Journal of Drug Delivery Science and Technology 35 (2016) 24–29

Contents lists available at ScienceDirect

Journal of Drug Delivery Science and Technology

journal homepage: www.elsevier.com/locate/jddst

Preparation and characterization of polymeric micelles loaded with a potential anticancer prodrug

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ARTICLE INFO

Article history:
Received 6 May 2016
Received in revised form
13 June 2016
Accepted 15 June 2016
Available online 17 June 2016

Keywords:
Amphiphilic block copolymers
Drug delivery system
HPMA
R-(+)-MRJF4
Polymeric micelles

ABSTRACT

Polymeric micelles based on HPMA [*N*-(2-hydroxypropyl) methacrylamide] polymers were recently evaluated as drug delivery systems of several anticancer drugs. The development of polymeric micelles to solubilize R-(+)-MRJF4, a potential anticancer prodrug, is reported in this paper. Two different amphiphilic block copolymers based on PEG-HPMA [(ω-methoxypoly (ethylene glycol)-*b*-(*N*-(2-benzoyloxypropyl) methacrylamide)-*co*-(*N*-(2-lactoyloxypropyl) methacrylamide) (PEG-HPMA-Bz-L) and (ω-methoxy poly (ethylene glycol)-*b*-(*N*-(2-benzoyloxypropyl) methacrylamide (PEG-HPMA-Bz)] were synthesized and investigated for this purpose. Results showed that both polymers were able to efficiently solubilize the drug at concentrations of 2 and 4 mg/mL and polymer concentration of 9 mg/mL yielding polymeric micelles with a size of 53–83 nm. Release studies showed that the formulation obtained using PEG-HPMA-Bz-L slowly released R-(+)-MRJF4 for 7–8 days. Moreover, cytotoxicity studies performed on C6 glioma cells revealed that, after 48 h, R-(+)-MRJF4-loaded PEG-HPMA-Bz and PEG-HPMA-Bz-L micelles possessed a higher antiproliferative activity when compared to free R-(+)-MRJF4, implying that the formulations could be internalized by the cells. Taken together, our results suggest that PEG-HPMA-Bz-L polymeric micelles are interesting to optimize the therapeutic efficacy of R-(+)-MRJF4.

1. Introduction

Amphiphilic block copolymers have been extensively studied for biomedical applications due to their capability to associate and organize, in aqueous media, in core-shell structures above a certain concentration [1–4]. These nanometer systems have favorable features, such as prolonged circulation time, increased drug availability, and controlled drug release resulting in improved therapeutic effectiveness [5–10]. Such systems are characterized by a peculiar structure composed of a hydrophobic core surrounded by a hydrophilic shell.

The micellar core, due to its chemical nature, has a good capability to solubilize significant amounts of poorly water-soluble chemotherapeutics [11–14], ameliorate their unfavorable pharmacokinetics, as well as their stability.

The hydrophilic corona, most frequently composed of PEG, is responsible for their colloidal stability and protection against protein absorption and opsonization in the circulation, thus resulting in a prolonged circulation time [15–17].

After systemic administration, polymeric micelles, typically ranging between 10 and 100 nm, can accumulate in tumor and diseased tissues through the EPR effect [18,19] avoiding fast removal by macrophages particularly present in liver and spleen. Block copolymers with a molecular weight below 50000 g/mol can be excreted via renal elimination [20], thus they are preferred for the design of polymeric micelles for drug delivery purposes.

Recently, we synthesized R-(+)-MRJF4, a novel haloperidol metabolite II (HP-mII) prodrug (a sigma-1 antagonist and sigma-2 agonist), obtained through conjugation with 4-phenylbutyric acid

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http://dx.doi.org/10.1016/j.jddst.2016.06.006
1773-2247/

(PhBA) [a histone deacetylase inhibitor (HDACi)] endowed with anticancer activities (Fig. 1) [21,22]. However, its poor water solubility (1.2 μg/mL) is a main restriction for administration.

To overcome this problem, in the present study we encapsulated R-(+)-MRJF4 into polymeric micelles based on HPMA polymers [23]. pHPMA is a synthetic, water-soluble, biocompatible, non-immunogenic, and highly multifunctional polymer that has been evaluated in clinical trials as doxorubicin-conjugated polymeric prodrug [24–28]. Notably, two chemically modified HPMA polymers, PEG-HPMA-Bz (ω-methoxy poly (ethylene glycol)-*b*-(*N*-(2-benzoyloxypropyl) methacrylamide and PEG-HPMA-Bz-L (ω-methoxypoly (ethylene glycol)-*b*-(*N*-(2-benzoyloxy-propyl) methacrylamide)-*co*-(*N*-(2-lactoyloxypropyl) methacrylamide) were recently investigated to improve the solubility of curcumin [14,26]. The first polymer is not water soluble since it contains only benzoyl groups as side chains that confer it a high grade of hydrophobicity; the second one contains hydrolytically sensitive moieties, such as lactic acid esters side groups, that improve the hydrophilicity of the polymer and thus its water solubility [29]. Moreover, the two polymers have another feature: PEG-HPMA-Bz is not thermosensitive while PEG-HPMA-Bz-L is a thermosensitive polymer that forms micelles above its cloud point [29].

In this study we used the two above mentioned polymers to solubilize the hydrophobic prodrug R-(+)-MRJF4 and improve its potential anticancer activity against C6 glioma cells (the structures of these polymers are shown in Scheme 1). The prodrug-loaded polymeric micelles were subjected to physico-chemical characterization (size, encapsulation efficiency, loading capacity), *in vitro* release studies, and *in vitro* cytotoxic assays against C6 glioma cells to assess their potential as formulations of an anticancer drug.

2. Materials and method

Acetonitrile (ACN), tetrahydrofuran (THF), dichloromethane (DCM), dimethylformamide (DMF), and molecular sieves (0.4 nm) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). The syntheses of R-(+)-MRJF4 and the polymers PEG-HPMA-Bz and PEG-HPMA-Bz-L were performed as previously reported [22,26,29].

The identity of the synthesized compounds was confirmed by ^{1}H NMR spectroscopy and their purities were evaluated by analytical HPLC. ^{1}H NMR spectra were recorded on a Gemini 300 MHz spectrometer (Varian Associates Inc. NMR Instruments, Palo Alto, CA). The weight average molecular weight (M_w), the number average molecular weight (M_n), and the Polydispersity Index (PDI) of the synthesized polymers were determined by gel permeation chromatography (GPC)[29]HPLC equipment was a Waters 600 HPLC pump (Waters Corporation, Milford, MA, USA), provided with a Waters 2996 photodiode array detector, a 20 μL Rheodyne injector loop and a computer-integrating apparatus.

Size and PDI of the polymeric micelles were analyzed by dynamic light scattering (DLS). DLS analysis was carried out using a Malvern 4700 system (Malvern Ltd., Malvern, U.K.) consisting of an Autosizer 4700 spectrometer, a pump/filter unit, a model 2013 air-cooler argon ion laser (75 mW, 488 nm, equipped with a 2500 remote interface controller, Uniphase) and a water bath, and a computer with DLS software (PCS, version 3.15, Malvern), operating at 25 °C at a fixed angle of 90°.

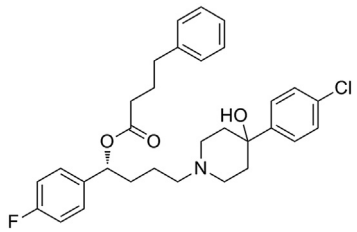


Fig. 1. Structure of R-(+)-MRJF4.

3. Experimental

3.1. Synthesis and characterization of block copolymers

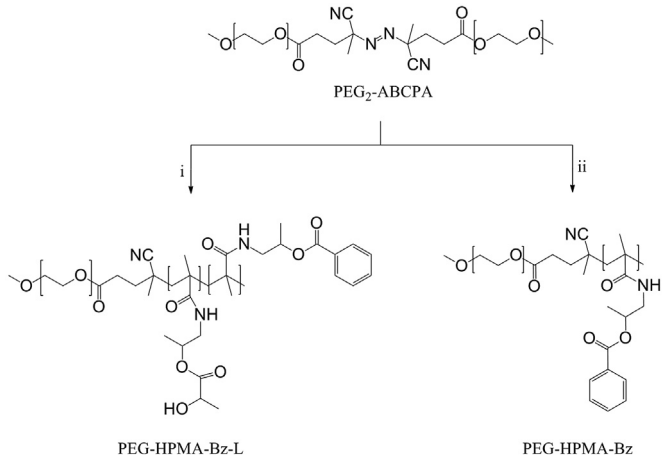
Block copolymers were prepared following free radical polymerization using different monomers (HPMAm-L, HPMAm-Bz $mPEG_2$-ABCPA (M_n of mPEG = 5000 g mol^{-1}) as macroinitiator [30]. The monomer/initiator ratio was 150:1 mol/mol. The monomers and the macroinitiator, at a concentration of 300 mg/mL, were dissolved in ACN. The resulting solution was flushed with N_2 for at least 20 min, heated at 70 °C and stirred for 24 h. Next, the polymers were precipitated by dropwise addition of the mixture to an excess of diethyl ether. After centrifugation, different procedures were applied to obtain the final polymers: the thermosensitive block copolymer (PEG-HPMA-Bz-L) was dissolved in water and dialyzed for two days at 4 °C, using membranes with a cut-off of 12–14 kDa while the non-thermosensitive copolymer (PEG-HPMA-Bz) was recovered without purification.

The polymers were fully characterized by ^{1}H NMR spectroscopy and GPC measurements. The NMR signals were assigned as previously reported [29].

GPC was performed using samples at the concentration of 5 mg/mL using a PL gel 5 μm polystyrene packed MIXED-D (Polymer Laboratories) column characterized by 5 μm particle size, 300 mm × 8 mm i.d. using DMF containing 10 mM of LiCl as eluent with a flow of 0.7 mL/min and the temperature was set at 40 °C [30]. PEG of defined molecular weights, ranging from 238 to 1015000 Da, were used for calibration.

3.2. Critical micelle concentration (CMC)

The CMC of the block copolymers was determined using pyrene as a fluorescent probe. Fluorescence excitation spectra of pyrene were obtained as a function of the polymer concentration using Jasco FP-6500 spectrofluorimeter. The excitation spectra were recorded at 37 °C from 300 to 360 nm with the emission



Scheme 1. Synthesis of PEG-HPMA-Bz-L and PEG-HPMA-Bz: (i) HPMAm-Bz/HPMAm-Lac_1 25/75; (ii) HPMAm-Bz by free radical polymerization (ACN, 70 °C) using PEG_2-ABCPA as macroinitiator.

Table 1
Characteristics of the synthesized block copolymers.

Polymer	Feed molar (%)	Yield (%)	HPMAm-Bz (%)	HPMA m-L (%)	Mn[a] (KDa)	Mn[b] (KDa)	PDI[a]	Mw[a] (KDa)
PEG-HPMA-Bz-L	25/75	80	21	79	18	16	1.5	27
PEG-HPMA-Bz	100	70	100	–	18	18	1.6	29

[a,b] Values obtained by GPC and NMR measurements, respectively.

wavelength set at 390 nm. The excitation and emission band slits were 5 and 1 nm, respectively. The intensity ratio I_{338}/I_{333} was plotted against polymer concentration to determine the CMC [30].

The same solutions were analyzed by DLS at a scattering angle of 90°, equipped with a 35 mW He–Ne laser at the wavelength of 660 nm at 37 °C. The scattered light intensity (Kcps), plotted as function of the polymer concentration, was used to determine the CMC.

3.3. Critical micelle temperature (CMT)

The thermosensitive polymer was dissolved at a concentration of 10 mg/mL in pH 4 ammonium acetate buffer (AAB, 120 mM) and left at 0 °C overnight. After micelles formation, following the fast heating procedure [31], the light scattering intensity of the micellar dispersion was recorded while the formulation was slowly cooled down to 0 °C. CMT was determined using DLS through graphical extrapolation by the interception point in a graph obtained plotting LSI against temperature.

3.4. Preparation of R-(+)-MRJF4 -loaded micelles

R-(+)-MRJF4-loaded PEG-HPMA-Bz-L micelles were prepared, using the fast heating method, by adding 0.1 mL of the drug solution in EtOH to 0.9 mL of the polymer solution in pH 4 ammonium acetate buffer (AAB, 120 mM) previously left at 0 °C for 16 h. The mixture was subsequently shaken for 1 min at 50 °C, and then cooled down to room temperature. The obtained micellar dispersions were filtered using 0.45 μm filters to remove the non-entrapped drug [30,31].

R-(+)-MRJF4-loaded PEG-HPMA-Bz micelles were prepared by adding 1 mL of a solution of polymer and drug in THF to 1 mL of AAB (pH = 4) buffer under stirring. After evaporation of the organic solvent for 16 h at room temperature, both the non-entrapped drug and aggregated micelles were removed by filtration using 0.45 μm filter.

3.5. Characterization of R-(+)-MRJF4 -loaded micelles

The dispersions of drug-loaded micelles were mixed with ACN (1:5), vortexed to destabilize the micelles and dissolve the drug, and subsequently centrifuged at 12000 rpm for 10 min. Aliquots (10 μL) of the supernatant were analyzed by HPLC to assess the amount of entrapped drug. Samples were eluted on a Symmetry RP-C18 column (150 × 4.6 mm, 5 μm Waters) employing a mobile phase composed of a mixture of ACN, water, and formic acid. The flow rate was 1 mL/min and the UV-detector operative at 264 nm. Solutions with different prodrug concentrations were used for calibration. Encapsulation efficiency (EE) and loading capacity (LC) were calculated from previously described equations [29].

3.6. In vitro release of R-(+)-MRJF4 from micelles

In *vitro* release studies were performed using a dialysis method. In detail, R-(+)-MRJF4-loaded micelles (1 mL) were transferred into a dialysis bag having a 12–14 molecular weight cut-off and placed in 1 L of release buffer (PBS, 170 mM, pH 7.4) under constant magnetic stirring at 100 rpm and thermostated at 37 ± 0.5 °C. Aliquots of 100 μL of micellar dispersions were taken at different time points, diluted with ACN and vortexed. After centrifugation the drug content was assayed by HPLC analysis. The percentage of released drug was calculated using the following equation:

$$\%\ \text{released drug} = 100\ \% - (\%\ \text{remaining drug})$$

3.7. In vitro cytotoxicity of R-(+)-MRJF4-loaded micelles against C6 glioma cells

The effect of R-(+)-MRJF4 and its PEG-HPMA-Bz-L and PEG-HPMA-Bz micellar formulations was assessed on the viability and proliferation of mouse C6 glioma cells by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay [32]. The cells, seeded in 96-well plates at a density of 20000 cells/cm^2, were treated with R-(+)-MRJF4, unloaded PEG-HPMA-Bz-L micelles, unloaded PEG-HPMA-Bz micelles, R-(+)-MRJF4-loaded PEG-HPMA-Bz-L micelles and R-(+)-MRJF4-loaded PEG-HPMA-Bz-micelles at feed drug concentration of 2 mg/mL. After 48 h of incubation, a solution of MTT in PBS was added to each well to a final concentration of 0.5 mg/mL. After 3 h of incubation, 200 μL of dimethyl sulfoxide was added to solubilize the formed formazan crystals. The plate was put in the dark at 37 °C for 30 min and subsequently the spectrometric absorbance at 540 nm was read using a microplate reader (SpectraMAX 190, Molecular Devices).

3.8. Statistical analysis

All experiments were performed in triplicate. All results are expressed as mean ± standard deviation. P values < 0.05 are considered as statistically significant.

4. Results and discussion

4.1. Preparation and characterization of polymers

The two different block copolymers, PEG-HPMA-Bz-L and PEG-HPMA-Bz, were synthesized as previously reported (Scheme 1, Table 1) [26,29]. The polymers were obtained in high yields after purification (70–80%) (Table 1). GPC analysis revealed that the M_n

Table 2
CMC values of polymers (37 °C).

	CMC (mg/mL)	
Polymer	I_{338}/I_{333}	DLS
PEG-HPMA-Bz	0.0083	0.0080
PEG-HPMA-Bz-L	0.0316	0.0334

of the synthesized polymers was comparable to that calculated by ^{1}H NMR analysis, and the PDIs were around 1.5 (Table 1). [29]. The two polymers present different characteristics: a) PEG-HPMA-Bz is not water-soluble; the presence of benzoyl groups in PEG-HPMA-Bz highlights the hydrophobic features of the polymer; b) on the other hand, the presence of 75% lactate side groups in PEG-HPMA-Bz-L confers it more hydrophilicity and the polymer is thermosensitive, meaning that it is soluble in water at low temperature, while precipitates above a certain temperature, referred to as the cloud point (CP) (Table 1).

4.2. CMC determination

The CMC was determined by DLS and fluorescence spectroscopy. Results obtained with both methods are in agreement with each other (Table 2); notably, PEG-HPMA-Bz showed CMC values equal to 0.0083 mg/mL and 0.0080 mg/mL using fluorescence and DLS analysis, respectively. These lower values, as compared to those found for PEG-HPMA-Bz-L polymer (CMC were 0.0316 mg/mL by fluorescence excitation and 0.0334 mg/mL by DLS analysis, respectively), are attributed to a stronger hydrophobicity of PEG-HPMA-Bz polymer(Fig. 2).

4.3. CMT determination

The CMT of the PEG-HPMA-Bz-L was determined using DLS. As shown in Fig. 3, the CMT is 6–7 °C in pH 4 ammonium acetate buffer (AAB, 120 mM).

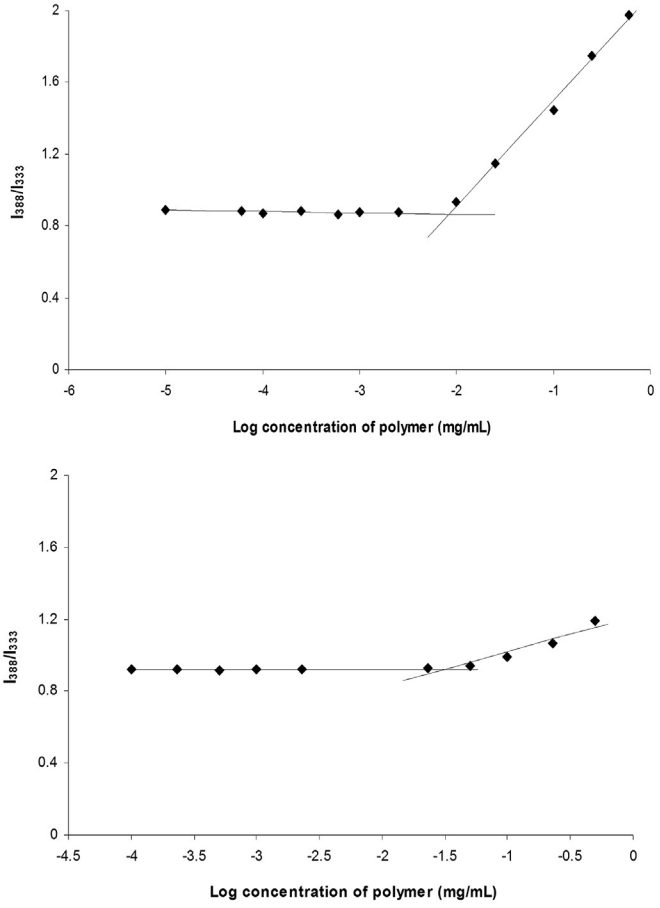


Fig. 2. CMC determination by fluorescence excitation spectra for PEG-HPMA-Bz (top) and PEG-HPMA-Bz-L (bottom) in 120 mM AAB.

4.4. Preparation and characterization of R-(+)-MRJF4-loaded micelles

R-(+)-MRJF4, a potential anticancer prodrug, has high hydrophobicity (cLogP = 6.95 ± 0.45) and a low water solubility (1.2 ± 0.2 μg/mL) [21]. We therefore selected two polymers – PEG-HPMA-Bz (non-thermosensitive) and PEG-HPMA-Bz-L (thermosensitive) – to solubilize this prodrug. R-(+)-MRJF4-loaded micelles were prepared at pH 4 in AAB (120 mM) using both fast heating and nanoprecipitation methods.

The encapsulation efficiencies (EE) of R-(+)-MRJF4 in the micellar dispersion were greater than 80% for PEG-HPMA-Bz-L and PEG-HPMA-Bz in the range of feed concentration between 0.5 and 4.0 mg/mL (Table 3). At pH 4, the EE was 98% when PEG-HPMA-Bz was used as polymer (at feed drug concentrations of 2 and 4 mg/mL). PEG-HPMA-Bz-L also showed a good entrapment efficiency (EE > 95%) at a feed drug concentration of 2 mg/mL.

The drug loading capacities (LC) of PEG-HPMA-Bz and PEG-HPMA-Bz-L were directly correlated with the EE values (Table 3). For the tested polymers the LC augmented at increasing feed drug concentrations, hence suggesting that we did not exceed the LC limit. LC of PEG-HPMA-Bz polymeric micelles for R-(+)-MRJF4 were 17.9 ± 1.4 and 30.5 ± 0.10 at feed concentration of 2 and 4 mg/mL, respectively. Similar results were obtained for R-(+)-MRJF4-loaded micelles prepared with PEG-HPMA-Bz-L.

The size of R-(+)-MRJF4-loaded micelles was greater than those of empty micelles. Table 3 shows that the particle size of the micelles increased with the LC. The results further showed that the size of R-(+)-MRJF4-loaded micelles at pH 4.0 ranged from 52 to 83 nm, suggesting that these formulations could take advantage of the EPR effect after intravenous administration.

The PDI, measured by DLS, was below 0.11 at all feed drug concentrations suggesting that particles had a relatively narrow size distribution.

Our data suggest that both polymers efficiently encapsulate R-(+)-MRJF4 and increase the solubility of the prodrug from 1.2 μg/mL to 4 mg/mL. The high EE and LC of the tested polymers can be explained by the strong hydrophobic π-π interactions between their benzoyl groups and the aromatic moieties of R-(+)-MRJF4. Furthermore, at pH 4 an important role could be played by the charged nitrogen of the piperidine ring of R-(+)-MRJF4 that makes a cation-π interaction with the negatively charged cloud of π systems of polymers. Literature data reported that these electrostatic interactions are the strongest among non-covalent interactions [33]. π-π and cation-π

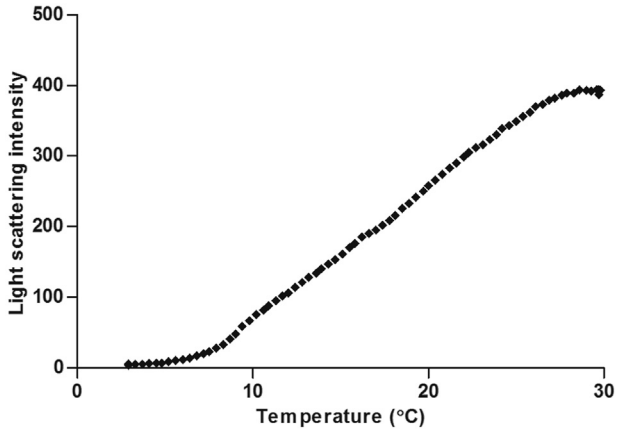


Fig. 3. CMT determination of PEG-HPMA-Bz-L.

Table 3
Size, PDI, EE, and LC of R-(+)-MRJF4-loaded micelles prepared at pH 4 ($n = 3$) at different feed concentrations.

Polymer	Feed drug concentration (mg/mL)	Size (nm)	PDI	EE %	LC%
PEG-HPMA-Bz	0	65 ± 3.9	0.11 ± 0.02	–	–
	0.5	72 ± 3.6	0.09 ± 0.02	91.4 ± 4.8	4.8 ± 0.4
	1	67 ± 9.4	0.12 ± 0.07	93.1 ± 10.4	9.4 ± 0.95
	2	63 ± 4.5	0.10 ± 0.09	98.4 ± 9.5	17.9 ± 1.4
	4	83 ± 7.4	0.18 ± 0.08	98.8 ± 0.4	30.5 ± 0.10
PEG- HPMA-Bz-L	0	46 ± 3.0	0.04 ± 0.01	–	–
	0.5	57 ± 0.1	0.06 ± 0.01	80.5 ± 9.7	4.3 ± 0.5
	1	52 ± 2.1	0.05 ± 0.01	83.4 ± 0.1	8.5 ± 0.01
	2	61 ± 4.0	0.11 ± 0.01	95.3 ± 11.3	17.5 ± 1.7
	4	71 ± 1.6	0.06 ± 0.01	98.3 ± 8.6	30.4 ± 1.9

interactions between PEG-HPMA-Bz-based micelles and R-(+)-MRJF4 contribute to the amelioration of solubility parameters of the prodrug.

Analyzing all data, micelles with a solubilized drug concentration of 2 mg/mL are considered attractive formulations because of their small size (61–63 nm), high EE% (between 95 and 98%), and low PDI (0.1); therefore these systems were further evaluated for prodrug release.

MRJF4-loaded micelles were also prepared at pH 7.4 in PBS but at the concentration of 2 mg/mL, the EE% was slightly lower than that at pH 4 (between 62 and 80%), while other parameters were comparable (Table 1S).

4.5. In vitro release studies

In vitro release studies of R-(+)-MRJF4 were carried out using dialysis method at physiological conditions (37 ± 0.5 °C and PBS at pH = 7.4). After 24h (Fig. 4), about 20% of R-(+)-MRJF4 was released from PEG-HPMA-Bz-L micelles while, after 3 days, the released percentage increased to 50%. A slower release of the drug was observed from PEG-HPMA-Bz micelles: only 5% and 20% of drug was released after 1 and 3 days, respectively. This different behavior can be ascribed to the nature of the two polymers: PEG-HPMA-Bz is more hydrophobic than PEG-HPMA-Bz-L, so it releases the lipophilic drug more slowly, due to stronger hydrophobic and π-π stacking interactions between the polymer and the drug which ensure a slower diffusion of the encapsulated drug. These data are in line with a previous study on the release of curcumin from the same type of micelles [26].

R-(+)-MRJF4-loaded PEG-HPMA-Bz-L micelles are a promising formulation because they show a sustained release of the prodrug over 7–8 days.

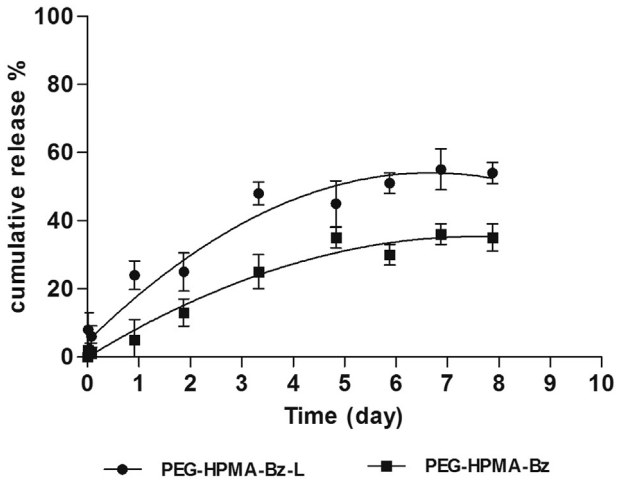


Fig. 4. Release studies of R-(+)-MRJF4-loaded micelles at pH 7.4 and 37 °C.

4.6. In vitro cytotoxicity of R-(+)-MRJF4-loaded micelles

The cytotoxicity of R-(+)-MRJF4-loaded PEG-HPMA-Bz-L and PEG-HPMA-Bz micelles was assessed on mouse C6 glioma cells using an MTT assay. To this end, cells were incubated for 48 h with R-(+)-MRJF4-loaded micelles with a feed drug concentration of 2 mg/mL. *In vitro* results showed that the unloaded micelles were not toxic for C6 glioma cells (Fig. 5). This figure also shows that R-(+)-MRJF4-loaded micelles possessed a higher cytotoxic activity when compared to free R-(+)-MRJF4 after incubation for 48 h. In fact, a significant reduction ($p < 0.05$) of cellular proliferation and viability was observed when R-(+)-MRJF4 was entrapped into PEG-HPMA-Bz and PEG-HPMA-Bz-L micelles, suggesting that the formulations could be internalized by the cells and release their payload intracellularly [34,35].

5. Conclusions

The aim of this study was the design of polymeric micelles loaded with the poorly soluble anticancer agent R-(+)-MRJF4. Two polymers (PEG-HPMA-Bz and PEG-HPMA-Bz-L) endowed with different features were chosen to entrap R-(+)-MRJF4. Results showed that the most hydrophobic polymer (PEG-HPMA-Bz) interacts more strongly with the drug, thus retarding its release. On the other hand, the most hydrophilic polymer releases the drug

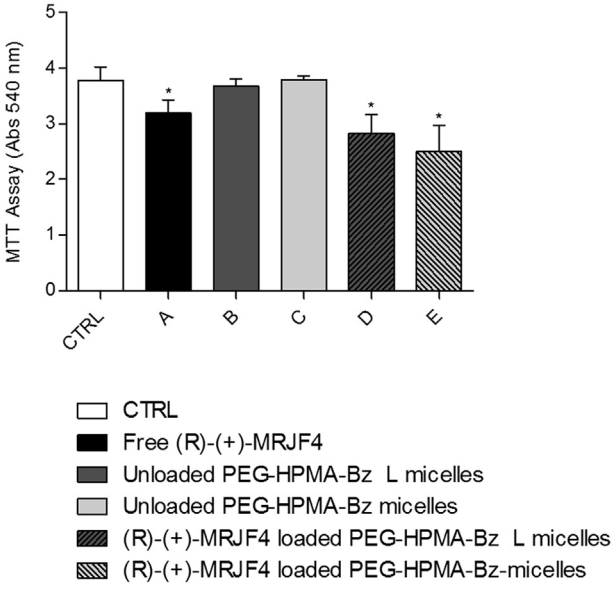


Fig. 5. Cytotoxicity of R-(+)-MRJF4-loaded micelles tested on C6 glioma cells.

more fastly. *In vitro* cell studies showed that the drug-loaded micelles had a higher cytoxicity than the free drug, suggesting that the micelles could be internalized by cells. Taken together, our results suggest that the micellar dispersion based on PEG-HPMA-Bz-L could be used as potential sustained release system for hydrophobic prodrugs. This encourages further evaluation of these micellar formulations in relevant animal models of cancer.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

The authors are grateful to MIUR (2015) for the financial support.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jddst.2016.06.006.

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