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Research Paper

HPMA-based polymeric micelles for curcumin solubilization and inhibition of cancer cell growth

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ABSTRACT

Curcumin (CM) has been reported as a potential anticancer agent. However, its pharmaceutical applications as therapeutic agent are hampered because of its poor aqueous solubility. The present study explores the advantages of polymeric micelles composed of block copolymers of methoxypoly(ethylene glycol) (mPEG) and *N*-(2-hydroxypropyl) methacrylamide (HPMA) modified with monolactate, dilactate and benzoyl side groups to enhance CM solubility and inhibitory activity against cancer cells. Amphiphilic block copolymers, ω-methoxypoly(ethylene glycol)-*b*-(*N*-(2-benzoyloxypropyl) methacrylamide) (PEG-HPMA-Bz) were synthesized and characterized by ^{1}H NMR and GPC. One polymer with a molecular weight of 28,000 Da was used to formulate CM and compared with other aromatic substituted polymers. CM was loaded by a fast heating method (PEG-HPMA-DL and PEG-HPMA-Bz-L) and a nanoprecipitation method (PEG-HPMA-Bz). Physicochemical characteristics and cytotoxicity/cytocompatibility of the CM loaded polymeric micelles were evaluated. It was found that HPMA-based polymeric micelles significantly enhanced the solubility of CM. The PEG-HPMA-Bz micelles showed the best solubilization properties. CM loaded polymeric micelles showed sustained release of the loading CM for more than 20 days. All of CM loaded polymeric micelles formulations showed a significantly potent cytotoxic effect against three cancer cell lines. HPMA-based polymeric micelles are therefore promising nanodelivery systems of CM for cancer therapy.

1. Introduction

Curcumin (CM), a natural major compound, has been reported to have many pharmacological activities such as anti-inflammatory, antioxidant, anti-arthritic, and anti-amyloid [1,2]. The *in vitro* studies on cancer cells reported that CM possesses cytotoxicity against many cell lines [3]. In spite of such high activities, previous studies on CM demonstrated poor systemic bioavailability after oral dosing which was related to its inadequate absorption [4]. A limiting factor that hampers its pharmaceutical and medicinal applications is its extremely low aqueous solubility [5]. It was reported that CM solubility in aqueous buffer (pH 5.0) was only 11 ng/mL, whereas its solubility at neutral pH was undetectable [6]. It is therefore essential to enhance the aqueous solubility of this compound.

Polymeric micelles have been recently recognized as an important and attractive class of drug carriers. They have shown great promises in solubilization and (targeted) delivery of hydrophobic drugs for chemotherapy [7–9]. Polymeric micelles are composed of amphiphilic block copolymers which spontaneously assemble into nano-sized structures in aqueous solution. Hydrophobic drugs can be accommodated into their hydrophobic core whereas their hydrophilic shell, most commonly polyethylene glycol (PEG), can provide stealth properties ensuring long circulation behavior required for tumor targeting exploiting the EPR effect [10,11]. Importantly, pharmaceutical production of polymeric micelles has been proven and some formulations have entered clinical evaluations [12–14]. The incorporation of hydrophobic drugs into the micellar core can be done by chemical conjugation or the physical entrapment. Chemical conjugation provides a high drug loading and stability [15,16] but the chemical conjugation is not always practicable or sometimes impossible when no functional group

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on the target molecule for conjugation is available. Therefore, physical entrapment of drugs is preferred to prepare drug-loaded polymeric micelles but in many case the drug is insufficiently retained in the micellar formulation [17,18].

Poly[*N*-(2-hydroxypropyl) methacrylamide] (pHPMA) is a hydrophilic polymer currently under investigation for pharmaceutical and biomedical applications because of its good biocompatibility, non-immunogenicity and possibilities for chemical functionalization [19–21]. HPMA has also been investigated as a building block of polymeric micelles. It can serve as hydrophilic part of a micellar stealth corona, while it can also be chemically modified with hydrophobic moieties to serve as a micellar core in which hydrophobic drugs can be solubilized [22–24]. In this study three derivatives of methoxypoly(ethylene glycol)-*N*-(2-hydroxypropyl) methacrylamide (PEG-HPMA) based block copolymers were synthesized and investigated for solubility enhancing and carrier systems of CM. The physicochemical properties and releasing behavior of CM in each carrier system were evaluated. Moreover, the cytotoxicity of these CM loaded micelles against various cancer cell lines was investigated in order to get insight into the use of these formulations for cancer therapy.

2. Materials and methods

2.1. Materials

N-(2-benzoyloxypropyl) methacrylamide (HPMAm-Bz), methoxy poly(ethylene glycol)$_2$-4,4-azobis(4-cyanopentanoic acid) (mPEG$_2$-ABCPA) macroinitiator (M_n of mPEG = 5000 g/mol), ω-methoxy poly(ethylene glycol)-b-(*N*-(2-hydroxypropyl) methacrylamidedilactate) (PEG-HPMA-DL) and ω-methoxy poly (ethylene glycol)-b-(*N*-(2-benzoyloxypropyl) methacrylamide)-co-(*N*-(2-lactoyloxypropyl) methacrylamide) (PEG-HPMA-Bz-L) were synthesized and characterized as described previously [23,25]. CM, bovine insulin and 3-(4,5-dimethylthiazolyl-2)-2,5-di phenyl tetrazolium bromide (MTT) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Human ovarian carcinoma cells (OVCAR-3), human colorectal adenocarcinoma (Caco-2) and human lymphoblastic leukemia (Molt-4) were originally obtained from the American Type Culture Collection (ATCC) (Maryland, USA). Dulbecco's modification of Eagle's medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640, fetal bovine serum (FBS), penicillin, streptomycin and trypsin/EDTA were purchased from PAA Laboratories GmbH (Pasching, Austria). Acetonitrile (ACN), diethyl ether, N,N-dimethylformamide (DMF), tetrahydrofuran (THF), dimethylsulfoxide (DMSO) and Triton X-100 were purchased from Merck (Darmstadt, Germany). All other chemicals were of the highest grade available.

2.2. Synthesis of PEG-HPMA-Bz

Block copolymers of PEG-HPMA-Bz were synthesized by free radical polymerization using HPMAm-Bz as the monomer and mPEG$_2$-ABCPA as the macroinitiator essentially as described previously [26]. In short, HPMAm-Bz was dissolved at a concentration of 0.3 g/mL in dried ACN also 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, 500:1). The solutions were degassed by flushing with nitrogen for 15 min. Polymerizations were conducted at 70 °C for 24 h under a nitrogen atmosphere. Next, the synthesized polymers were precipitated by dropping the ACN solution in an excess diethyl ether. The precipitate was collected by filtration and subsequently dried under vacuum.

2.3. Characterization 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 a Gemini 300 MHz spectrometer (Varian Associates Inc. NMR Instruments, Palo Alto, CA), using d^6-DMSO as solvent and the DMSO peak at 2.50 ppm was used as the reference line. ^{1}H NMR of PEG-HPMA-Bz: 8.0 (b, 2H, aromatic CH), 7.55 (b, 1H, aromatic CH), 7.65 (b, 2H, aromatic CH), 7.35 (b, CO—NH—CH$_2$), 5.1 (b, NH—CH$_2$—CH(CH$_3$)—O—(Bz)), 3.40–3.60 (b, mPEG5000 methylene protons, O—CH$_2$—CH$_2$), 3.2 (b, NH—CH$_2$—CH), 0.6–2.2 (b, the rest of the protons are from the methyl and backbone CH$_2$ protons). The ratio HPMAm-Bz:mPEG was determined by the integral value of aromatic protons of HPMAm-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:

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

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) 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.

2.4. Critical micellar concentration (CMC) determination of PEG-HPMA-Bz

The CMC of PEG-HPMA-Bz was determined using pyrene as a fluorescent probe essentially as described by Soga et al. [27]. In short, the 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 the evaporation of acetone. Fluorescence excitation spectra of pyrene were obtained by a Fluorolog fluorometer (Horiba Jobin Yvon, Japan) 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.

2.5. Preparation of CM loaded polymeric micelles

CM loaded PEG-HPMA-Bz micelles were prepared by a nanoprecipitation method [18]. Briefly, the polymer (10 mg/mL) and CM with different concentrations ranging from 0.5, 1, 2 and 4 mg/mL were dissolved in THF and these solutions (500 μL) 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 CM was removed by centrifugation (5000 rpm, 20 min) and the supernatant was filtered through a 0.45 μm membrane. CM loaded PEG-HPMA-DL and PEG-HPMA-Bz-L micelles were prepared using the fast heating method [28]. Briefly, the polymers were dissolved in 120 mM

ammonium acetate buffer (pH 5.0) at a concentration of 10 mg/mL at 0 to 4 °C. Next, 100 μL of THF for unloaded polymeric micelles or 100 μL of 5, 10, 20 or 40 mg CM dissolved in THF was added to 900 μL of the polymer solutions. Then, micelles were formed by rapidly heating (in about 1 min) the mixtures from 4 to 50 °C in a water bath. Next, the mixtures were slowly cooled to room temperature and the non-entrapped precipitated CM was removed by centrifugation (5000 rpm, 20 min). Finally, the micellar dispersion was filtered through a 0.45 μm membrane.

2.6. Determination of loading efficiency and loading capacity

The amount of loaded CM 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 CM. The absorbance of the obtained solutions was measured at 428 nm using a UV–Vis spectrophotometer. CM dissolved in DMF was used for calibration (concentration from 0.3 μg/mL to 5.0 μg/mL). The encapsulation efficiency (EE) and loading capacity (LC) were calculated as follows:

$$\text{EE} = (\text{amount of loaded CM}/\text{amount of CM used for loading}) \times 100\%$$

$$\text{LC} = (\text{amount of loaded CM}/\text{amount of copolymer used for loading}) \times 100\%$$

2.7. Size and size distribution determination

The CM loaded and 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 dynamic light scattering (DLS) using a Malvern system (Malvern Instruments Ltd., Malvern, UK). The size measurements were taken at a fixed angle of 173 °C.

2.8. Morphological investigation

Transmission electron microscopy (TEM) using Tecnai12 equipped with a Biotwin lens and a LaB6 filament (Philips, The Netherlands) was performed in order to investigate the morphology of CM loaded 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. FT-IR and X-ray diffractometry analysis of polymeric micelles

Freeze dried samples of 10% feeding CM loaded micelles were investigated for molecular interactions between polymers and CM by means of Fourier transform infrared spectrometry (FT-IR) using Nicolet/470FT-IR (International Equipment Trading Ltd, USA). Control samples were CM powder and freeze dried samples of unloaded 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}.

Possible crystallinity of CM powder, freeze dried samples of 10% feeding of CM loaded micelles and unloaded micelles was investigated by means of X-ray diffractometry (XRD) using a Miniflex II desktop X-ray diffractometer (Rigaku, Japan). The XRD diffractograms were registered at Bragg angle (2θ) of 5–60° at a scanning rate of 12°/min.

2.10. Release of CM from polymeric micelles

The release of CM from the polymeric micelles was examined by a dialysis method. In detail, 3 mL of CM 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 15,000). 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 CM in the different samples was measured at 428 nm using a UV–Vis spectrophotometer. Calibration was done using CM (concentration from 0.3 μg/mL to 5.0 μg/mL) in 2% Triton X-100. At the end of the experiment, the amount of the remaining CM in polymeric micelles in dialysis bags was measured using a UV–Vis spectrophotometer at 428 nm, and the size of the micelles was determined by DLS.

2.11. Cytotoxicity of CM loaded polymeric micelles against cancer cells

Three different cancer cell lines, human ovarian carcinoma cells (OVCAR-3), human colorectal adenocarcinoma (Caco-2) and human lymphoblastic leukemia (Molt-4) were used to investigate the cytotoxicity of the different formulations. The OVCAR-3 and Molt-4 cell lines were cultured in RPMI 1640 supplemented with 10% FBS, 100 IU/mL of penicillin and 0.1 mg/mL of streptomycin, 1% sodium pyruvate; 0.01 mg/mL of bovine insulin was also present in the medium of the OVCAR-3 cells. The Caco-2 cells were cultured in DMEM supplemented with 10% FBS, 100 IU/mL of penicillin and 0.1 mg/mL of streptomycin. The cytotoxicity of CM loaded micelles was determined using a MTT assay [29] and compared with free CM. In detail, the cells at a density of 10,000 cells/well were cultured in 96-well plates at 37 °C and in a 5% CO_2 humidified atmosphere for 24 h. Next, 100 μL of free CM in DMSO or CM loaded polymeric micelles with concentrations of CM (either in its free form or micellar formulations) that ranged from 1 to 37 μg/mL as well as unloaded polymeric micelles (concentration ranging from 20 to 600 μg/mL) was added the medium and plates were incubated as described above for 72 h. The final concentration of DMSO that dissolved CM 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. After that, 100 μL of media was removed from each well and 15 μL of MTT in PBS (5 mg/mL) was added to cell media and incubated for 4 h. Then, the media were removed and the formed formazan crystals were dissolved by the addition of 200 μL of DMSO. The absorbance was measured at 570 nm using a microplate reader. The cell viability was calculated using the following equation:

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

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

2.12. Statistical analysis

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

3. Results and discussion

3.1. Synthesis and characterization of the synthesized polymers

PEG-HPMA-Bz block copolymers synthesized in this study were composed of 5 kDa mPEG as hydrophilic block and HPMAm-Bz as hydrophobic block. ^{1}H NMR spectrum of PEG-HPMA-Bz obtained is shown in Fig. 1. The polymers synthesized at low monomer:initiator ratios (50:1 and 150:1) were obtained in a very high yield (>95%) as presented in Table 1. The results indicate that the molecular weight of the polymer increases with *M*/*I* ratio, as expected. At extremely higher *M*/*I* ratios (300:1 and 500:1), hardly any further increase in molecular weight was found which can likely be ascribed due to their lower yield. The results further indicate that the M_n's of the synthesized polymers determined by GPC were close to the value calculated using ^{1}H NMR analysis. The polymer synthesized at the feed ratio of 150:1 was obtained with the highest yield and was therefore selected for further studies. In order to investigate the effect of hydrophobic composition on the polymer characteristics, PEG-HPMA-Bz block copolymers were further modified with substitution or the addition of lactate moiety to yield PEG-HPMA-DL and PEG-HPMA-Bz-L. The characteristics of these two block copolymers, PEG-HPMA-DL and PEG-HPMA-Bz-L with the different aromatic content (benzoyl side group) are reported in Table 2. pHPMA is a non-degradable water-soluble polymer which has been evaluated in clinical trials as a polymeric prodrug formulation of doxorubicin [19,20,30]. pHPMAm with molecular weight <30 kDa can be cleared by the kidneys [31,32]. PEG-HPMA-DL, PEG-HPMA-Bz-L and PEG-HPMA-Bz copolymers used in the present study are not soluble in water, but hydrolysis of the ester bonds between the pendant groups and the hydrophobic block converts this block into the hydrophilic block copolymer PEG/p(HPMAm) [23,33] that, likely similar as pHPMAm, can be cleared by the kidneys if the molecular weight is below 30 kDa. PEG-HPMA-DL has dilactate side chains which is the thermosensitive block whereas PEG-HPMA-Bz-L has monolactate (75%) and benzoyl (25%) side groups. It was found that PEG-HPMA-Bz could not soluble in water whereas PEG-HPMA-Bz-L and PEG-HPMA-DL could completely soluble at low temperature. The increase of water soluble property of these polymers is due to the decrease of aromatic content. The hydrophobicity of PEG-HPMA-Bz is higher than PEG-HPMA-Bz-L and PEG-HPMA-DL, respectively. The cloud point of PEG-HPMA-DL, PEG-HPMA-Bz-L and PEG-HPMA-Bz decreased respectively due to their hydrophobic content. The results show that PEG-HPMA-DL and PEG-HPMA-Bz-L have a cloud point below 10 °C which means that they are soluble in water below this temperature and self-assemble in micellar structures above this temperature [25] whereas PEG-HPMA-Bz does not have a cloud point

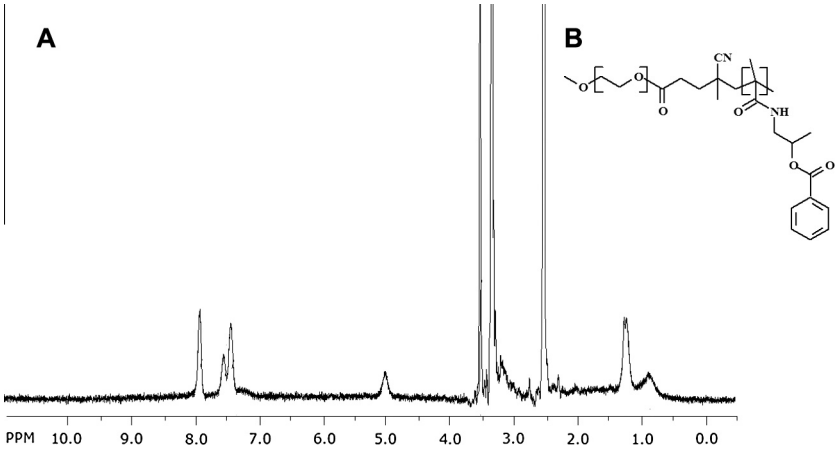

Fig. 1. ^{1}H NMR spectrum of PEG-HPMA-Bz (A) and chemical structure of PEG-HPMA-Bz (B).

Table 1
Characteristics of PEG-HPMA-Bz copolymers as determined by ^{1}H NMR and GPC.

Ratio of monomer:initiator	Yield (%)	HPMAm-BZ units/mPEG chain (NMR)	M_n (NMR)	M_n (GPC)	M_w (GPC)	PDI (M_w/M_n)
50:1	98	16	8400	13,000	18,000	1.4
150:1	101	76	21,300	21,400	28,300	1.3
250:1	56	68	19,600	21,000	36,000	1.7
300:1	56	74	20,800	23,400	43,800	1.9
500:1	65	75	21,000	23,800	44,200	1.9

Table 2
Characteristics of PEG-HPMA-DL, PEG-HPMA-Bz-L and PEG-HPMA-Bz.

Polymer	Monolactate content (%)	Dilactate content (%)	Benzoyl content (%)	M_w (GPC)	Cloud point (°C)
PEG-HPMA-DL	0	100	0	27,000	3
PEG-HPMA-Bz-L	75	0	25	29,000	2
PEG-HPMA-Bz[a]	0	0	100	28,000	–[b]

[a] From Table 1, PEG-HPMA-Bz were synthesized at the ratio of monomer:initiator = 150:1.
[b] Insoluble in water.

since it is very hydrophobic and insoluble in water. It was previously reported that the cloud point of the block copolymer decreased with the increasing content of HPMA-Bz [23].

3.2. Determination of PEG-HPMA-Bz CMC

Pyrene is a very hydrophobic compound and its fluorescent properties depend on the microenvironment in which it is present. Using fluorescence spectroscopy, a shift of the excitation peak spectra is observed when pyrene partitions from a hydrophilic to a more hydrophobic environment of polymeric micelles [34]. By using pyrene, the CMC values of PEG-HPMA-DL and PEG-HPMA-Bz-L were previously reported as 0.080 mg/mL and 0.023 mg/mL, respectively [23]. In the present study, the CMC of PEG-HPMA-Bz was found to be 0.0006 mg/mL which is substantially lower than that of PEG-HPMA-DL and PEG-HPMA-Bz-L, respectively. This is in line with the high hydrophobicity of the HPMA-Bz block. At very low PEG-HPMA-Bz concentrations (below the CMC) the maximum peak of the fluorescence of pyrene was observed at 333 nm and the peak shifted to 338 nm when the concentration of PEG-HPMA-Bz increased above the CMC (Fig. 2A). The 338/333 intensity ratio can thus be used to determine the CMC of polymeric micelles. The CMC in Fig. 2B is calculated by the intersection of the two lines. Since the concentration is on a log scale, a small difference in intersection results in a bigger difference in CMC. The intersection depends on how the lines are drawn and

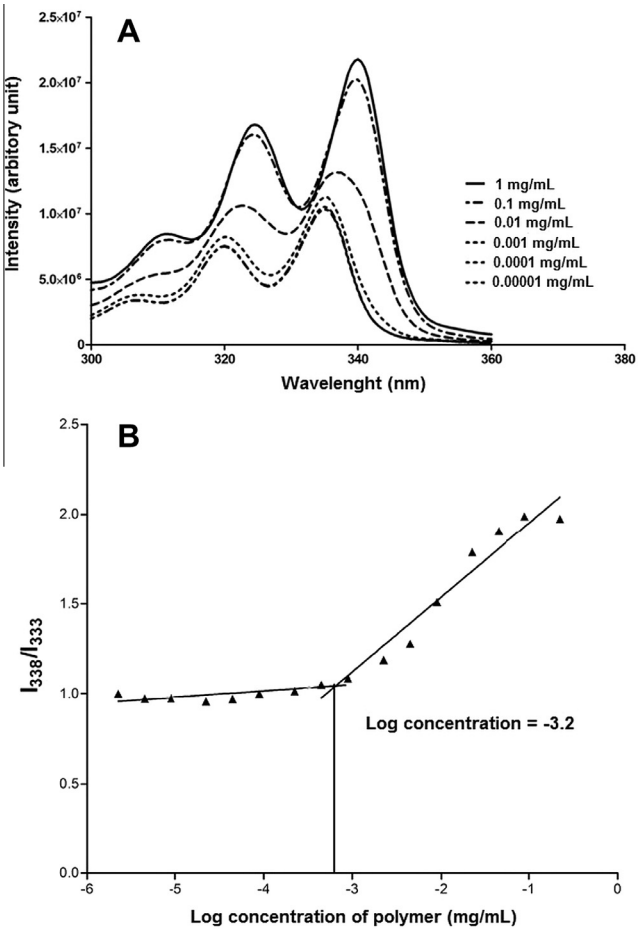

Fig. 2. Fluorescence excitation spectra of pyrene in 120 mM ammonium acetate buffer (pH = 5.0) containing PEG-HPMA-Bz at different concentrations. Emission wavelength = 390 nm (A) and I_{338}/I_{333} fluorescence ratio for pyrene as a function of the log concentration of PEG-HPMA-Bz (B).

varies between −3.2 and −3.0 yielding a CMC value between 0.6 and 1.0 μg/mL. PEG-HPMA-Bz self-assembled to form micelles by a simple nanoprecipitation method and demonstrated the lowest CMC compared to PEG-HPMA-Bz-L and PEG-HPMA-DL indicating a better thermodynamic stability.

3.3. Solubilization and loading capacities

In the previous studies, PEG-HPMA-DL has been used to solubilize hydrophobic molecules such as paclitaxel [25], vitamin K [35] and xanthone [36]. Recently, it has been reported that PEG-HPMA-Bz-L micelles showed an excellent loading and retention for both paclitaxel and docetaxel [23]. CM is an active molecule which has high hydrophobicity [38]. The previous *in vitro* studies demonstrated that CM possessed a strong cytotoxic agent against a variety of cancer cells. However, it is important to consider that CM used in those *in vitro* studies was fully dissolved in DMSO, ethanol or methanol which cannot be used in animal trials or *in vivo* clinical studies because of the toxicity of those organic solvents. The low aqueous solubility of CM leads to poor bioavailability and that is highlighted as a major limiting factor to use as therapeutic agent in human [37]. Therefore, the micelles of PEG-HPMA-DL and PEG-HPMA-Bz-L as well as that of the higher hydrophobic polymer, PEG-HPMA-Bz, were investigated for their solubilization capacity for CM. Micellar formation was done by the fast heating method for PEG-HPMA-DL and PEG-HPMA-Bz-L due to their thermosensitive properties and a nanoprecipitation method for PEG-HPMA-Bz since PEG-HPMA-Bz is insoluble in water. CM was successfully loaded into PEG-HPMA-DL, PEG-HPMA-Bz-L and PEG-HPMA-Bz micelles and solubility of CM was obviously enhanced. The results demonstrate that the dispersions of all polymeric micelles loaded with CM were clear, transparent, yellowish orange color indicating that all loaded CM was indeed solubilized in the core of the different micelles. The loading capacity of PEG-HPMA-Bz increased from 4% to 19% (entrapment efficiency of 87–97%, respectively) with increasing CM feed (Fig. 3) which was higher than that of PEG-HPMA-DL (ranging from 5% to 11%) and PEG-HPMA-Bz-L (ranging from 5% to 17%). This result clearly indicates that the synthesized polymeric micelles with aromatic group could distinctly enhance the solubilization of CM. The previous study has demonstrated that aqueous solubility of CM was only 11 ng/mL [6]. However, the present results show that the highest solubility of CM increased to 2 mg/mL by loading with PEG-HPMA-Bz micelles. Our results are in good agreement with the previous report that the introduction of benzoyl group to these micelles yielded an excellent loading and retention of the hydrophobic molecules [23]. The very high CM loading capacity of the PEG-HPMA-Bz micelles can likely be explained by the better compatibility of CM with the core of these micelles because of the stabilizing π–π stacking interactions between the aromatic groups of CM and the benzoyl groups of the polymer. It was further observed that the formulations of CM loaded PEG-HPMA-DL and PEG-HPMA-Bz-L prepared at a high feed of CM (20–40%) showed severe precipitation of CM upon incubation at room temperature for 24 h, whereas that of PEG-HPMA-Bz micelles with the same CM concentrations remained homogeneous and clear. Therefore, these three different micellar formulations with a 10% feeding of CM were used for further studies of release and cytocompatibility and cytotoxicity.

3.4. Size and size distribution determination

The particle size of unloaded as well as CM loaded polymeric micelles determined by DLS is shown in Fig. 4. It was observed that, independent of the micellar forming polymer, the size of the micelles increased with CM loading. The higher CM encapsulation

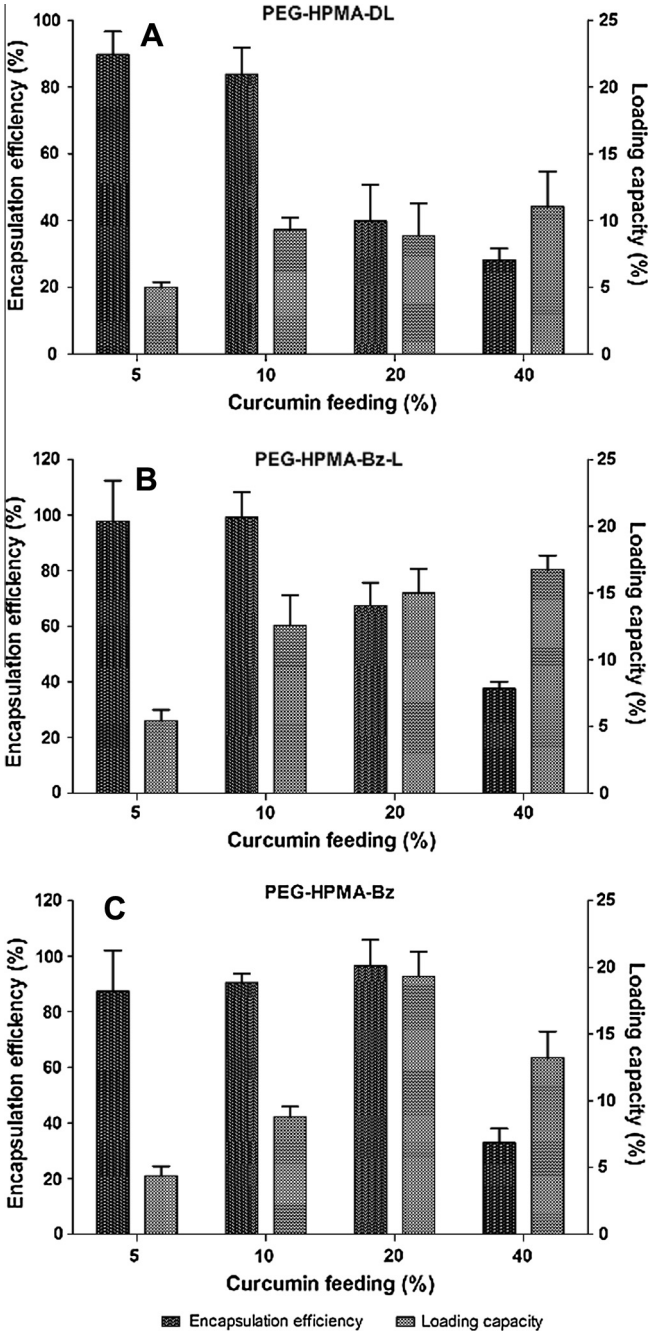

Fig. 3. The loading capacity/encapsulation efficiency of micelles at different feeding of CM ($n = 3$).

caused the particles to be bigger size than polymeric micelles without the loading of CM. It was in the agreement of Khonkarn et al. [39]. The result further shows that the size range of CM loaded PEG-HPMA-Bz micelles (57–62 nm) was smaller than that of CM loaded PEG-HPMA-DL micelles (62–85 nm) and PEG-HPMA-Bz-L micelles (46–84 nm). This can be ascribed to the more condensation by the stacking of aromatic ring and hydrophobic effect in the micellar core of the PEG-HPMA-Bz micelles. The small size around 10–100 nm of drug delivery systems could enable passively tumor accumulation through the EPR effect. Therefore, these HPMA-based polymeric micelles, particularly of PEG-HPMA-Bz polymer showed potential ability to enhance the solubility of CM and were expected to be suitable for targeting tumors exploiting the EPR effect.

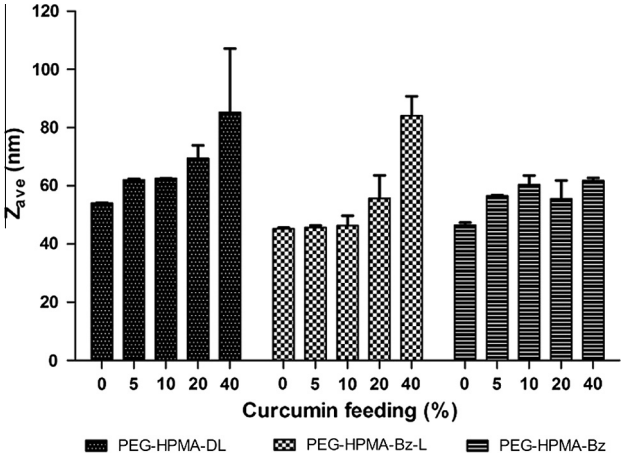

Fig. 4. Z-average hydrodynamic diameter (Z_{ave}) and polydispersity index (PDI) of micelles at different feeding of CM ($n = 3$).

3.5. Morphological investigation

All of unloaded and 10% feeding CM loaded polymeric micellar formulations appeared to be transparent dispersions. The different intensity of a yellowish orange color of each CM loaded formulation (small photographs in the right corner of Fig. 5) was depended on the type of the polymers. Morphological investigation of unloaded and CM loaded polymeric micelles by TEM showed that all particles were spherically shaped with a relatively narrow size distribution (Fig. 5). Further, the size of the micelles obtained from TEM was similar to that determined by DLS.

3.6. FT-IR and X-ray diffractometry analysis of polymeric micelles

FT-IR analysis of the three different formulations of CM loaded polymeric micelles was done to get insight into possible CM–polymer interactions through the band shifts in the FT-IR spectra [40]. As shown in Fig. 6A, the broad band in the spectrum of CM at 3508 cm^{-1} can be ascribed to the OH stretching vibration of the phenolic hydroxyl groups. The peak at 1627 cm^{-1} is ascribed to the C—O stretching vibration of the diketonic bonds [41] and the sharp peak at 1509 cm^{-1} is assigned to C=C bond of the aromatic ring [42]. The FT-IR spectra of the unloaded and CM loaded polymeric micelles showed the characteristic peaks at 3404–3422 cm^{-1} (N—H stretching) of HPMA [27] that shifted a few cm^{-1} in CM loaded polymeric micelles indicating the enhanced hydrogen bonding between CM and polymer [43]. The peaks at 1744 and 1734 cm^{-1} (C—O stretching) are ascribed to the lactic acid side groups. The disappearance of CM characteristic peak of CM loaded polymeric micelles by the FT-IR spectrum demonstrated the successful loading of CM by physical assembly without chemical interaction. XRD analysis was performed to investigate the physical state (amorphous or crystalline) in which CM was present in the core of the micelles. The XRD pattern of CM as shown in Fig. 6B demonstrates that CM powder is highly crystalline. The XRD patterns of all unloaded (control) and CM loaded micelles revealed no diffraction peaks of CM which demonstrates that CM is present in amorphous form in the core of the micelles and most likely molecularly dissolved in it. As evidence by XRD patterns, CM loaded polymeric micelles indicated the molecular dispersion of CM in the amorphous phase of polymers arranged as polymeric micelles. The halo patterns indicating amorphous form might be the reason for the improved solubility of CM. These XRD results are in parallel with the XRD patterns of CM and CM loaded MPEG-P(CL-co-PDO) micelles which also showed the absent of

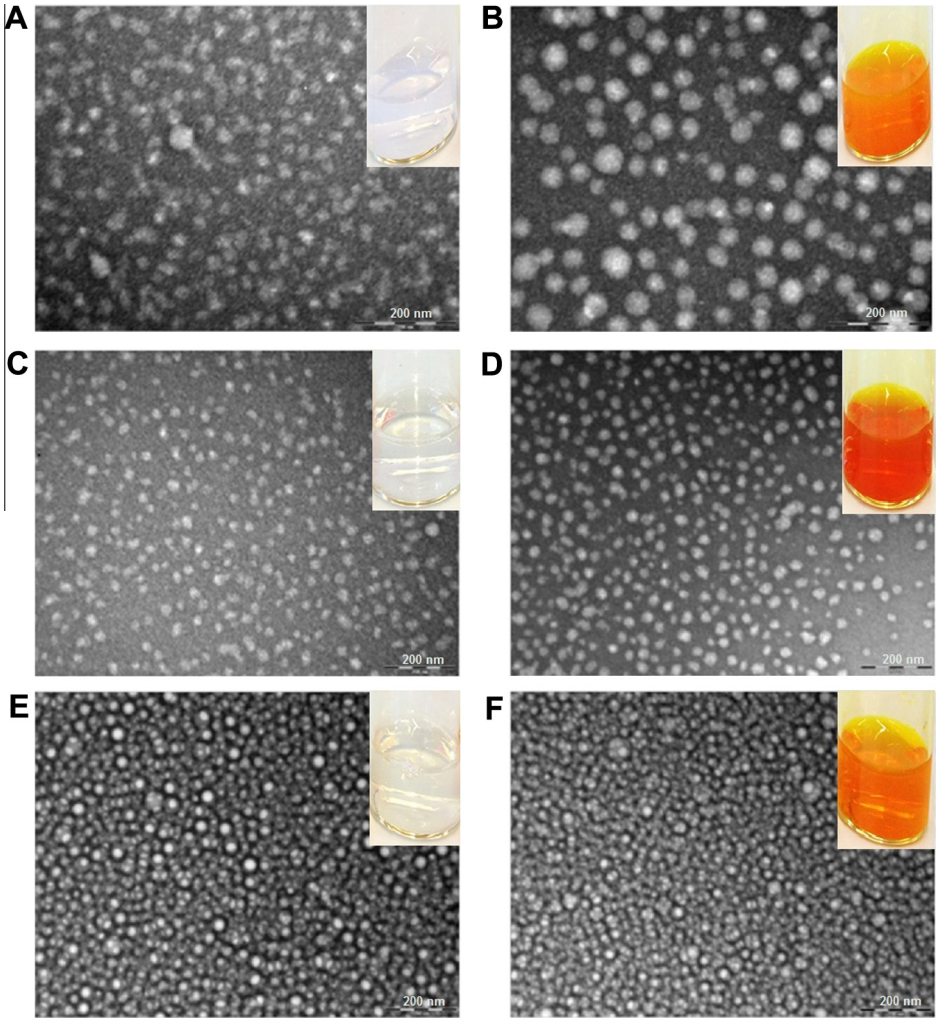

Fig. 5. TEM images of unloaded PEG-HPMA-DL micelles (A), CM loaded PEG-HPMA-DL micelles (B), unloaded PEG-HPMA-Bz-L micelles (C), CM loaded PEG-HPMA-Bz-L (D), unloaded PEG-HPMA-Bz micelles (E) and CM loaded PEG-HPMA-Bz micelles (F). The small photograph in the right corner of each TEM image shows the visual appearance of each formulation.

crystalline peak of CM loaded micelles [44]. Therefore, these explained how excellent enhancement effect of PEG-HPMA-DL polymer, PEG-HPMA-Bz-L polymer and PEG-HPMA-Bz polymer on CM solubility.

3.7. Release of CM from the polymeric micelles

The *in vitro* release of CM from the loaded polymeric micelles was determined by a dialysis method. The release study was conducted under the sink condition. Due to the very low aqueous solubility of CM, it is hard to maintain the molecular dispersion of the released CM by using buffer alone. The large amount of release media was required to keep CM solubilized, but it might result in too low CM detection and the polymer concentration might be below the CMC. Therefore, adding of suitable surfactant like Triton X-100 to solubilize the released CM was considered to be a better option. This low molecular weight surfactant does not interfere with polymeric micelles (no formation of mixed micelles occurred) since DLS showed the presence of separate peaks in the mixture (Fig. 7). Moreover, mixed micelle formation is not likely because of the different chemical natures of the surfactant and the polymers. It was confirmed that adding of non-ionic surfactant to the release media did not effect to the micelles destabilization and mixed micelles forming [36]. Fig. 8 shows that CM loaded PEG-HPMA-DL micelles released their full contents in about 20 days. On the other hand, PEG-HPMA-Bz-L micelles showed a better retention of CM and 50% of the loading was released in 20 days. PEG-HPMA-Bz micelles showed the slowest release and the best retention. Only 27% of the CM loading was released from these micelles during 20 days. At day 20, a sample of the micelles in the dialysis bags was solubilized in DMF and the remaining (non-released) CM was determined by UV–Vis spectroscopy. Table 3 shows that the sum of the amount of CM in the polymeric micelles inside and the released CM in external media corresponded, within the experiment error, with the loaded amount of CM at the start of the release experiments. DLS analysis showed that the size of micelles in dialysis bag was bigger than that of the freshly prepared micelles. Moreover, PEG-HPMA-DL and PEG-HPMA-Bz-L micelles showed a higher polydispersity (>0.2) than that of PEG-HPMA-Bz. The release profile of CM loaded polymeric micelles showed a sustained release manner. The CM content in polymeric micelles in the dialysis bag confirmed the results of cumulative release of CM from polymeric micelles and the particles size of these polymeric micelles in the dialysis bag was larger than 200 nm. This increase in size is likely due to chemical hydrolysis of the lactate acid side groups which in turn result

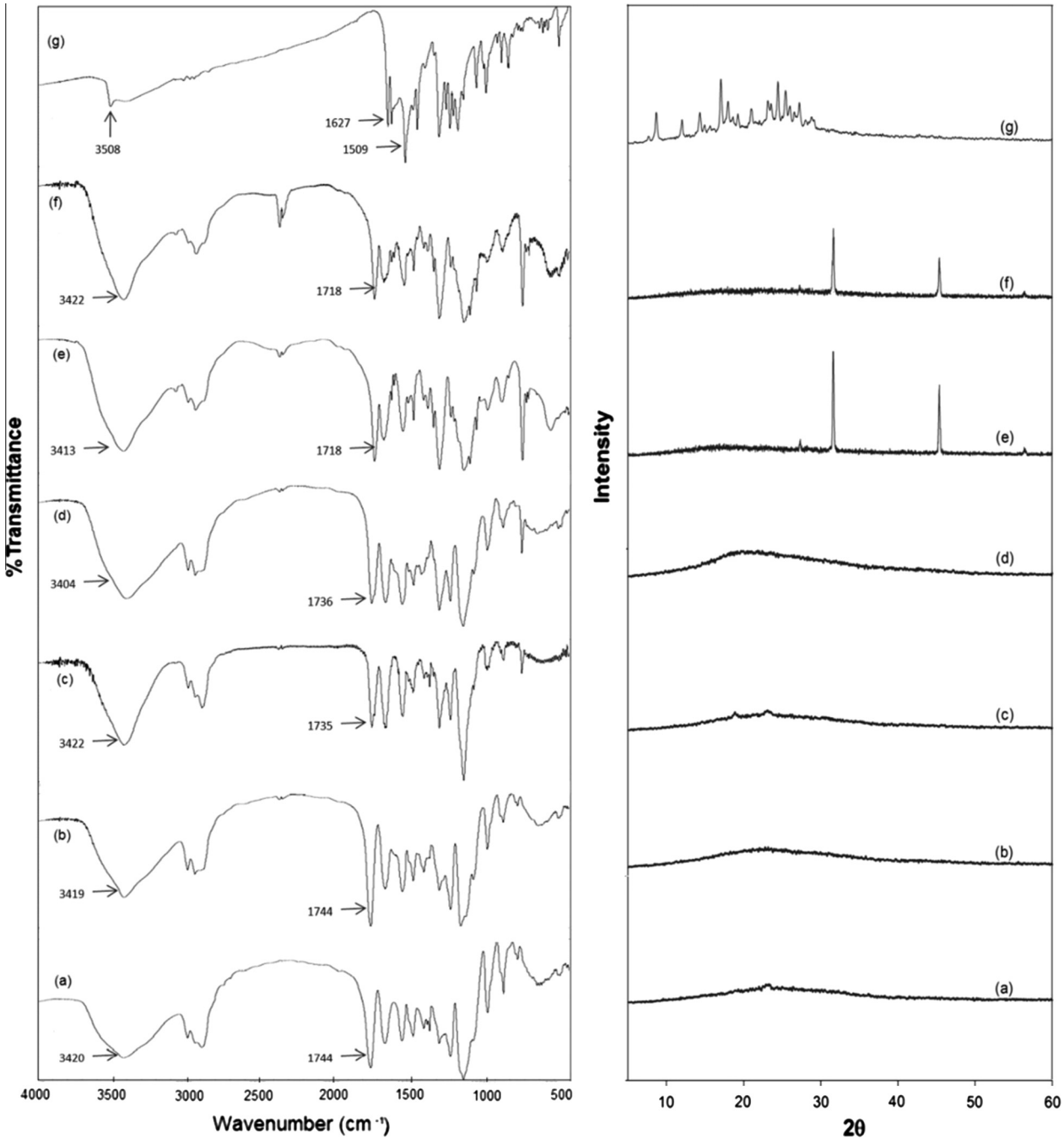

Fig. 6. FT-IR spectrum (A) and XRD patterns (B) of unloaded PEG-HPMA-DL micelles (a), CM loaded PEG-HPMA-DL micelles (b), unloaded PEG-HPMA-Bz-L micelles (c), CM loaded PEG-HPMA-Bz-L micelles (d), unloaded PEG-HPMA-Bz micelles (e), CM loaded PEG-HPMA-Bz micelles (f), and CM (g).

in hydrophilization and subsequently swelling of the core of the micelles [27,45]. It is obviously seen that the polymeric micelles with the aromatic group (PEG-HPMA-Bz-L micelles and PEG-HPMA-Bz micelles) showed the slower release than that of micelles without the aromatic group (PEG-HPMA-DL micelles). The result was in good agreement with the previous report that the slower release of hydrophobic molecules was occurred in polymers with the aromatic ending groups [39]. This better drug retention of CM in polymeric micelles with aromatic groups was considered to be due to the π–π stacking and hydrophobic interactions between the polymer and CM.

3.8. Cytotoxicity against cancer cells

Possible cytotoxic effects of unloaded polymeric micelles, CM loaded polymeric micelles as well as free CM against cancer cells were investigated using the MTT assay. The results revealed that the unloaded polymeric micelles did not shown cytotoxicity to the cells even at the high concentration of 600 μg/mL. Besides, the effect of DMSO was studied by adding the same amount that used for dissolving CM in cell medium and there was no difference between the cell viability of cell medium containing DMSO and cell medium without DMSO. On the other hand, free CM and CM loaded

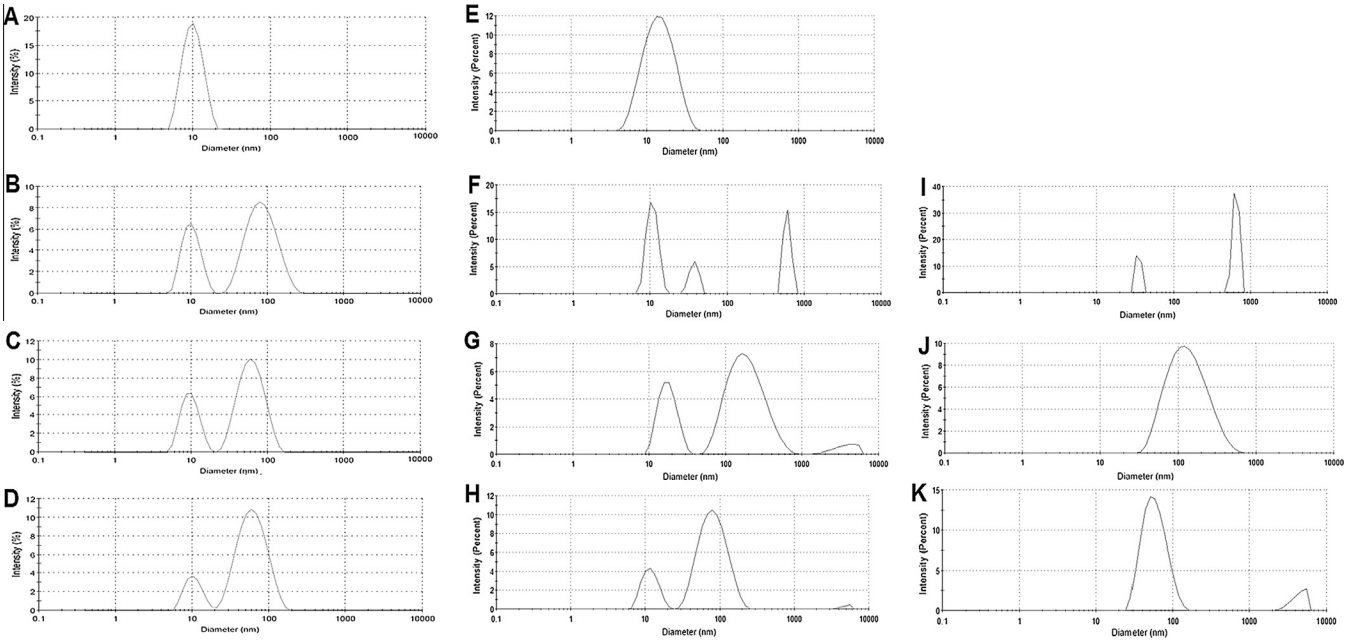

Fig. 7. Size distribution as measured by DLS at day 0 of 2% Triton X-100 [A], a mixture of CM loaded PEG-HPMA-DL micelles and 2% Triton X-100 [B], a mixture of CM loaded PEG-HPMA-Bz-L micelles and 2% Triton X-100 [C], a mixture of CM loaded PEG-HPMA-Bz micelles and 2% Triton X-100 [D] and at day 20 of 2% Triton X-100 [E], a mixture of CM loaded PEG-HPMA-DL micelles and 2% Triton X-100 [F], a mixture of CM loaded PEG-HPMA-Bz-L micelles and 2% Triton X-100 [G], a mixture of CM loaded PEG-HPMA-Bz micelles and 2% Triton X-100 [G], CM loaded PEG-HPMA-DL micelles [I], CM loaded PEG-HPMA-Bz-L micelles [J], CM loaded PEG-HPMA-Bz micelles [K].

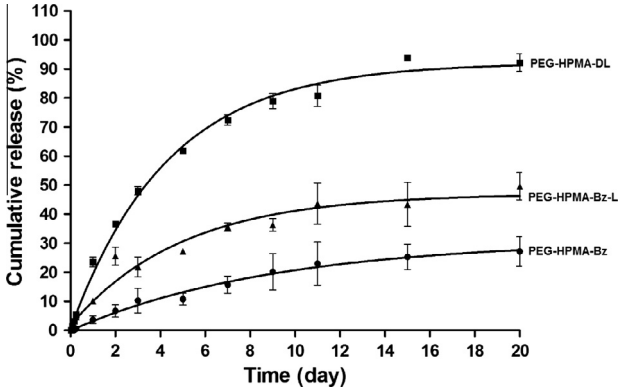

Fig. 8. CM release from the three different micelles in PBS with 2% of Triton X-100 pH 7.4, 37 °C (n = 3).

polymeric micelles showed cytotoxicity against the three tested cancer cell lines (Caco-2, OVCAR-3, and Molt-4) as shown in Fig. 9. It was found that the cytotoxic effect against Caco-2 in all of CM loaded polymeric micelles formulations and free CM was similar. The results showed that the 50% inhibition concentration (IC_{50}) of CM loaded PEG-HPMA-Bz micelles, CM loaded PEG-HPMA-Bz-L micelles, CM loaded PEG-HPMA-DL micelles and free CM shows no significant differences regarding Caco-2 cells (15 ± 3 μg/mL, 17 ± 1 μg/mL, 17 ± 1 μg/mL and 14 ± 2 μg/mL, respectively). Our results of the equipotential cytotoxicity of CM loaded and unloaded formulations are in good agreement with the previous studies of the CM loaded chitosan/polycaprolactone nanoparticles [46]. According to the cytotoxicity against OVCAR-3 and Molt-4, it was found that the IC_{50} of CM loaded PEG-HPMA-DL micelles (4 ± 1 μg/mL and 7 ± 0 μg/mL) and CM loaded PEG-HPMA-Bz-L (5 ± 1 μg/mL and 6 ± 0 μg/mL) micelles shows comparable cytotoxic effects to free CM (4 ± 1 μg/mL and 6 ± 0 μg/mL) whereas the IC_{50} of CM loaded PEG-HPMA-Bz micelles (10 ± 2 μg/mL and 13 ± 2 μg/mL) showed a significant difference to other micellar systems and free CM ($p < 0.05$). Our results indicated that the different phenotypes of cancer cells were responsible to the different IC_{50} of CM loaded polymeric micelles. The comparable cytotoxicity of free CM and CM loaded PEG-HPMA-DL at CM concentration <5 μg/mL can be explained by the concentration of PEG-HPMA-DL which is below the CMC, meaning that CM was presented in its free form in the culture media. However, all concentrations of the other two polymers were above the CMC indicating that micelles were present. In previous studies we have reported that PEG-HPMA-DL micelles [25] and PEG-HPMA-Bz-L micelles [26] were taken up by cells. Therefore, the cytotoxicity of CM loaded micelles is likely due to the cellular uptake of the micelles rather than the release of CM in the tissue culture medium. However, the cellular uptake of HPMA-Bz micelles has not been investigated yet, but it might be expected that similar as the other micelles they are also internalized. The lower toxicity of CM loaded PEG-HPMA-Bz micelles might be caused by the slower intracellular release of CM [47].

Table 3
Curcumin content and size of micelles in the dialysis bag after 20 days.

Polymer	Curcumin in bag (%)	Cumulative release (%)	Total mass (%)	Z_{ave} (nm)	PDI
PEG-HPMA-DL	0.3 ± 0.5	92.2 ± 3.1	92.6 ± 3.0	263 ± 194	0.8 ± 0.1
PEG-HPMA-Bz-L	48.1 ± 6.2	49.7 ± 4.7	97.8 ± 3.3	258 ± 27	0.2 ± 0.0
PEG-HPMA-Bz	74.9 ± 8.4	27.2 ± 5.1	102.1 ± 12.1	56 ± 4	0.1 ± 0.0

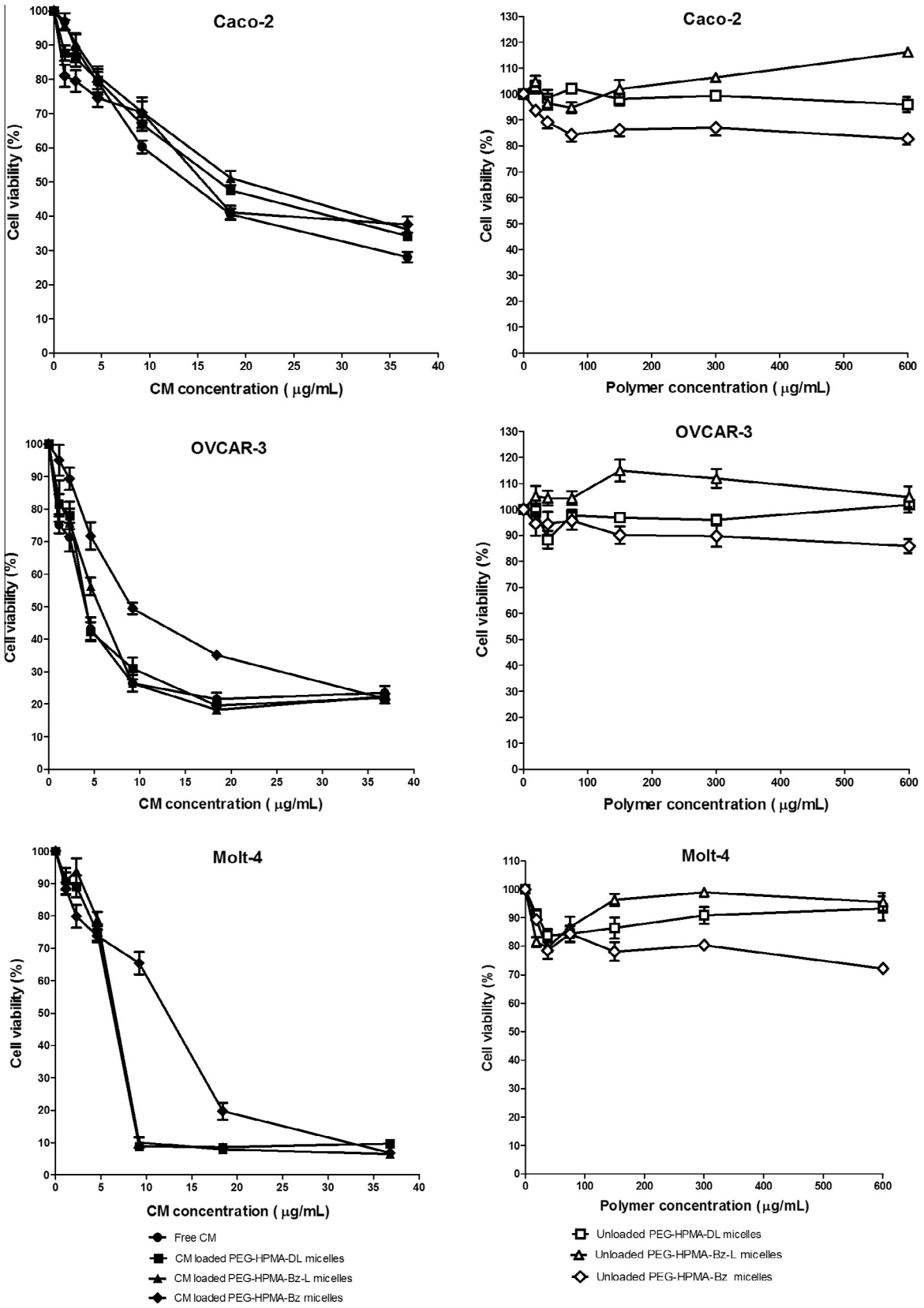

Fig. 9. Cytotoxicity of CM loaded micelles and unloaded micelles against Caco-2, OVCAR-3 and Molt-4 after 72 h of incubation ($n = 3$).

4. Conclusion

The present study demonstrates that HPMA-based polymeric micelles substantially increased the solubility of CM up to 2 mg/mL. All micellar systems showed sustained release of the CM loading and the release from the polymeric micelles with aromatic groups was slower than the polymeric micelles without aromatic groups which are likely because of π–π stacking and hydrophobic interactions between the polymer and CM. Cell tests showed good cytotoxic effects of CM loaded polymeric micelles against cancer cells. The CM loaded polymeric micelles described in this study are therefore attractive systems for *in vivo* studies.

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