 µM in PBS pH 7.4) was added to the aqueous suspension of CCNs (90 µL containing 100 µg CCNs) and then incubated at 4°C for 24 h. A drop of sample was placed on glass slide and covered with cover slip. Then the slide was subjected to CLSFM. The PNA loading were calculated using equation (2).

3.10. Cell culture

3.10.1 Cell line

The human embryonic kidney (HEK293T) and Mouse leukaemic monocyte macrophage cell line (RAW 264.7) (purchased from ATCC TIB-71) were seeded in 25 cm^2 vented culture flasks and untreated petri dish, respectively. The human epidermoid cervical carcinoma (CaSki) cell lines (purchased from ATCC CRL-1550) were seeded in untreated petri dish. The cells were incubated at 37 °C in a humidified atmosphere of 95:5 % (v/v) air: CO_2, in 7 mL of complete medium (CM). CM was comprised of either Dulbecco's Modified Eagle medium (for HEK293T cells and RAW 264.7 cells) or Roswell park memorial institute medium 1640 (for CaSki cells) as the base and then supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin, 0.4 mg/mL streptomycin, 1% (w/v) sodium pyruvate and 1% (w/v) N-2-hydroxyethylpiperazine-N'-2- ethanesulfonic acid (HEPES).

3.10.2 Cell preparation

For CaSki cells and RAW 264.7 cells, cells were cultured in untreated petri dish in 7 mL CM. To prepare cells for experiment, cells were collected and washed with phosphate buffered saline (PBS) pH 7.4 (PBS;137 mM NaCl, 2.7 mM KCl, 10 mM $NaHPO_4/NaH_2PO_4$). Cells were then centrifuged at 1,000 rpm (95 g) for 5 min. The culture supernatant was discarded and cell pellets were re-suspended in CM. Viable cell number were counted by trypan blue dye exclusion method and plated into the tissue culture treated plates. The CM was changed every 2-3 days and cells were repassaged when they reached 80-85% confluency.

For HEK293T cells, cells were seeded in 25 cm^2 vented culture flask in 7 mL CM. To prepare cells for experiment, the CM was removed from flask using appropriate sized serological pipette. Cells were washed with PBS pH 7.4 before trypsinized by typsin and then incubated for 3 min at 37 °C. After that cells were centrifuged at 1,000 rpm (95 g) for 5 min. The culture supernatant was discarded and cell pellets were re-suspended in CM. Viable cell number were counted by trypan blue dye exclusion method and plated into the tissue culture treated plates. The CM was changed every 2-3 days and cells were repassaged when they reached 80-85% confluency.

3.10.3 Cell preservation for storage

Cell preservation, cells were centrifuged at 1,000 rpm (95 g) for 5 min. The culture supernatant was discarded and cell pellets were re-suspended in CM. The cell was then re-suspended in CM freezing medium containing 10% DMSO (v/v) in CM and kept in cryogenic vials (Coring Incorporation, USA). The cryogenic vials were stored at -80 °C for short term storage. For long term storage, the cryogenic vials were kept at liquid nitrogen tank (Taylor Wharton, USA).

3.10.4 Thawing cells

Stored cell line was rapidly thawed in the water bath (Memmert, Germany) at 37 °C and then added into 9 mL of serum-free CM. Cells suspension was centrifuged at 1,000 rpm (95 g) for 5 min. The culture supernatant was discarded and cell pellets were resuspended in CM. cells were maintained and cultured as described above.

3.11. Cellular uptake of CCNs nanoparticls

3.11.1 Cellular uptake of curcumin-loaded CCNsT (CCNsT-C)

HEK293T cells, at a density of 3 x 10^5 cells per well, were seeded in 6-well plates with placed cover slip and incubated overnight at 37°C in humidified atmosphere (5% CO_2). The samples, free curcumin or CCNsT-C and PBS pH 7.4 as negative control, were introduced to to cells in each well at a final concentration of 10 ppm and then incubated for 2 h at 37 °C in a humidified atmosphere (5% CO_2). After incubaltion, the culture medium was discarded and the cells were washed three times with PBS pH 7.4. Then 1 mL of 4% paraformaldehyde in 0.1 M PBS pH 7.4

was added and incubated for 15 min. after that the solution was removed and the cells were washed with PBS pH 7.4. Fifty microlitter of 0.1 mg/ mL acridine orange were added and incubated for 10 min. The cells were washed with PBS and then the cover slip was placed on glass slide before being subjected to CLSFM analysis. The fluorescent signal of free curcumin was excited by 405 nm laser. The fluorescent signal of acridine orange and TAMRA was excited by 561 nm laser. The CLSFM images were analyzed using a Nikon Digital Eclipse C1-Si equipped with Plan Apochromat VC 100×, Diode Laser and 85 YCA-series Laser (405 nm and 561 nm, Melles Griot, Carlsbad, CA USA), a Nikon TE2000-U microscope, a 32-channel-PMT-spectral-detector and Nikon-EZ-C1 Gold Version 3.80 software).

3.11.2 Cellular uptake of PNA_{flu}-loaded CCNsT (CCNs-PNA_{flu})

RAW 264.7 cells, at a density of 2×10^6 cells per well, were seeded in 6-well plates with placed cover slip and incubated overnight at 37°C in humidified atmosphere (5% CO_2). The samples, free random PNA_{flu} or free NF-κB_{flu}, random PNA_{flu}-loaded CCNsT or NF-κB_{flu}-loaded CCNsT (0.5 μM of final PNA concentration) and PBS pH 7.4 as negative control, were introduced to cells in each well (10 ppm of final CCNs concentration) and the mixtures were incubated for 4 h at 37 °C in a humidified atmosphere (5% CO_2). After incubaltion, the culture medium was discarded and the cells were washed three times with PBS pH 7.4. Then 1 mL of 4% paraformaldehyde in 0.1 M PBS pH 7.4 was added and incubated for 15 min. After that the solution was removed and the cells were washed with PBS pH 7.4. One mililitter of 0.01 mg/ mL dapi solution were added and incubated for 10 min. The cells were washed with PBS and then the cover slip was placed on glass slide before being subjected to CLSFM analysis. The fluorescent signal of fluorescein-labeled PNA and Dapi were excited by 405 nm laser. The fluorescent signal of TAMRA-CCNs was excited by 561 nm laser. The CLSFM image was analyzed using a Nikon Digital Eclipse C1-Si equipped with Plan Apochromat VC 100×, Diode Laser and 85 YCA-series Laser (405 nm and 561 nm, Melles Griot, Carlsbad, CA, USA), a Nikon TE2000-U microscope, a 32-channel-PMT-spectral-detector and Nikon-EZ-C1 Gold Version 3.80 software).

3.12. Assessment of cell viability

3.12.1 Assessment of cell viability for CCNs

Assessment of cell viability was performed using the 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT) mitochondrial activity assay as a surrogate measure of cell viability. HEK293T and CaSki cells were seeded into 96-well plates at a density of 1×10^4 cells per well in CM and incubated for 24 h. After removal of the CM, cells were exposed to 100 µL of CCNs suspension in CM at various concentrations (range of 0.01-10 µg/mL) and PBS in CM as negative control for 48 h under standard culture conditions. Then, 10 µL of PBS containing 1 mg/mL MTT was added to each well and incubated as above for 4 h before removing the media. The cell membranes were permeabilized with MTT after that the formazan crystals dissolved by aspirating in 200 µL/well of isopropanol. Then the 96-well plates were centrifuged at 4840 g for 10 min. Fifty microliter of the supernatant was placed on new 96-well plates for measurement of the absorbance at 540 nm using a microplate reader (Anthos 2010, Anthos Labtec Instrument, Austria). All conditions were tested in triplicate. Cell viability was calculated using the following equations.

$$\% \text{ Cell viability} = \frac{OD_{test} - OD_{blank}}{OD_{control} - OD_{blank}} \times 100 \quad (3)$$

3.12.2 Assessment of cell viability for NF-κB PNA

Assessment of cell viability was carried out by a MTT assay. RAW 264.7 cells were seeded into a 96 well plates at density of $1x10^4$ cells in DMEM and allowed to adhere for 24 h. After removal of the culture medium, cells were exposed to 100 µL of free NF-κB PNA, CCNs and CCNs-NF-κB PNA at various concentrations, in range of 0.1-10 µg/mL in DMEM media, for 24 h. After incubation, 10 µL of PBS containing 1 mg/mL MTT solution was added to each well and was followed by 4 h of incubation. After removing of the media, formazan crystals were dissolved by adding 200 µL/well of isopropanol. Plates were spinned at 4840 g for 10 min and 50 µl of the supernatant was collected for absorbance measurement at 540 nm by microplate reader. All conditions were tested in triplicate. Cell viability was calculated using equations (3).

3.13. Anticancer activities

The anticancer activities of the CCNs-C were evaluated by MTT assay. The pre-incubated for 24 h, CaSki cells were seed into a 96 well plates at density of $1x10^4$ cells per well. After removal of the CM, cells were exposed to 100 µL of CCNs suspension in CM (10 µg/mL), CCNs-C suspension in CM (10 µg/mL) and PBS in CM as negative control for 24 h. After incubation, 10 µL of PBS containing 1 mg/mL MTT was added to each well and incubated as above for 4 h before removing the media. After removing of the media, formazan crystals were dissolved by adding 200 µL/well of isopropanol. Then the 96-well plates were centrifuged at 4840 g for 10 min. Fifty microliter of the supernatant was placed on new 96-well plates for measurement of the absorbance at 540 nm using a microplate reader. All conditions were tested in triplicate. Cell viability was calculated using the following equations. All conditions were tested in triplicate. The anticancer activity was calculated using the following equations below.

$$\% \text{ Anticancer activity} = \frac{OD_{test} - OD_{blank}}{OD_{control} - OD_{blank}} \times 100 \quad (4)$$

3.14. *il-6* gene expression

3.14.1 *il-6* mRNA suppression

The IL-6 mRNA suppression of the NF-κB-loaded CCNs (CCNs- NF-κB) was evaluated by real time PCR. For pre-incubation, RAW 264.7 cells were seeded into a 24 well plates at density of $2x10^5$ cells per well. Then 100 µL of the aqueous solutions of free random PNA, free NF-κB PNA, CCNs, random CCNs-PNA and CCNs-NF-κB PNA (final concentration of 5 ppm of CCNs and 0.5 µM of PNA or NF-κB PNA) were introduced into cells and incubated for 2 h and 4 h. After incubation, the cells were stimulated with lipopolysaccharide (LPS) plus murine recombinant interferon gamma (rmIFN-γ) at final concentration 100 ng/mL and 10 ng/mL, respectively, for 6 h before RNA extraction.

3.14.2 RNA extraction

After stimulation, the cells were washed twice with PBS pH 7.4 before 500 µL of TriZol reagent was added for RNA extraction and incubated for 5 min at room temperature. Then the aqueous solution was collected in to microtube after that 100 µL of chloroform was added. The aqueous solution was mixed by vortex and incubated for 15 min at room temperature. After incubation, the sample which contains the interphase/organic phase with TRIzol was spin down by centrifugation at 12,000 g for 15 min at 4 °C. Then 200 µL of chloroform fraction was collected using a P-100 autopipettor to new microtube and carefully removed any remaining organic phase which would contaminate RNA with DNA. The aqueous phase was added 250 µL of isopropyl alcohol, mixed gently, and incubated at room temperature for 10 min. After that the aqueous phase was centrifuged at 12,000 g for 10 min at 4 °C and supernatant was removed. The RNA precipitate will form a gel-like pellet at the bottom of the tube. To wash the RNA pellets, 500 µL of 75% Ethanol (ice cold) was added and mixed using vortex. The RNA solution was centrifuged at 7,500 g for 5 min at 4°C. Then the supernatant was removed and RNA precipitate was dried in PCR hood. The RNA precipitate should form a visible, white pellet, at the bottom of the tube. Allow to dry completely so there is no residual ethanol. To RNA redissolve, RNA precipitate was dissolved by Rnase-free water (DEPC WATER) at 55°C for 10 min. RNA concentration was measured by nanodrop spectrophotometer (Nanodrop 2000, Thermo Scientific, USA). RNA solution was stored at -80°C.

3.14.3 Reverse transcription polymerase chain reaction (RT-PCR)

To generate cDNA, 100 ng of total RNA was used to generate complementary DNA (cDNA) using random hexamer primers, deoxyribonucleotide triphosphate (dNTP) mix, RNase inhibitor and reverse transcriptase, as recommended by the supplier's instructions (Fermentas, Glen Burnie, MD, USA). Firstly, RNA was incubated with 0.2 µg random hexamer primers. DEPC water was used to adjust the volume to 12.5 µL. The solutions were heated at 65 °C for 5 min to denature RNA secondary structure and then quickly chilled on ice to let the primer anneal to the RNA. Other components of reverse transcription (RT) were added to the reaction including 1 mM dNTPs mix,

20U of RNase inhibitor, 200U of reverse transcriptase and 1xRT buffer. The sample was mixed well before analyzed by PCR machine Bioer Life Express (Bioer technology, China). The RT reaction was started with incubation at 25 °C for 10 min. Then the reaction was extended at 42 °C for 1 h and heated at 70 °C for 10 min to inactivate the enzyme. Finally, cDNA was stored at -20 °C until use. The RT-PCR reaction was done 30 cycles.

3.14.4 Quantitative real time polymerase chain reaction (qRT-PCR)

To determine quantitative real time PCR (qRT-PCR), PCR amplification of the 474-bp murine *il-6* gene was performed with MIL-6F/R primers (5'-CATGTTCTCTGGGAAATCGTGG-3'/ 5'-AACGCACTAGGTTTGCCGAGTA-3'), with amplification of a 380-bp fragment of the mβ-actin gene (using bACTF/R primers) as a control (5'-ACCAACTGGGACGACATGGAGAA-3'/ 5'-GTGGTGGTGAAGCTGTAGCC-3'). Two µL of cDNA solution was added to qPCR solution that composed of 12.5 µL iQ™ SYBR Green Supermix, 0.3 µM of forward and reverse primer and 9 µL of nuclease free water (each sample was performed in triplicate). The quantitative PCR (qPCR) amplification was performed using an MJ Mini personal Thermal cycler (BioRad, Richmond, VA, USA), according to the manufacturer's protocol (Fermentas, Burlington, Ont., Canada). Briefly, cDNA was specifically amplified m*il-6* for 40 cycles and mβ-actin for 30 cycles. The amplified m*il-6*, the condition was using 95 °C for denaturing, 57 °C for annealing and 72 °C for extension. The amplified mβ-actin, the condition was using 94 °C for denaturing, 52 °C for annealing and 72 °C for extension. The relative expression levels were calculated and analyzed by $2^{\Delta\Delta C_T}$ [182].

2.14.5 10% acrylamide gel electrophoresis

DNA which is double stand was dissociated by heat at 95 °C for 10 min in the heat box (Thermomixer Compact, Eppendorf, Germany). After that the excess samples; free PNA, free NF-κB PNA and free NF-κB_{flu} PNA, were immediately added into DNA solution. The mixture solution was incubated for 2 h on ice bath. The mixture solution was then separated among PNA, DNA and PNA-DNA complexes, by gel electrophoresis. Ten present of acrylamide gel was prepared follow;...

Xx µl of samples was loaded into acrylamide gel. The samples were separated using a constant volt for at least 90 min in TEA running buffer for 10% acrylamide gel. 10%

acrylamide gel was visualized by ImageScanner™ II (GE Healthcare) with fluorescence exposed. After that the 10% acrylamide gel was then stained with ethidium bromide (EtBr) and visualized by ImageScanner™ II.

3.14.6 Statistical analysis

All data were presented as means ± standard deviation (SD). Statistical significant difference between experiment and control groups was determined by one-way analysis of variance (ANOVA), followed by Duncan's multiple range test. The SPSS Statistic Base 17.0 (SPSS Co., Ltd., Thailand, Server IP Address; dc1.win.chula.ac.th) was used. $P < 0.05$ was accepted as statistically significant.