CHAPTER III

EXPERIMENTAL

3.1 General procedures

3.1.1 Analytical measurements

Nuclear Magnetic Resonance (NMR) spectra were recorded on a Varian Mercury Plus 400 NMR spectrometer. All chemical shifts were reported in part per million (ppm) using the residual proton signals in deuterated solvents as internal references. Particle morphology was determined by scanning electron microscopy (SEM) performed on a JEOL JSM-6510A with a high resolution of 3.0 nm at 30 kV. Sample was grounded on aluminuim stub. Transmission electron microscopy (TEM) was performed on a JEOL JEM 2010 with a field emission gun operated at 200 kV. The TEM micrographs were used to determine size of the deposited particles by counting approximately 200 particles (Scion Image, Scion Corporation). Dynamic light scattering (DLS) was used to measure the particle size by using Malvern Zetasizer. IR spectrophotometric measurement of the dried particle samples was performed on Thermo, Nicolet 6700 FT-IR. Absorption spectra were measured by a Varian Cary 50 UV-Vis spectrophotometer. Fluorescence spectra were performed on Varian spectrofluorometer by personal computer data processing unit. The light source is Cary Eclipse a pulsed xenon lamp and a detector is a photomultiplier tube. X-ray absorption near-edge structure (XANES) measurement was performed in transmission mode at beamline BL8 of the Synchrotron Light Research Institute (SLRI) Thailand. The *L*III-edge spectra of the lanthanides Gd at energies of 7.241 keV were recorded using a Si (111) double-crystal monochromator. The concentration of Gd^{3+} in the solution was measured by inductively coupled plasma-atomic emission spectroscopy (ICP-AES) model iCAP 6000 series (Thermo scientifid). X-ray diffraction patterns (XRD) were determined on D4 X-ray diffractometer using Cu Kα radiation (λ=0.1541). Fluorescence images were obtained by using Inverted fluorescence microscope, Olympus IX71, Mercury Lamp U-RFL-T. The confocal

laser scanning microscopy (CLSM) model Nikon eclipse Ti with λ_{ex} at 488, 405 and 561 nm was used to study cellular uptake of SW620 cells.

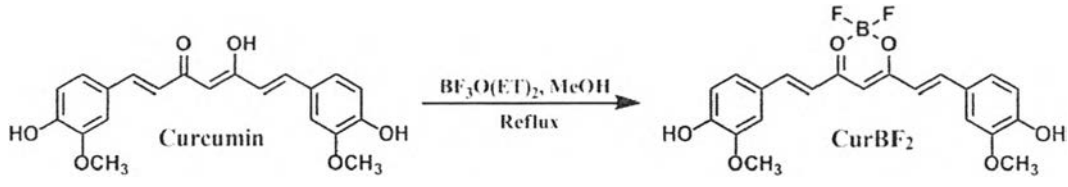
3.1.2 Materials

All materials, solvents and chemicals were purchased from Aldrich, Fluka and Merck as standard analytical grade and were used without further purification. curcumin powder; **Cur** was provided from THAI-CHINA FLAVORS & FRAGRANCES INDUSTRY CO., LTD. Difluoroboron curcumin derivative, **$CurBF_2$** was obtained by the replacement of borontrifluoride diethytherate to enolate unit of curcumin [60]. Mono and di-tosylated (**$CurBF_2OTs$** and **$CurBF_2(OTs)_2$**) were synthesized by the reaction between **$CurBF_2$** and tosyl chloride [58]. Commercial grade solvents such as dichloromethane, ethyl acetate, hexane, and methanol were purified by distillation. Column chromatography was carried out on silica gel (Kieselgel 60, 0.063-0.200 mm, Merck). Thin-layer chromatography (TLC) was performed on silica gel plates (Kieselgel 60 F_{254}, 1 mm, Merck). Dimethyl sulfoxide, spectrophotometric grade used in UV-Visible and fluorescence measurement was purchased from Merck.

All synthesized compounds were characterized by ^{1}H-NMR spectroscopy.

3.2 Synthesis of curcumin derivatives

3.2.1 Synthesis of curcumin borondifluoride ($CurBF_2$)

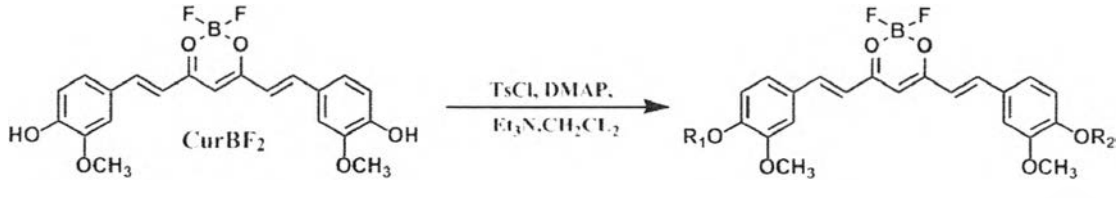


Into a 250 mL two-neck round bottom flask, **curcumin** (2.947g, 8 mmol) was dissolved in methanol (200 ml) to give a bright yellow solution. Borontrifluoride diethyl etherate (1.02 ml, 8 mmol) was added and the solution turned to red suddenly. The reaction was stirred at 25 °C for 16 h under N_2. Solvent was removed under reduced pressure to obtain a red powder. The desired product, **$CurBF_2$** 63% yield, was obtained as a red solid after recrystallization in MeOH:EtOAc (1:3).

Characterization data for $CurBF_2$

^{1}H-NMR spectrum (400 MHz, DMSO-d_6) : δ (in ppm) = 10.09 (s, -O*H*, 2H), 7.90 (*J* = 15.6 Hz, d, -olifinic *H*, 2H), 7.45 (s, -Ar*H*, 2H), 7.32 (*J* = 8.0 Hz, d, -Ar*H*, 2H), 6.99, 6.85 (*J* = 8.0 Hz, d, -Ar*H*, 2H), 6.42 (s, -Ar*H*, 1H), 3.83 (s, -OCH_3, 6H,)

3.2.2 Synthesis of tosylation curcumin borondifluoride ($CurBF_2OTs$ and $CurBF_2(OTs)_2$)



Into a 100 mL two-neck round bottom flask, a solution of **$CurBF_2$** (0.277 g, 0.67 mmol), DMAP (4-dimethylaminopyridine) and triethylamine (0.084 mL, 2.01 mmol) was mixed and stirred for 30 minutes in dichloromethane (30 mL) at 0 °C under nitrogen atmosphere. A solution of *para*-toluenesulfonyl chloride (0.284 g, 1.49 mmol) in dichloromethane (10 mL) was added dropwise through the addition funnel over 30 minutes into the mixture. Upon completion of the reaction, a solution of HCl (3 M) was added into the mixture to adjust pH to be 1 and the mixture solution was stirred for 30 minutes. The reaction was extracted with water and the organic solvent was evaporated. The crude product was purified by column chromatography using 5% ethyl acetate in dichloromethane to afford the yellow compound of **$CurBF_2OTs$** and **$CurBF_2(OTs)_2$** (21 and 65% yield, respectively)

Characterization data for $CurBF_2OTs$

^{1}H-NMR spectrum (400 MHz, CD_3Cl) : δ (in ppm) = 7.97 (*J* = 15.6 Hz, d, -olifinic *H*,-, 1H), 7.85 (*J* = 15.2 Hz, d, -olifinic *H*, 1H), 7.70 (*J* = 8.0 Hz, d, -Ar*H*, 2H), 7.26 (*J* = 8Hz, d, -Ar*H*, 2H), 7.07 (d, -Ar*H*, 2H), 7.03 (s, -Ar*H*, 1H), 6.93 (d, -Ar*H*, 1H), 6.90 (s, -ArO*H*, 1H), 6.54 (*J* = 15.6 Hz , d, -C*H*=C*H*-, 3H), 6.01 (*J* = 17.2 Hz , d, -C*H*=C*H*-, 2H), 3.90 (s, -OCH_3, 3H), 3.56 (s, -OCH_3, 3H), 2.42 (s, -CH_3, 3H)

Characterization data for $CurBF_2(OTs)_2$

^{1}H-NMR spectrum (400 MHz, CD_3Cl) : δ (in ppm) = 7.91 (*J* = 16 Hz, d, -olifinic *H*,-, 2H), 7.70 (*J* = 8.4 Hz, d, -Ar*H*, 4H), 7.26 (*J* = 8.0 Hz, d, -Ar*H*, 4H), 7.15 (d, -Ar*H*, 4H), 6.96 (s, -C*H*=C*H*-, 2H), 6.59 (*J* = 15.6 Hz , d, -C*H*=C*H*-, 2H), 6.04 (s, -C*H*=C*H*-, 1H), 3.57 (s, -OCH_3, 6H), 2.42 (s, -CH_3, 6H)

3.2.3 UV-visible studies of Cur, $CurBF_2$, $CurBF_2OTs$ and $CurBF_2(OTs)_2$

Typically, a $2x10^{-4}$ M solution of **Cur**, **$CurBF_2$**, **$CurBF_2OTs$** and **$CurBF_2(OTs)_2$** in spectrophotometric grade DMSO was prepared in a 5 mL volumetric flask (shown in Table 3.1). The stock solution (0.25 mL) was pipetted into volumetric flask (5 mL) then adjusted to 5 mL by DMSO for preparing the 1×10^{-5} M solution.

The 2 mL of 1.0×10^{-5} M **Cur**, **$CurBF_2$**, **$CurBF_2OTs$** and **$CurBF_2(OTs)_2$** in DMSO was placed in a 1-cm quartz cuvette and the absorption spectra were recorded from 200-800 nm at ambient temperature. Amounts of **Cur**, **$CurBF_2$**, **$CurBF_2OTs$** and **$CurBF_2(OTs)_2$** were used in UV-visible spectrophotometry studies as shown in Table 3.1.

Table 3.1 Amounts of **Cur**, **$CurBF_2$**, **$CurBF_2OTs$** and **$CurBF_2(OTs)_2$** used in spectrophotometry studies and their absorption wavelengths.

	Cur	**$CurBF_2$**	**$CurBF_2OTS$**	**$CurBF_2(OTs)_2$**
Molecular-weight ($gmol^{-1}$)	368.13	416.18	570.37	724.55
Weight (mg)	0.37	0.42	0.57	0.72
λ_{ab} (nm)	429	517	506	444 and 467

3.3 Self-assembled coordination nanoparticles from surfactants and gadolinium in buffer solution

3.3.1 Preparation of coordination nanoparticles (CNPs)

The stock solutions of gadolinium (Gd^{3+}) were prepared in spectrophotometric grade DMSO at concentrations of 2.00 x 10^{-2} mol/L. The stock solutions of sodium dodecyl sulfate (SDS) was prepared in 0.1 M HEPES buffer pH 7.4 at the concentration of 1.00 x 10^{-2} mol/L. In a 10 mL volumetric flask, 1 mL of the SDS stock solution was added. Then 0.5 mL of the gadolinium (Gd^{3+}) stock solution and 0.5 mL spectrophotometric grade DMSO were added to the solution mixture. After that, a volume was adjusted to 10.00 mL with 0.1M HEPES buffer pH 7.4 to give a final concentration of SDS surfactant and gadolinium (Gd^{3+}) at 1.00 x 10^{-3} mol/L in 10% DMSO aqueous solution. The mixtures were transferred to 15 mL vial and stirred at room temperature for 3 h. Other surfactants and buffers were prepared in the same method. Samples name and their components were shown in Table 3.2.

Table 3.2 Composition of samples prepared at room temperature

Sample name	Lanthanide	Surfactant	Buffer
GdCH	Gd^{3+}	CTAB	HEPES
GdTxH	Gd^{3+}	Triton X-100	HEPES
GdSH	Gd^{3+}	SDS	HEPES
GdSM	Gd^{3+}	SDS	MOPS
GdST	Gd^{3+}	SDS	Tris
GdSP	Gd^{3+}	SDS	Phosphate

3.3.2 Morphology of coordination nanoparticles

The coordination nanoparticles were prepared as follows. After keeping the mixtures for 3 h, the precipitates formed were ultrasonicated for 1 minute before collected by high speed centrifugation (10,000 rpm/10 min). The obtained materials were washed with MilliQ water (5.00 mL) around 3 times. Then the resulting aqueous suspensions were redispersed in MilliQ water by ultrasonicating and subjected to scanning electron microscope measurements (SEM). The transmission electron microscope (TEM) specimen of coordination nanoparticles was prepared as follows. The surfactant/lanthanide nanoparticles collected by centrifugation was redispersed in MilliQ water (10.00 mL) by ultrasonicating for 1 minute. A drop of the obtained dispersion was placed on carbon-coated copper grid. After 1 minute, the droplet was removed by adsorbing to a piece of filter paper. The specimen was dried in vacuum and monitored by TEM measurements. The TEM micrographs were used to determine size of the deposited particles by counting approximately 200 particles (Scion Image, Scion Corporation).

Dynamic light scattering (DLS) technique was also used to determine the particle size of the coordination nanoparticles. The **CNPs** samples were prepared after washing with MilliQ water (5.00 mL) around 3 times. They were redispersed in MilliQ water (10.00 mL) by ultrasonicating for 1 minute. Then the samples were measured by Dynamic light scattering (DLS) using Malvern Zetasizer.

The dried specimens of coordination nanoparticles, prepared after the last washing and centrifugation, were dried in a vacuo for 48 h. The samples were characterized by FT-IR, X-ray diffractometer and X-ray absorption near-edge (XANES) techniques. The dried samples in dried KBr were homogeneously grinded in a mortar and then, were examined by FT-IR.

Crystal structure of coordination nanoparticles were studied by X-ray diffraction (XRD) using a D4 X-ray diffractometer operating with Cu Kα radiation (λ=0.1541) radiation with 2θ angles in the range 2–70° in steps of 0.02°.

The XAS technique was usually employed to analyze the oxidation state of Gd^{3+} and the binding mode of donor atom based SDS and HEPES for adaptive self-assembled nanoparticles [61,62]. The XANES spectra of Gd L_{III}-edge were verified in transmission mode at room temperature by X-ray absorption. The storage ring was

operated with the electron energy of 1.2 GeV and electron current between 90 and 140 mA. Using a synchrotron X-ray monochromator equipped with double Ge (2 2 0) crystals, the photon energy was scanned from 7223 to 7323 eV with a step of 0.3 eV. Each sample powder was pressed into a frame and then covered by polyimide tape and mounted onto a sample holder. Internal energy calibration was performed by Gd foil at 7147 eV. Linear combination fitting of XANES spectra was processed and analyzed with Athena program [63,64].

3.3.3 Inductively coupled plasma-atomic emission spectroscopy

ICP-AES was used to quantify gadolinium ion concentration in the supernatant and to study the machanism of SDS and HEPES buffer in the novel coordination nanoparticles. The results of ICP-AES experiment focused on an investigation of effects from various SDS in water system and HEPES buffer systems.

In water system, SDS (dissolved in Milli-Q water) in different concentrations from 0 to 18 mM was added to a 1 mM gadolinium ion solution. The conditions of compound, which were studied in each system, were shown in Table 3.3.

In HEPES buffer system (**GdSH CNPs** system), SDS was dissolved in 0.1 M HEPES buffer solution pH 7.4 in different concentrations from 0 to 18 mM, then it was added to a 1 mM gadolinium ion solution. The conditions of compound, which were studied in each system, were shown in Table 3.4.

Table 3.3 Amount of SDS, Gd^{3+}, DMSO and Milli-Q water used in ICP-AES studies

[SDS]/[Gd^{3+}]	Volume of			
	20 mM SDS stock sol^{n}	20 mM Gd^{3+} stock sol^{n}	DMSO	Milli-Q water
0	0.00	0.5	0.5	9.00
0.5	0.25	0.5	0.5	8.75
1	0.50	0.5	0.5	8.50
1.5	0.75	0.5	0.5	8.25
2	1.00	0.5	0.5	8.00
2.5	1.25	0.5	0.5	7.75
3	1.50	0.5	0.5	7.50
3.5	1.75	0.5	0.5	7.25
4	2.00	0.5	0.5	7.00
4.5	2.25	0.5	0.5	6.75
5	2.50	0.5	0.5	6.50
5.5	2.75	0.5	0.5	6.25
6	3.00	0.5	0.5	6.00
6.5	3.25	0.5	0.5	5.75
7	3.50	0.5	0.5	5.50
7.5	3.75	0.5	0.5	5.25
8	4.00	0.5	0.5	5.00
9	4.50	0.5	0.5	4.50
10	5.00	0.5	0.5	4.00
12	6.00	0.5	0.5	3.00
14	7.00	0.5	0.5	2.00
16	8.00	0.5	0.5	1.00
18	9.00	0.5	0.5	0.00

Table 3.4 Amount of SDS, Gd^{3+}, DMSO and 0.1 M HEPES buffer solution pH 7.4 used in ICP-AES studies

[SDS]/[Gd^{3+}]	Volume of			
	20 mM SDS stock sol^{n}	20 mM Gd^{3+} stock sol^{n}	DMSO	HEPES
0	0.00	0.5	0.5	9.00
0.5	0.25	0.5	0.5	8.75
1	0.50	0.5	0.5	8.50
1.5	0.75	0.5	0.5	8.25
2	1.00	0.5	0.5	8.00
2.5	1.25	0.5	0.5	7.75
3	1.50	0.5	0.5	7.50
3.5	1.75	0.5	0.5	7.25
4	2.00	0.5	0.5	7.00
4.5	2.25	0.5	0.5	6.75
5	2.50	0.5	0.5	6.50
6	3.00	0.5	0.5	6.00
7	3.50	0.5	0.5	5.50
8	4.00	0.5	0.5	5.00
9	4.50	0.5	0.5	4.50
10	5.00	0.5	0.5	4.00
12	6.00	0.5	0.5	3.00
14	7.00	0.5	0.5	2.00
16	8.00	0.5	0.5	1.00
18	9.00	0.5	0.5	0.00

All of samples were continuously stirred for 3 h and then left to equilibrate for 12 h. The samples exhibited a clear two phase separation, which showed the occurrence of a flocculation. The aliquots of supernatant were taken for ICP-AES analysis. All solutions were pipetted into a polypropylene tube diluted with a 2% (w/w) nitric acid aqueous solution to obtain the necessary final analytical metal concentration between 0.1 and 100 ppm. Before each set of measurements, the calibration was performed using metal-containing standard solutions and an aqueous solution of 2% (w/w) HNO_3 was used for a blank and for rinsing the instrument after the highest standard solution measurement. Each sample was injected in triplicate and the reported value related to an average value of those three measurements.

3.3.4 UV-Vis and Fluorescence measurements of coordination nanoparticles (CNPs)

The coordination nanoparticles were prepared as follows. After keeping the mixtures for 3 h, the resulting aqueous suspensions were ultrasonicating for 1 minute before the measurement. Then the UV-Vis spectra were measured in the range of 200-800 nm. The fluorescence spectra were recorded at room temperature under the following condition:

	Fluorescence
Start (nm)	*465*
End (nm)	*800*
Excitation (nm)	*445*
Excitation Slit	*10.0*
Emission Slit	*10.0*
PMT	*600*
Scan rate	*600 nm/min*

3.4 Self-assembled coordination nanoparticles from surfactants and gadolinium to stabilize curcumin derivatives in buffers solution

3.4.1 The effects of various surfactants and various buffers to the curcumin derivative ($CurBF_2$) encapsulated in CNPs

The stock solutions of gadolinium (Gd^{3+}) were prepared in spectrophotometric grade DMSO at concentrations of 2.00 x 10^{-2} mol/L. The stock solutions of surfactants (Triton X-100, CTAB and SDS) were prepared in 0.1 M HEPES buffer pH 7.4 at the concentration of 1.00 x 10^{-2} mol/L. The stock solutions of SDS were prepared in 0.1 M MOPS, Tris and Phosphate buffers solution pH 7.4 at the concentration of 1.00 x 10^{-2} mol/L. The stock solution of **$CurBF_2$** was prepared in DMSO at the concentration of 2.00 x 10^{-4} mol/L.

In a 10 mL volumetric flask, 1 mL each surfactant in stock solution was added. Then, 0.5 mL **$CurBF_2$** in stock solution and 0.5 mL gadolinium (Gd^{3+}) in stock solution were added to the solution mixture. After that, a volume was adjusted to 10.00 mL with 0.1M HEPES buffer pH 7.4 to give a final concentration of SDS surfactant and gadolinium (Gd^{3+}) at 1.00 x 10^{-3} mol/L, and **$CurBF_2$** at 1.00 x 10^{-5} mol/L in 10% DMSO aqueous solution. The mixtures were transferred to 15 mL vial and stirred at room temperature for 3 h. Then, the resulting aqueous suspensions were ultrasonicating for 1 minute before collected by high speed centrifugation (10000 rpm/10 min). The resulting aqueous supernatants were placed in a 10.0 mm width quartz cell. UV-Vis spectra were recorded at 25°C after each addition. The dyed-doped samples name and their components were shown in Table 3.5.

Table 3.5 Dyed-doped samples name and their components

Sample name	Lanthanide	Surfactant	Buffer	Derivative
GdCH-$CurBF_2$	Gd^{3+}	CTAB	HEPES	**$CurBF_2$**
GdTxH-$CurBF_2$	Gd^{3+}	Triton X-100	HEPES	**$CurBF_2$**
GdSH-$CurBF_2$	Gd^{3+}	SDS	HEPES	**$CurBF_2$**
GdSM-$CurBF_2$	Gd^{3+}	SDS	MOPS	**$CurBF_2$**
GdST-$CurBF_2$	Gd^{3+}	SDS	Tris	**$CurBF_2$**
GdSP-$CurBF_2$	Gd^{3+}	SDS	Phosphate	**$CurBF_2$**

3.4.2 The effect of various concentrations of SDS surfactant to the curcumin derivative ($CurBF_2$) encapsulated in CNPs

The stock solutions of gadolinium (Gd^{3+}) were prepared in spectrophotometric grade DMSO at concentrations of 2.00 x 10^{-2} mol/L. The stock solutions of SDS surfactants were prepared in 0.1 M HEPES buffer pH 7.4 at the concentration of 1.00 x 10^{-2} mol/L. The stock solution of **$CurBF_2$** was prepared in DMSO at the concentration of 2.00 x 10^{-4} mol/L.

In a 10 mL volumetric flask, each studied volume of SDS surfactant in stock solution was added. Then, 0.5 mL **$CurBF_2$** in stock solution and 0.5 mL gadolinium (Gd^{3+}) stock solution were added to the solution mixture. After that, a volume was adjusted to 10.00 mL with 0.1M HEPES buffer pH 7.4 to give a final concentration of gadolinium (Gd^{3+}) at 1.00 x 10^{-3} mol/L, and **$CurBF_2$** at 1.00 x 10^{-5} mol/L in 10% DMSO aqueous solution. The final concentration of SDS and the volume of each component were shown in Table 3.6. The mixtures were transferred to 15 mL vial and stirred at room temperature for 3 h. Then, the resulting aqueous suspensions were ultrasonicating for 1 minute before collected by high speed centrifugation (10000 rpm/10 min). The resulting aqueous supernatants were placed in a 100.0 mm width quartz cell. UV-Vis spectra were recorded at 25°C after each addition.

Table 3.6 Amount of SDS, Gd^{3+}, **$CurBF_2$** and 0.1 M HEPES buffer solution pH 7.4 used in the study of effect of SDS concentration

[SDS] (mM)	Volume of			
	10 mM SDS stock sol^n	20 mM Gd^{3+} stock sol^n	0.2 mM **$CurBF_2$** stock sol^n	0.1 M HEPES buffer sol^n
0.25	0.25	0.5	0.5	8.75
0.5	0.50	0.5	0.5	8.50
0.75	0.75	0.5	0.5	8.25
1	1.00	0.5	0.5	8.00
1.5	1.50	0.5	0.5	7.50
1.75	1.75	0.5	0.5	7.25
2	2.00	0.5	0.5	7.00
2.5	2.50	0.5	0.5	6.50
3.5	3.50	0.5	0.5	5.50
5	5.00	0.5	0.5	4.00

3.4.3 The stability of curcumin derivatives dissolved in HEPES buffer solution

The stability of curcumin derivatives including, **Cur**, **$CurBF_2$**, **$CurBF_2OTs$** and **$CurBF_2(OTs)_2$** was studied by using UV-Vis and fluorescence techniques. The stock solutions of the **Cur**, **$CurBF_2$**, **$CurBF_2OTs$** and **$CurBF_2(OTs)_2$** derivatives were dissolved in spectrophotometric grade DMSO at the concentration of 2.00×10^{-4} mol/L. Each substance solution was diluted by 0.1 M HEPES buffer solution pH 7.4, the final concentration substance was 1.00×10^{-5} mol/L in 10% DMSO/HEPES. Then the **Cur**, **$CurBF_2$** and **$CurBF_2(OTs)_2$** fluorescence spectra were recorded at room temperature as a function of time under the following condition. In addition, the **$CurBF_2OTs$** absorbance spectra were measured at room temperature.

	Cur	*$CurBF_2$*	*$CurBF_2(OTs)_2$*
Start (nm)	*465*	*537*	*460*
End (nm)	*800*	*800*	*800*
Excitation (nm)	*445*	*517*	*440*
Excitation Slit	*10.0*	*10.0*	*10.0*
Emission Slit	*10.0*	*10.0*	*10.0*
PMT	*600*	*700*	*550*
Scan rate	*600 nm/min*	*600 nm/min*	*600 nm/min*
Emission (nm)	*553*	*652*	*614*

3.4.4 The stability of curcumin derivatives encapsulated in GdSH CNPs

The **GdSH CNPs** prepared from gadolinium ion, SDS and HEPES buffer solution was selected to encapsulate the curcumin derivatives including, **Cur**, **$CurBF_2$**, **$CurBF_2OTs$** and **$CurBF_2(OTs)_2$**. Each curcumin derivative encapsulated in coordination nanoparticle was prepared as the previous experiment. After each sample was stirred for 3 h, the resulting aqueous suspensions were ultrasonicated for 1 minute before collected by high speed centrifugation (10000 rpm/10 min). Each filtrate was washed with 5 mL Milli-Q water and collected by high speed centrifugation at the same rpm, then repeated it twice. Then the coordination nanoparticles filtrates were

redispersed in HEPES buffers solution. The final concentration of the components in the preparation of **CNPs** was summarized in Table 3.7.

Table 3.7 The final concentration of the components to the prepared **CNPs**

GdSH-curcumin derivatives	Concentration of (M)			
	Gd^{3+}	SDS	HEPES	derivatives
GdSH-Cur	$1x10^{-3}$	$1x10^{-3}$	$8x10^{-2}$	$1x10^{-5}$
GdSH-CurBF	$1x10^{-3}$	$1x10^{-3}$	$8x10^{-2}$	$1x10^{-5}$
GdSH-CurBF(OTs)	$1x10^{-3}$	$1x10^{-3}$	$8x10^{-2}$	$1x10^{-5}$
GdSH-CurBF(OTS)$_2$	$1x10^{-3}$	$1x10^{-3}$	$8x10^{-2}$	$1x10^{-5}$

Then, fluorescence spectra of **GdSH-Cur**, **GdSH-CurBF$_2$** and **GdSH-CurBF$_2$(OTs)$_2$** were recorded at room temperature as a function of time under the following condition. In addition, the **GdSH-CurBF$_2$OTs** absorbance spectra were recorded at various times under the room temperature. The maximum intensity of each spectrum as a function of time was normalized by a value of 100 at zero time.

GdSH of	***Cur***	***CurBF$_2$***	***CurBF$_2$(OTs)$_2$***
Start (nm)	*465*	*537*	*460*
End (nm)	*800*	*800*	*800*
Excitation (nm)	*445*	*517*	*440*
Excitation Slit	*10.0*	*10.0*	*10.0*
Emission Slit	*10.0*	*10.0*	*10.0*
PMT	*600*	*700*	*550*
Scan rate (nm/min)	*600*	*600*	*600*
Emission (nm)	*532*	*653*	*605*

3.4.5 Entrapment efficiency and loading efficiency

All curcumin derivatives encapsulated in coordination nanoparticles were prepared as the previous experiment. After each sample was stirred for 3 h, free **curcumin** was separated from **GdSH CNPs** by centrifugation (at 10,000 rpm x 10 min) and quantified by using UV-visible technique at λ_{ab} of 429 nm. The calibration curves were performed using each curcumin derivative dissolved in 10% DMSO/HEPES buffer solutions. Entrapment efficiency and loading efficiency of curcumin derivatives incorporated in **CNPs** were calculated by equations (1) and (2), respectively [7]. The weight of **CNPs** was determined after centrifugation.

$$\text{Entrapment efficiency}(\%) = \frac{(\text{Total amount of curcumin} - \text{free curcumin})}{(\text{Total amount of curcumin})} \times 100 \ldots (1)$$

$$\text{Loading efficiency}(\%) = \frac{(\text{Total amount of curcumin} - \text{free curcumin})}{(\text{Total amount of nanopartides})} \times 100 \ldots\ldots\ldots (2)$$

3.4.6 Drug release studies

The **curcumin** release profiles from **CNPs** were observed at pH 7.4 and 6.5 of 0.1 M HEPES buffer solution. In a 10 mL volumetric flask, 1 mL of 1.00 x 10^{-2} mol/L SDS surfactant stock solution was added. Then, 0.5 mL **curcumin** at concentration of 2.00 x 10^{-4} mol/L and 0.5 mL gadolinium (Gd^{3+}) of 2.00 x 10^{-2} mol/L stock solution were added to the solution mixture. After that, a volume was adjusted to 10.00 mL with 0.1M HEPES buffer pH 7.4 to give a final concentration of SDS surfactant and gadolinium (Gd^{3+}) at 1.00 x 10^{-3} mol/L, and **curcumin** at 2.00 x 10^{-4} mol/L (20-folds of previous preparation for improving the UV-Vis absorption) in 10% DMSO aqueous solution. The mixtures were transferred to 15 mL vial and stirred at room temperature for 3 h. After the preparation and centrifugation of **GdSH-Cur CNPs** at 10,000 rpm for 10 minutes, the **CNPs** were redispersed in 10 mL of HEPES buffer solution pH 7.4 and 6.5. After that, the suspension was divided into eppendorf tubes with 0.5 mL per tube. The eppendorf tubes were kept at 37 °C under tender shaking upon period of 4 days. At timed interval, the **GdSH-Cur CNPs** were collected by centrifugation and extracted in methanol. Then the quantification of **curcumin** was determined by UV-

Visible spectrophotometry. **Curcumin** releases (%) were investigated by using the following equation [7].

$$\text{Release (\%)} = \frac{\text{Released curcumin}}{\text{Total curcumin}} \times 100 \qquad \text{..... (3)}$$

3.5 Cytotoxic assay

3.5.1 Cell line and media

SW620 cancer cell lines and L293T normal cell lines were purchased from American Type Culture Collection (ATCC). SW620 cancer cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, FBS (HyClone, Logan, UT). L293T normal cells were cultured in DMEM medium supplemented with 10% fetal bovine serum. Both of cell types were kept at 37 °C in a controlled humidified atmosphere of 5% CO_2 (v/v) incubator (Thermo Electron Corperation, USA).

3.5.2 Cell preservations

SW620 cancer cell lines and L293T normal cell lines were collected from cell culture flasks and centrifuged at 1000 rpm for 5 minutes (Hettich, Germany). Cell pellets were resuspended in 1 mL of freezing media (10% v/v of DMSO/ RPMI 1640 complete media for SW620 cancer cell lines and 10% v/v of DMSO/ DMEM complete media for L293T normal cell lines) and transfer to cryogenic vialx (Coming Incorperation, USA). Cells were instantly stored at -80 °C and transferred to store in Liquid Nitrogen Tank model 34 HC Taylor Wharton Cryogenic (Harsco Corporation, USA) at least 24 h at -80 °C for long term storage.

3.5.3 Cell preparation

After removing culture media, SW620 and L293T cells were washed by using PBS 3 mL and collected from cell culture flasks by using trypsin. After that, they were centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded and the SW620 and L293T cell pellets were resuspended in RPMI 1640 and DMEM complete media, respectively. Viable cells were diluted in trypan blue dye and counted by using

a hemacytometer. The cell numbers were calculated by using the following equation [65].

$$\text{Cell numbers(cells/mL)} = (\text{number of counted cells in 16 - large square}) \times (\text{dillution factor}) \times 10^4 \quad \text{.....(4)}$$

3.5.4 MTT assay

Before the MTT studies, **Cur** was studied in 2 separated conditions: (1) **Cur** dissolved in DMSO and (2) **Cur** dissolved in 10% DMSO/0.1 M HEPES buffer solution pH 7.4. The case of **$CurBF_2$, $CurBF_2(OTs)_2$, $CurBF_2(OTs)_2$** were dissolved in DMSO. Finally, **Cur** and **$CurBF_2$** in **CNPs** with equivalent dose concentration of drug was dissolved in 10% DMSO/0.1 M HEPES buffer solution pH 7.4.

Cytotoxicity study was determined using MTT assay in cell lines. Cells were seeded onto 96-well plates with a density of 10,000 cells per well and incubated at 37 °C under 5% CO_2 for 24 h. The medium in each well was replaced by 100 µL of culture medium containing a treatment group (curcumin derivatives with and without **CNPs**) and medium containing 1% HEPES to a negative control then cultured for 4 days. The medium in each well was added with 10 µL of 5 mg/mL of MTT reagent and incubated for 4 h. After that, the medium was removed. Each well was added and mixed by 200 µL of 0.04 N HCl in isopropanol. The optical density (OD.) of solution at 540 nm was measured on Antros 2010 microplate reader. The percentage of cell viability was calculated by the following formula [10,65].

$$\text{Cell viability}(\%) = \frac{(\text{OD.treatment} - \text{OD.blank})}{(\text{OD.control} - \text{OD.blank})} \times 100 \quad \text{..... (5)}$$

3.5.5 Statistical Analysis

An independent *t*-test of SPSS Statistics Base 17.0 software (IBM SPSS Software, USA) was used to analyze the comparison of anti-cancer effect between curcumin derivatives and curcumin derivatives incorporated in **CNPs**. The p-value < 0.05 was considered to be statistically significant [65,66].

3.6 Cellular uptake of GdSH-Cur CNPs

SW620 cells were seeded with 10,000 cells per cover slip in 24 well plates and incubated for 24 h at 37 °C under 5% CO_2. After 24 h, the cells were incubated in RPMI medium containing **GdSH-Cur CNPs** and **Cur** at the same equivalence dose, 25 μg/mL and **GdSH CNPs** without **curcumin** for 4 h under 5% CO_2. After the incubation, the medium was removed and the wells were washed with PBS buffer sufficiently to remove excess **CNPs**. Thereafter the cells were fixed in 4% paraformaldehyde and washed with PBS. After that, it was incubated in 0.2% triton x-100 in PBS for 2 minutes and washed with PBS. Then the nuclear dye DAPI was added to the wells and incubated for 3 minutes and washed with PBS again. The cover slips were mounted on to glass slide and subjected to a confocal laser scanning microscopy (CLSM). The **curcumin** in **CNPs** and the nuclear dye Dapi was examined by a green channel and a blue channel, respectively.