CHAPTER 2

Materials and Methods

2.1 Chemicals

2.1.1 Acetic acid (Merck, Germany)

2.1.2 Acetone (Merck, Germany)

2.1.3 Acetronitrile (ACN) (Biosolve, the Netherlands)

2.1.4 DL–1–amino–2–propanol (ACROS Organics, Belgium)

2.1.5 Ammonium acetate ($CH_3CO_2NH_4$) (Merck, Germany)

2.1.6 Ammonium chloride (NH_4Cl) (Merck, Germany)

2.1.7 Ammonia (NH_3) (Merck, Germany)

2.1.8 4,4´–azobis–(4–cyanopentanoic acid) (ABCPA) (Sigma–Aldrich, the Netherlands)

2.1.9 Benzoyl chloride (Sigma–Aldrich, the Netherlands)

2.1.10 Boric acid (Sigma–Aldrich, the Netherlands)

2.1.11 Bovine insulin (Sigma–Aldrich, the Netherlands)

2.1.12 Bovine serum albumin (Sigma–Aldrich, the Netherlands)

2.1.13 Chloroform, deuterated ($CDCl_3$), (Sigma–Aldrich, the Netherlands)

2.1.14 Curcumin (28260, mixture of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin, ≥95.0% (TLC), Fluka–Sigma–Aldrich, USA)

2.1.15 Dichloromethane (Biosolve, the Netherlands)

2.1.16 N,N´–dicyclohexylcarbodiimide (DCC) (Acros Organics, Belgium)

2.1.17 Diethyl ether (Biosolve, the Netherlands)

2.1.18 4–dimethylaminopyridine (DMAP) (Acros Organics, Belgium)

2.1.19 4–(dimethylamino) pyridinium–4–toluene sulfonate (DPTS) (synthesized from Utrecht University, the Netherlands)

2.1.20 N,N–dimethylformamide (DMF) (Biosolve, the Netherlands)

2.1.21 3–(4,5–dimethylthiazolyl–2)–2,5–diphenyl tetrazolium bromide (MTT) (Sigma–Aldrich, USA)

2.1.22 Dimethylsulfoxide (DMSO) (Merck, Germany)

2.1.23 Dimethylsulfoxide, deuterated (d^6–DMSO), (Sigma–Aldrich, the Netherlands)

2.1.24 2–diphenyl–1–picrylhydrazyl (DPPH) (Sigma–Aldrich, USA)

2.1.25 Disodium hydrogen phosphate (Na_2HPO_4) (Sigma–Aldrich, USA)

2.1.26 Dulbecco's modification of Eagle's medium (DMEM)

2.1.27 Ethanol, absolute (Merck, Germany)

2.1.28 Ethyl acetate (Biosolve, the Netherlands)

2.1.29 Ethylenediamine tetraacetic acid (EDTA) (Sigma–Aldrich, USA)

2.1.30 Fetal bovine serum (FBS) (Invitrogen™ life, USA)

2.1.31 Ficoll–Paque solution (Sigma–Aldrich, USA)

2.1.32 Goat anti–rabbit IgG conjugated with HRP (Promega, USA)

2.1.33 L–glutamine (Invitrogen™ life, USA)Hydrochloric acid (HCl) (Merck, Germany)

2.1.34 Hexane (Biosolve, the Netherlands)

2.1.35 Hydroquinone monomethyl ether (Sigma–Aldrich, the Netherlands)

2.1.36 Hyflo®Super cell (Sigma–Aldrich, the Netherlands)

2.1.37 L–lactide (Purac Biochem, the Netherlands)

2.1.38 Luminata™ Forte Western HRP Substrate (Millipore Corporation, USA)

2.1.39 Magnesium sulfate, desiccate (MgSO4) (Sigma–Aldrich, the Netherlands)

2.1.40 Methacryloyl chloride (Sigma–Aldrich, the Netherlands)

2.1.41 Methanol (Biosolve, the Netherlands)

2.1.42 Penicillin(Invitrogen™ life, USA)

2.1.43 Poly(ethylene glycol) monomethylether (mPEG) (Sigma–Aldrich, the Netherlands)

2.1.44 Potassium Bromide (KBr) (Sigma–Aldrich, USA)

2.1.45 Potassium chloride (KCl) (Sigma–Aldrich, the Netherlands)

2.1.46 Potassium dihydrogen phosphate (KH_2PO_4) (Sigma–Aldrich, the Netherlands)

2.1.47 Primary rabbit polyclonal anti–Wilms' tumor 1 (WT1) (C–19, Santa Cruz Biotechnology, USA)

2.1.48 *n*–propanol (RCI Labscan Limited, Thailand)

2.1.49 Propidium iodide (PI) (US Biological, USA).

2.1.50 Protease inhibitor cocktail (Sigma–Aldrich, USA)

2.1.51 Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen™ life, USA)

2.1.52 Rabbit polyclonal anti–GAPDH (Glyceraldehyde 3–phosphate dehydrogenase) (FL–335; Santa Cruz Biotechnology, USA)

2.1.53 Sodium Chloride (NaCl) (Sigma–Aldrich, USA)

2.1.54 Sodium Hydroxide (NaOH) (Merck, Germany)

2.1.55 Sodium dodecyl sulfate (SDS) (Sigma–Aldrich, USA)

2.1.56 Sodium pyruvate (Sigma–Aldrich, the Netherlands)

2.1.57 Streptomycin (Invitrogen™ life, USA)

2.1.58 Tetrahydrofuran (THF) (Biosolve, the Netherlands)

2.1.59 Triethylamine (TEA) (Sigma–Aldrich, the Netherlands)

2.1.60 Tris–HCl (Sigma–Aldrich, USA)

2.1.61 Triton X–100 (Merck, Germany).

2.1.62 Trypsin/EDTA (PAA Laboratories GmbH, Austria)

2.1.63 Tween 80 (Sigma–Aldrich, USA)

2.1.64 Uranyl acetate (Sigma–Aldrich, the Netherlands)

2.2 Cell lines

2.2.1 Human colorectal adenocarcinoma (Caco–2) (the American Type Culture Collection (ATCC), Maryland, USA)

2.2.2 Human breast adenocarcinoma (MCF–7) (the American Type Culture Collection (ATCC), Maryland, USA)

2.2.3 Human cervical carcinoma cells (HeLa) (the American Type Culture Collection (ATCC), Maryland, USA)

2.2.4 Human ovarian carcinoma cells (OVCAR–3) (the American Type Culture Collection (ATCC), Maryland, USA)

2.2.5 Human lymphoblastic leukemia (Molt–4) (the American Type Culture Collection (ATCC), Maryland, USA)

2.2.6 Human chronic myelogenous leukemia cell line (K562) (RIKEN BRC Cell Bank (Ibaraki, Japan).

2.3 Cell culture conditions

The HeLa, OVCAR–3, Molt–4, and K562 cell lines were cultured in RPMI 1640 supplemented with 10% FBS, 1 mM L–glutamine, 100 IU/mL of penicillin, and 0.1 mg/mL of streptomycin, 1% sodium pyruvate, and 0.01 mg/mL of bovine insulin were added in the medium of the OVCAR–3 cells. The Caco–2 and MCF–7 cells were cultured in DMEM supplemented with 10% FBS, 100 IU/mL of penicillin, and 0.1 mg/mL of streptomycin. All cell lines were incubated at 37°C, 5% CO_2, and 80% humidity.

2.4 Determination of antioxidant activity of curcumin

The DPPH free radical scavenging method modified from Okonogi *et al*. [111] was used to measure the free radical scavenging activity of curcumin and explore the comparative antioxidant power of curcumin in comparison with gallic acid, ascorbic acid, and xanthone individually. A stock solution of curcumin was prepared by dissolving in ethanol and diluted to get the concentrations of 1000, 500, 250,125, and 62.5 μg/mL. Trolox was used as positive control. Stock standard of Trolox solution was prepared to have the concentration series of 125, 100, 75, 50, 25, and 12.5μg/mL. Then, 20 μL of the ethanolic solution of test substance was added into a 96–well plate. Next, 180 μL of 1×10^{-4} M DPPH in ethanol or 180 μL of absolute ethanol as a negative control was added and mixed rapidly. The mixtures were kept for 30 min then the absorbance of each sample was measured at 540 nm by using microtiter plate reader. The percentage of inhibition of free radical was expressed as % antioxidant activity and was calculated by the equation as follows:

$$\% \text{ Antioxidant activity} = [(Abs_{control} - Abs_{sample}) / Abs_{control}] \times 100$$

Where $Abs_{control}$ is the absorbance value of DPPH solution containing all reagents except test samples and Abs_{sample} is the absorbance value of sample at each time.The antioxidant activity was expressed as the 50% effective concentration (EC_{50}) which 50% free radicals was scavenged by this concentration and as Trolox equivalent antioxidant capacity (TEAC) which obtained from 1 mg of the test sample. The lower the EC_{50} value or the higher the TEAC value, the higher the antioxidant capacity of the compound.

2.5 Solubility study of curcumin

2.5.1 Solubility study of curcumin in different solvents

Curcumin was added at the fixed amount of 1 mg. The exact volume of each solvent (hexane, THF, DMF, DMSO, DCM, ethyl acetate, acetone, *n*–propanol, ethanol, methanol, and water) was gradually added to a 15 mL or 100 mL test tube or and mixed by a vortex mixture until curcumin was completely soluble. The minimum of solvent that completely solubilized curcumin by naked eyes observation was recorded and compared to solubility parameter.

2.5.2 Solubility study of curcumin in water/methanol mixtures

In detail, 20 µL of curcumin in methanol stock solutions (1.25 and 2.50 mg/mL) was added to 980 µL of 100 mM ammonium acetate buffer, pH = 5.0 (final concentration of curcumin in the buffer solution was 25 and 50 µg/mL). The samples were separately kept at room temperature for 6 h, 3, and 7 days in tightly closed vials. At different time points, samples of 1 mL were taken and centrifuged at 5000 rpm for 20 min and 200 µL of the supernatant were mixed with 800 µL of methanol and stored at –20°C prior to the HPLC analysis as described in section 2.5.3. Calibration was done using curcumin stock solutions in methanol (0.0625–10 µg/mL) with an injection volume of 20 µL.

The solubility of curcumin in different volume fraction of water/methanol mixtures was studied to select the appropriate solvent for the stability study. Curcumin was dissolved in methanol at a concentration of 5 mg/mL and 5 µL of this stock

solution was added to 995 µL of 100 mM ammonium acetate buffer pH = 5.0 that contained 10–50% (*v/v*) of methanol (final concentration of curcumin in the buffer solution was 25 µg/mL). All samples were separately kept at room temperature for 6 h, 3, and 7 days in tightly closed vials. At different time points, samples of 1 mL were taken and centrifuged at 5000 rpm for 20 min and 200 µL of the supernatant were mixed with 800 µL of methanol and stored at –20°C prior to the RP–HPLC analysis.

Photon correlation spectroscopy (PCS) analysis was performed to investigate the possible formation of (nano) precipitates. In detail, 20 µL of stock solutions of curcumin in methanol (37µg/mL (100 µM) and 50 µg/mL (136 µM)) was added to 980 µL of 100 mM ammonium acetate buffer, pH = 5.0 or 100 mM phosphate buffer, pH = 8.0 or buffer (pH = 5.0 or 8.0) containing 50% (*v/v*) of methanol. These mixtures were incubated at 25°C and 37°C. The appearances of the mixtures were visually inspected. The scattering intensity of the solutions/dispersions as well as the size of the particles was measured using a particle size analyzer at 0 h and 24 h. The size measurements were taken at a fixed angle of 173°.

2.5.3 Reversed–phase high performance liquid chromatography (RP–HPLC)

HPLC analysis was carried out on a Waters system using an analytical C18 column, SunFire^{TM} (5 µm, 150 × 4.6 mm). A gradient system was run from 5:95 (*v/v*) acetonitrile/water as eluent A and acetonitrile as eluent B. Both eluents were adjusted by the addition of acetic acid to pH = 3.0. The gradient was run from 45% A to 60% B in 10 min as shown in Table 2.1. The flow rate was 1.2 mL/min, the injection volume was 20 µLand the detection wavelengths were 425 and 254 nm.

Table 2.1 The gradient condition of RP–HPLC analysis

Time (min)	Eluent A (%)	Eluent B (%)
0.00	45	55
7.00	40	60
8.00	45	55

2.6 Stability study of curcumin

2.6.1 Stability of curcumin in media of different pH

A stock solution of 5 mg/mL curcumin in methanol (50 µL) was added to 10 mL of buffer/methanol mixture (the final concentration of curcumin was 25 µg/mL). The buffers used were ammonium acetate (pH = 5.0), phosphate (pH = 7.0 and 8.0), borate (pH = 9.0 and 10.0), and ammonium buffers (pH = 11.0 and 12.0). The buffer concentrations were 100 mM and the methanol volume fraction was 50% (*v/v*). The samples were incubated at 37°C for at least 24 h or until no curcumin was detected. Samples of 200 µL were withdrawn at regular time intervals and added to 800 µL of methanol. The pH of the solution was checked regularly and adjusted if necessary. The withdrawn samples were stored at –20°C prior to the analysis using RP–HPLC. The reaction rate constant (k_{obs}) was calculated from the slope of the plot of the logarithm (log) of the curcumin concentration versus time.

2.6.2 Stabilty of curcumin as a function of temperature

The influence of temperature on the degradation of curcumin was studied using curcumin solution in a 50:50 (*v/v*) mixture of phosphate buffer/methanol pH = 8.0. The final concentration of curcumin was 25 µg/mL. The samples were incubated at 37, 50, and 60°C for at least 24 h or until no curcumin was detected. Samples of 200 µL were withdrawn at different time points, added to 800 µL of methanol and stored at –20°C prior to analysis using RP–HPLC.

2.6.3 Stability of curcumin in media of different dielectric constants

A stock solution of curcumin in methanol (5 mg/mL) was diluted to a final concentration of 25 µg/mL in solutions of 100 mM phosphate buffer pH = 8.0 with different volume fractions of methanol (25:75, 40:60, 50:50, 60:40, and 75:25 (*v/v*)). The pH of the buffer/methanol mixtures was adjusted prior to the degradation study. The samples were incubated at 37°C for at least 5 days or until no curcumin was detected. Samples of 200 µL were withdrawn at different time points, added to 800 µL of methanol, and stored at –20°C prior to analysis using RP–HPLC. The dielectric constants (∈) of the buffer/methanol mixture were calculated according to the formula:

$$\epsilon = [(\epsilon_{methanol} \times \text{methanol (\%)}) + (\epsilon_{water} \times \text{water (\%)})]/100$$

Where $\epsilon_{methanol}$ is 32.7 and ϵ_{water} is 78.5 [112,113].

2.6.4 Determination of degradation products by liquid chromatography electrospray ionization mass spectrometry (LC–ESI–MS) analysis

Samples of 250 µg/mL of curcumin in 100 mM ammonium buffer/methanol mixtures (50:50 (*v/v*)) pH = 9.0, were incubated at 37°C and at 0, 4, 24, and 168 h. The samples of 200µL were added with 800 µL of methanol. The initial concentration of curcumin was 50 µg/mL. The samples were analyzed using a liquid chromatography electrospray ionization mass spectrometer (LC–ESI–MS). An HPLC column (5 µm, 150 × 4.6 mm, SunFire™) was coupled to the mass spectrometer. A gradient system was run from 5:95 (*v/v*) acetonitrile/water as eluent A and acetonitrile as eluent B as shown in Table 2.2. Both eluents were adjusted by addition of acetic acid to pH = 3.0 (0.125% (*v/v*) of acetic acid). The gradient was run from 90% A to 70% B in 20 min with the flow rate of 1.2 mL/min, the injection volume of 100 µL, and the scan range of 140–415 m/z.

Table 2.2 The gradient condition of LC–ESI–MS analysis

Time (min)	Eluent A (%)	Eluent B (%)
0.00	90	10
15.00	30	70
16.00	90	10

2.7 Polymer synthesis

2.7.1 Synthesis and characterization of poly(ethylene glycol) monomethyl ether 4,4´–azobis–(4–cyanopentanoic acid) ($mPEG_2$–ABCPA) macroinitiator

Poly(ethylene glycol) monomethyl ether 4,4´–azobis–(4–cyanopentanoic acid) (ABCPA) was achieved by N,N´–dicyclohexylcarbodiimide (DCC)coupling [114]. Five g

of poly(ethylene glycol) monomethyl ether (mPEG), 0.14 mg (0.50 mmol) of ABCPA, and 0.78 mg (0.25 mmol) of 4–(dimethylamino) pyridinium–4–toluene sulfonate (DPTS) were dissolved in 27.64 mL of dried THF. The mixture was stirred at room temperature under nitrogen for 10 to 20 min. After that, 309 g (1.50 mmol) of DCC was dissolved in 10 mL of dried DCM and mixed together for 48 h in a nitrogen atmosphere. The mixture was filtered by a paper filter, and the solvent of mixture was removed in vacuum. The product was dialyzed in water and stirred for 2 h. Then, the product was filtered over Hyflo® and freeze–dried to obtain the powder. ^{1}H–NMR spectra was recorded using a nuclear magnetic resonance spectrometer (NMR). d^{6}–DMSO (1 mL) was used as a solvent to dissolve 20 mg of sample and the d^{6}–DMSO peak at 2.50 ppm was used as the reference line.

2.7.2 Synthesis and characterization of *N*–(2–hydroxylpropyl) methacrylamide (HPMA)

HPMA was synthesized as reported by Oupicky *et al.* [115]. Firstly, 26.25 g (0.35 mol) of DL–1–amino–2–propanol was mixed with 100 mL of deionized water, 5 mL of 1M of NaOH solution, 100 mL of DCM, and 0.087 g (0.70 mmol) of hydroquinone monomethyl ether. The mixture was cooled under the vigorous stirring by ice bath to 0°C. Next, 37 mL of methacryloyl chloride (0.38 mol) was loaded to a funnel and added drop wise to DL–1–amino–2–propanol mixture for 30 min with continue checking pH every 5 minutes. The pH was adjusted with 5M NaOH if it dropped below the pH range of 9–10 until it was constant. The reaction was continued under vigorous stirring for overnight. The mixture was extracted with 100 mL of DCM and water with 50 mg of sodium chloride for 3 times. Then, the DCM extract was dried over magnesium sulfate ($MgSO_4$), filtered off, and added with 0.087 g (0.70 mmol) of hydroquinone mono-methyl ether. After that, the DCM extract was evaporated with a rotary evaporator at 40°C. ^{1}H–NMR spectra was recorded using a nuclear magnetic resonance spectrometer (NMR). Chloroform (1 mL), deuterated ($CDCl_3$), was used as a solvent to dissolve 20 mg of sample, and the $CDCl_3$ peak at 7.25 ppm was used as the reference line.

2.7.3 Synthesis and characterization of *N*–(2–hydroxypropyl) methacrylamide monolactate and *N*–(2–hydroxypropyl) methacrylamide dilactate (HPMA–ML and HPMA–DL)

HPMA–ML and HPMA–DL were synthesized as described previously by Neradovic *et al.* [116]. In detail, 10 g (70 mmol) of HPMA, 4.97 g (35 mmol) of L–lactide, 8.5 g (70 mmol) of DMAP, and 0.087 g (0.70 mmol) of hydroquinone monomethyl ether were mixed together. The mixture was applied for 3 times alternating vacuum and nitrogen to remove water vapor and oxygen. Then, the mixture was put into the oil bath at 130°C with stirring until molten for 20 min and cooled down at room temperature overnight. The mixture was fractionated by a normal phase preparative column chromatography (silica) using an analytical column, VersaFlash system with a Supelco 80 × 300mm column, and 9:1 of ethyl acetate:hexane was used as the eluent with the flow rate of 40 mL/min. The fractions were collected by a volume size of 50 mL for 25 min and determined by UV detection. Then, the eluent was evaporated with rotary evaporator at 40°C. ^{1}H–NMR spectra was recorded using NMR. $CDCl_3$ (1 mL) was used as a solvent to dissolve 20 mg of sample, and the $CDCl_3$ peak at 7.25 ppm was used as the reference line.

2.7.4 Synthesis and characterization of *N*–(2–benzoyloxypropyl) (HPMA–Bz)

HPMA–Bz was synthesized as described previously by Shi *et al.*[104]. In detail, 10 g of HPMA (0.070 mol), 7.1 g (0.070 mol) of triethylamine (TEA)), and 0.087 g (0.70 mmol) of hydroquinone monomethyl ether were dissolved in 70 mL of dried DCM. This solution was added dropwise to a solution of 9.8 g (0.060 mol) of benzoyl chloride in 70 mL in dried DCM. The resulting solution was stirred for 24 h under a nitrogen atmosphere at room temperature. Then, the remaining solution was extracted for 3 times with the same volume of water. The DCM extract was collected and dried over $MgSO_4$, filtered off, and evaporated DCM with a rotary evaporator at 40°C. ^{1}H–NMR spectra was recorded using NMR. d^6–DMSO (1 mL) was used as a solvent to dissolve 20 mg of sample, and the d^6–DMSO peak at 2.50 ppm was used as the reference line.

2.7.5 Synthesis and characterization of *ω*–methoxy poly(ethylene glycol)–b–(*N*–(2–hydroxypropyl) methacrylamide dilactate) (PEG–HPMA–DL)

The block copolymers were synthesized by free radical polymerization according to Soga *et al.*[100]. Firstly, 2.0 g (7.66 mmol) of HPMAm–DL and 525.2 mg (0.05 mmol) of $mPEG_2$–ABCPA with a molar ratio of 150:1 (monomer:initiator) were added to 8 mL of dried ACN in a polymerization vial with a septum. The monomer/initiator solution was degassed by flushing with N_2 for at 15 min *via* 2 needles (one needle to put N_2 in and one needle to let the air out). Next, the monomer/initiator solution was placed in a stirring heated oil bath of 70°C for 24h. Then, synthesized polymers were precipitated by dropping the polymer mixture solution to 45 mL of dried diethylether with cyclic moves and vigorous shaking. The mixture was centrifuged at 3000 rpm for 20 min. The supernatant was removed, and the precipitated polymer was dried by a vacuum oven. ^{1}H–NMR spectra was recorded using NMR. d^6–DMSO (1 mL) was used as a solvent to dissolve 20 mg of sample. The d^6–DMSO peak at 2.50 ppm was used as the reference line. Gel permeation chromatography (GPC) was performed to determine the weight average molecular weight (M_w) using two serial Plgel 5 μm MIXED–D columns (Polymer Laboratories). Calibration was done by PEGs of different molecular weights and with narrow molecular weight distribution. The eluent was DMF containing 10 mM LiCl; the flow rate was 0.7 mL/min at 40°C.

2.7.6 Synthesis and characterization of *ω*–methoxy poly(ethylene glycol)–b–(*N*–(2–benzoyloxypropyl) methacrylamide)–co–(*N*–(2–lactoyloxypropyl) methacrylamide) (PEG–HPMA–Bz–L)

The block copolymers were synthesized by free radical polymerization according to Shi *et al.* [104]. In detail, 1.44 g (6.75 mmol) of HPMA–ML, 0.56 g (2.25 mmol) of HPMA–BZ, and 0.62 g (0.06 mmol) of $mPEG_2$–ABCPA with a molar ratio of 150:1 (monomer:initiator) were added to 8 mL of dried ACN in a polymerization vial with a septum. The synthesis and characteristic procedure were carried out as described in 2.7.5.

2.7.7 Synthesis and characterization of *ω*–methoxy poly(ethylene glycol)–b–(*N*–(2–benzoyloxypropyl) methacrylamide) (PEG–HPMA–Bz)

The block copolymers were synthesized by free radical polymerization according to Shi *et al.* [104]. In detail, 200 mg (0.81 mmol) of HPMA–Bz was dissolved in dried ACN containing different concentrations of $mPEG_2$–ABCPA. Polymers were synthesized at different feed ratios of HPMAm–Bz:$mPEG_2$–ABCPA (50:1, 150:1, 250:1, 300:1, and 500:1). The synthesis and characteristic procedure were carried out as described in 2.7.5. The reaction scheme is shown in Figure 2.1.

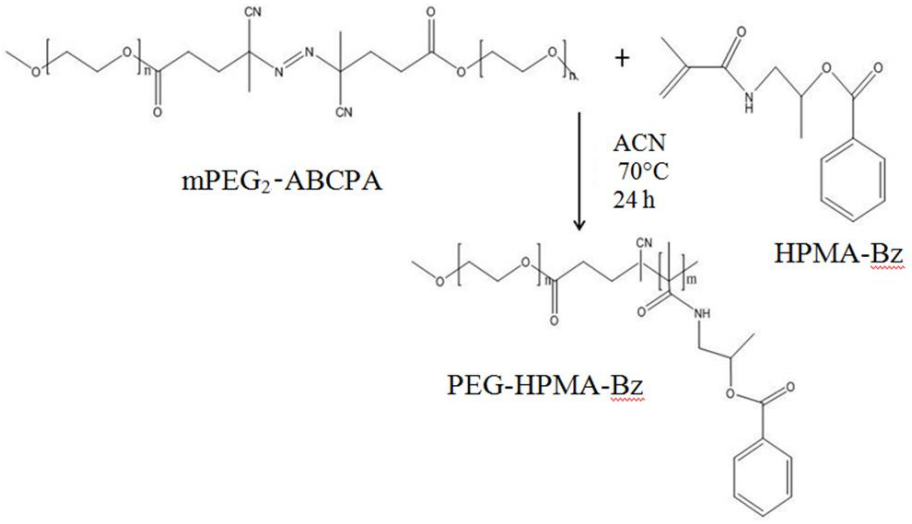

Figure 2.1 Synthesis of PEG–HPMA–Bz

The different PEG–HPMA–Bz polymers were characterized by ^{1}H–NMR spectroscopy and gel permeation chromatography (GPC). ^{1}H–NMR spectra were recorded using d^6–DMSO (1 mL) as a solvent to dissolve 20 mg of sample and the DMSO peak at 2.50 ppm was used as the reference line. The ratio HPMA–Bz : mPEG was determined by the integral value of aromatic protons of HPMA–Bz (8.0 ppm, 2H, aromatic CH) divided by two, and the integral value of mPEG protons divided by 448 (the average number of protons per one mPEG chain, M_n = 5000 g/mol) gives the integral value for one mPEG chain. The number average molecular weight (M_n) of the

block copolymers was determined by integral value of protons using the following equation:

$$M_n = \frac{(\text{integral (H at 8.0 ppm)}/2) \times \text{molar mass of HPMAm–Bz}}{\text{integral (methylene protons at 3.40–3.60 ppm)}/\ 448} + 5000 \text{ g/mol}$$

GPC was performed to determine the number average molecular weight (M_n), weight average molecular weight (M_w), and polydispersity (PDI, equal to M_w/M_n) as described in 2.7.5.

2.7.8 Critical micelles concentration (CMC) determination of PEG–HPMA–Bz

The CMC of PEG–HPMA–Bz was determined using pyrene as a fluorescence probe essentially as described by Soga *et al.* [117]. The PEG–HPMA–Bz block copolymer was dissolved in 500 μL THF and added slowly to 4.5 mL of 120 mM ammonium acetate buffer (final polymer concentration ranging from 1 to 2.5×10^{-6} mg/mL). The dispersions were stirred for 2 h at room temperature to evaporate THF. Next, 15 μL of pyrene dissolved in acetone (concentration 1.8×10^{-4} M) was added and the mixtures were incubated at room temperature for 20 h to allow evaporation of acetone. Fluorescence excitation spectra of pyrene were obtained by a spectrofluorometer at an angle of 90°. The excitation spectra were recorded at 37°C (from 300 to 360 nm with an emission wavelength at 390 nm). The excitation and emission band slits were 4 and 2 nm, respectively. The intensity ratio of I_{338}/I_{333} was plotted against the polymer concentration to determine the CMC.

2.8 Preparation of polymeric micelles

Curcumin–loaded PEG–HPMA–Bz micelles (CN) were prepared by a nanoprecipitation method [118]. In detail, the PEG–HPMA–Bz polymer (10 mg/mL) and curcumin with different concentrations ranging from 0.5, 1, 2, and 4 mg/mL were dissolved in acetone, and these solutions (500 μL) or PEG–HPMA–Bz polymer dissolved in 500 μL of acetone for curcumin–unloaded PEG–HPMA–Bz micelles (BN) were slowly dropped into 2 mL of 120 mM ammonium acetate buffer (pH = 5.0) under stirring and subsequently stirred for 2 h. Next, the non–entrapped, precipitated curcumin

was removed by centrifugation (5000 rpm, 20 min), and the supernatant was filtered through a 0.45 μm membrane.

Curcumin–loadedPEG–HPMA–DL (CD) and PEG–HPMA–Bz–L (CZ) micelles were prepared using the fast heating method [119]. The polymers were dissolved in 120mM ammonium acetate buffer (pH= 5.0) at a concentration of 10 mg/mL at 0 to 4°C. Next, 100 μL of acetone for curcumin–unloaded PEG–HPMA–DL micelles (BD) and curcumin–unloaded PEG–HPMA–Bz–L micelles (BZ) or 100 μL of 5, 10, 20, or 40 mg curcumin dissolved in acetone was added to 900 μL of the polymer solutions. Then, micelles were formed by rapidly heating the mixtures from 4 to 50°C in a water bath for 1 min. Next, the mixtures were slowly cooled to room temperature, and the non–entrapped precipitated curcumin was removed by centrifugation (5000 rpm, 20 min). Finally, the micellar dispersion was filtered through a 0.45 μm membrane.

2.9 Characterization of polymeric micelles

2.9.1 Determination of entrapment efficiency and loading capacity

The amount of loaded curcumin of the polymeric micelles was determined by diluting the different micellar dispersions (2.5–20 μL) in DMF (997.5–980 μL). Subsequently vortexing was done to destabilize the micelles and dissolve curcumin. The absorbance of the obtained solutions was measured at 425 nm using the UV–visible spectrophotometer. Curcumin dissolved in DMF was used for calibration (concentration from 0.3 – 5.0 μg/mL). The encapsulation efficiency (EE) and loading capacity (LC) were calculated as follows:

$$EE = \frac{\text{amount of loaded curcumin}}{\text{amount of curcumin used for loading}} \times 100\%$$

$$LC = \frac{\text{amount of loaded curcumin}}{\text{amount of copolymer used for loading}} \times 100\%$$

2.9.2 Size and size distribution determination

The curcumin–loaded and curcumin–unloaded polymeric micelles (20 μL) were diluted in deionized water (980 μL). The Z_{ave} particle size and size distribution of polymeric micelles were measured by PCS using a Zetasizer ZS. The size measurements were taken at a fixed angle of 173°C.

2.9.3 Morphological investigation

Transmission electron microscopy (TEM) using Tecnai12 equipped with a Biotwin lens and a LaB6 filament was performed in order to investigate the morphology of curcumin–unloaded and curcumin–loaded polymeric micelles. A copper 200 mesh grid with a carbon coated thin polymer film was placed on the 10 μL of micellar dispersions for 3 min. Then, the grid with the film was put onto a 5 μL of uranyl acetate 2% for 1 min and left for 5 min. Next, the grid was loaded into a TEM sample holder and analyzed by AnalySIS software.

2.9.4 Fourier transform infrared spectroscopy (FT–IR) analysis

Freeze dried samples of 10% feeding curcumin–loaded polymeric micelles were investigated for molecular interactions between polymers and curcumin by means of Fourier transform infrared spectrometry (FT–IR). Control samples were curcumin powder and freeze dried samples of curcumin–unloaded polymeric micelles. The samples were crushed with KBr and pellets were obtained by applying a pressure 500 kg/cm^2. The FT–IR spectra were obtained by scanning between 4000 and 400 cm^{-1}.

2.9.5 X–ray diffractometry (XRD) analysis

Possible crystallinity of curcumin powder, freeze dried samples of 10% feeding of curcumin–loaded polymeric micelles, and curcumin–unloaded polymeric micelles were investigated by means of XRD. The XRD diffractograms were registered at Bragg angle (2θ) of 5° to 60° at a scanning rate of 12°/min.

2.9.6 Differential scanning calorimetric (DSC) analysis

Curcumin powder, freeze dried samples of 10% feeding of curcumin–loaded polymeric micelles and curcumin–unloaded polymeric micelles were sealed in an aluminum pan. The samples were heated from 50°C to 200°C under nitrogen gas flow of 20 mL/min at a heating rate of 10°C/min. The temperature was calibrated with pure indium, with a melting point of 156.6°C. An empty pan was used as a reference.

2.10 Release of curcumin from polymeric micelles

The release of curcumin from the polymeric micelles was examined by a dialysis method. In detail, 3 mL of curcumin–loaded polymeric micelles in phosphate buffer saline pH = 7.4 (PBS) containing 137 mM NaCl, 10 mM Na_2HPO_4, 2.68 mM KCl, and 1.84 mM KH_2PO_4 was pipetted into a pre–swollen dialysis bag (MWCO = 15000). A solution of 2% Triton X–100 in the same PBS buffer was used as releasing medium. The dialysis bag was immersed into 20 mL of the releasing medium with stirring at 500 rpm at 37°C. Samples (5 mL) of the receiving medium were drawn periodically, and the volume was adjusted with fresh release medium. The concentration of curcumin in the different samples was measured at 425 nm using the UV–visible spectrophotometer. Calibration was done using curcumin (concentration from 0.3–5.0 µg/mL) in 2% Triton X–100. At the end of the experiment, the amount of the remaining curcumin in polymeric micelles in dialysis bags was measured using the UV–visible spectrophotometer at 425 nm, and the size of the micelles was determined by PCS.

2.11 Stability of curcumin–loaded polymeric micelles

2.11.1 Stability study under basic condition

The stability of curcumin–loaded polymeric micelles was monitored under basic condition in 100 mM phosphate buffer pH = 8.0. The curcumin–loaded polymeric micelles (1 mL) was added to 3 mL of 100 mM of phosphate buffer pH = 8.0 with a final concentration of curcumin of 25 µg/mL and incubated at 37°C for at least 7 days or until no curcumin was detected. At regular time points samples of 200 µL were

withdrawn, added with 800 μL of methanol and stored at –20°C prior to analysis using RP–HPLC analysis.

2.11.2 Stability study under storage condition

Curcumin–loaded polymeric micelles (2 mL)were prepared in ammonium acetate buffer pH = 5.0 and stored in closed containers at various temperatures of 4, 30, and 40°C for 90 days. Changes in physical appearances were investigated. The size of micelles was measured by dynamic light scattering,and the curcumin content was measured at various time intervals using RP–HPLC analysis.

2.12 Biological activity of polymeric micelles

2.12.1 Protein adsorption of polymeric micelles

Curcumin–loaded polymeric micelles (1 mg/mL) were dispersed in bovine serum albumin (BSA) (100 μg/mL in PBS pH = 7.4) for 2 h at 25°C, and the samples were centrifuged at 12,000 rpm for 15 min to obtain albumin–bound polymeric micelles. The particle size and zeta potential of albumin–bound curcumin polymeric micelles were measured by PCS.

2.12.2 Hemocompatibility study

Whole blood was obtained from healthy donor volunteers. Red blood cells (RBCs) from the whole blood (8 mL) were collected by centrifugation at 2,000 rpm for 10 min. Then, RBCs were resuspended in 8 mL of PBS. Different concentrations of curcumin–loaded polymeric micelles (0–100 μM, 100 μL) as well as the same dilution of 1 mg/mL of curcumin in DMSO (CM) were incubated at 37°C with 400 μL of RBCs. After 2 h of incubation at 37°C, the extent of hemolysis caused by all treatments was observed. The samples were centrifuged 2,000 rpm for 10 min, and the supernatant fluid was measured for optical density at λ_{max} of 570 nm and 630 nm as a reference wavelength using a microplate reader. 100 mM PBS and hypotonic solutions (75 mM KCl solution) were used as the negative and positive controls, respectively. The percent of hemolysis was calculated as follows:

$$\% \text{ Hemolysis} = (\text{Abs}_{sample}/\text{Abs}_{control}) \times 100$$

Where $\text{Abs}_{control}$ is the absorbance value of RBCs and hypotonic solution and Abs_{sample} is the absorbance value of RBCs and sample.

2.12.3 Cytotoxicity towards peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from 20 mL of whole blood and obtained from healthy human volunteers. The blood sample was anticoagulated with heparin and diluted with the same volume of PBS. The PBMCs were separated by Ficoll–Paque solution and centrifuged at 5,000 rpm for 30 min at 20°C. The PBMCs were collected, pelleted down, and resuspended in complete RPMI 1640 medium with 100 units/mL penicillin, 100 µg/mL streptomycin, and 10%(v/v) FBS. The cell viability of PBMCs was examined using a MTT assay [120]. In detail, 100 µL of PBMCs (1.0×10^5 cells) were transferred into a 96–well plate and incubated at 37°C, 5% CO_2, and 80% humidity for 24 h. Next, 100 µL of CM, CD, CZ, and CN (curcumin concentration ranged from 3 – 100 µM) as well as BD, BZ, and BN with the polymer concentrations ranged from 20 – 600 µg/mL were added to the cells and incubated under the same condition for 72 h. Then, 100 µL of medium was removed from each well, and 15 µL of MTT(5 mg/mL) in PBS solution was added and incubated for 4 h. After that, the media were removed and the formed formazan crystals were dissolved by 200 µL of DMSO. The absorbance was measured at the wavelength of 570 nm and 630 nm as a reference wavelength using a microplate reader. The percentage of cell viability was calculated as follows:

$$\% \text{ Cell viability} = (\text{Abs}_{sample}/\text{Abs}_{control}) \times 100$$

Where $\text{Abs}_{control}$ is the absorbance value for the control cells which contain all reagents except the test samples, and Abs_{sample} is the absorbance value of samples.

2.12.4 Cytotoxicity against cancer cells

Five different cancer cell lines, OVCAR–3, Caco–2, MCF–7, HeLa, and Molt–4 were used to investigate the cytotoxicity of the different formulations. The cell culture

conditions were described in section 2.3. The cytotoxicity of curcumin–loadedpolymeric micelles was determined using a MTT assay and compared to CM. In detail, the cells at a density of 1.0×10^4 cells/well were cultured in 96–well plates at 37°C, 5% CO_2, and 80% humidified atmosphere for 24 h. The cell viability was examined by MTT test as described in section 2.12.3. Next, 100 µL of CM or curcumin–loaded polymeric micelles with concentrations of curcumin (either in its free form or micellar formulations) that ranged from 3 – 100 µM as well as curcumin–unloaded polymeric micelles (concentration ranging from 20 – 600 µg/mL) were added the medium, and plates were incubated for 72 h. The final concentration of DMSO that dissolved curcumin in the cell medium was 0.4% (*v/v*), and the cell medium containing 0.4% (*v/v*) of DMSO was used as the vehicle control (VC). After that, 100 µL of media was removed from each well, and 15 µL of MTT (5 mg/mL) in PBS was added to cell media and incubated for 4 h. Then, the media were removed, and the formed formazan crystals were dissolved by addition of 200 µL of DMSO. The absorbance was measured at 570 nm and 630 nm as a reference wavelength using a microplate reader. The cell viability was calculated using the equation as described in section 2.12.3.

2.12.5 Inhibitory effect on K562 leukemic cell proliferation

1) Cytotoxicity against K562 cells

The cytotoxicity study of K562 cells was performed as described in section 2.12.4

2) Cellular uptake

K562 cells at 2.5×10^5cells/mL were added into a cell culture plate and incubated for overnight. Then, cells were incubated with various formulations (BD, BZ, BN, CM, CD, CZ, and CN) at a final concentration of 20 µM curcumin in each formulation. After 4 h of incubation, the culture medium was removed and carefully washed twice with PBS. For fluorescence microscope, the cells were fixed with 500 µL of cold 2% paraformaldehyde fixative solution for 20 min at room temperature. After the fixative solution was removed, cells were washed twice with PBS. Then, the cells were added into a 96–well glass bottom plate (In Vitro Scientific, USA) and observed under a fluorescence microscope (Inverted phase contrast microscope).

3) Cell cycle analysis

Cell cycle analysis was evaluated by flow cytometry using PI that implies the content of nuclear DNA. K562 cells were cultured in culture medium with non–cytotoxic concentration (the inhibition concentration at 20% growth value, IC_{20}) of curcumin–loaded polymeric micelles (CD, CZ, CN, and CM) for 72 h. BD, BZ, and BN treated cells and untreated cells were used as control groups. After treatment, cells were washed twice with 5 mL of PBS and prepared as a single cell suspension. After centrifugation at 1500 pm for 5 min, the supernatant was removed, and cells were fixed with 1 mL of ice cold 70% ethanol for 30 min. Cell pellets were washed with 1 mL of ice cold PBS and stained with 500 µL PI solution in the dark at 4°C. The red fluorescence was measured on a flow cytometer, and the data were analyzed by using FlowJo 7.6™ software.

4) Investigation on Wilms' tumor 1 (WT1) protein expression by Western blot analysis

K562 cells were treated with CM and curcumin–loaded polymeric micelles using the concentration at IC_{20} values. Total protein extracts after formulation treatments were prepared using RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 1% Triton X–100, 0.5 mM EDTA, 0.1% SDS, and protease inhibitor cocktail). The protein concentration was measured by Folin–Lowry method [121]. The protein was separated by 12% SDS–polyacrylamide gel electrophoresis (SDS–PAGE). WT1 protein level was performed by using primary rabbit polyclonal anti–WT1 at 1:100 dilution, and rabbit polyclonal anti–GAPDH at 1: 1,000 dilution was used as a loading control. The secondary antibody was goat anti–rabbit IgG conjugated with HRP at 1:10,000 dilution for WT1 and GAPDH protein detection. Proteins were visualized by Luminata™ Forte Western HRP Substrate and exposed to X–ray film. The protein bands were analyzed and quantified by program Quality One Basic 4.6.6, scan densitometer. Additionally, the total cell number of K562 cells after treatments for 72 h was measured by trypan blue exclusion method.

2.13 Statistical analysis

All experiments except FT–IR, XRD, and DSC were done at least in the triplicate. The results were expressed as mean ± SD, and the results of biological activity were expressed as mean ± SEM. Statistical analysis was done by using one–way ANOVA and p–value at a level of 95% confidence limit.