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ARTICLE TYPE

PEG based random copolymer micelles as drug carriers: Effect of hydrophobe content on drug solubilization and cytotoxicity

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Polymeric micelles (PMs) of block copolymers have been extensively used as drug delivery vehicle in recent times and polyethylene glycol (PEG) is being used as surface modification for these delivery systems, as PEG helps to escape from recognition by the reticuloendothelial system in the body. This work reports synthesis and characterization of three new PEG-based random copolymers poly(dodecyl methacrylate-co-polyethylene glycol methyl ether methacrylate), poly[DMA_x-co-mPEG_y] (where x:y = 1:2, 1:4, 1:6), with different hydrophobe (DMA) contents. These copolymers were observed to exhibit good surface activity in aqueous solution. The micelle formation by these random amphiphilic copolymers in aqueous medium was studied by steady-state fluorescence probe technique. The size and shape of the PMs were determined by use of light scattering and electron microscopic techniques, respectively. The aqueous solutions of all three copolymers exhibit temperature-induced phase transition above a critical temperature (LCST) forming a stable emulsion. The LCST value of the micellar solution was observed to increase with the increase of mPEG content of the copolymer, but decrease with the increase of polymer concentration. The PMs were successfully used to solubilize hydrophobic anticancer drug, (S)-(+)-camptothecin (CPT); the solubilization capacity of the PMs was observed to increase with the decrease of hydrophobe content (or with the substantial increase of hydrophile content) in these copolymers. However, all three copolymers have the potential to protect the labile lactone ring of CPT from hydrolysis. Though all three copolymers showed a considerable release of the guest at acidic pH, but the polymer having maximum hydrophile content showed maximum guest release. Furthermore, the PMs of all three copolymers were observed to be easily permeable to the cancer cell membrane. In the permissible concentration regime, the polymers were found to be nontoxic and hemocompatible, but the cell viability as well as hemocompatibility was observed to deteriorate with the increase of DMA content in the copolymers.

1. Introduction

Improvement of the therapeutic efficacy of drug molecule by developing a better delivery vehicle, rather than the drug molecule itself, is an emerging field in modern medication process. Polymeric nanostructures (polymeric micelles,¹⁻³ vesicles,⁴ nanoparticles,⁵ nanotubes,^{6, 7} etc.) have drawn a tremendous interest as drug delivery carrier due to their several advantageous properties like large surface area, various functionalities to conjugate drug or receptor, stimuli sensitivity, easy synthesis and low cost. Polymeric micelles (PMs) are one of the first self-assembled nanostructures used in drug delivery in early 1990.¹ Since then PMs have been successfully used as promising drug delivery systems (DDS) for chemotherapy because of their nano-range sizes, in vivo stability and structures similar to those of some viruses and lipoproteins that are natural carriers in biological systems.⁸⁻¹⁰ Since PMs are composed of two distinct domains, a hydrophobic core and a hydrophilic corona, they can protect fragile molecules in their hydrophobic core from being destroyed by environmental conditions and allow them to remain unrecognized in circulation. Due to the encapsulation within PMs, drug molecules can have prolonged circulation lifetime and do not cause any embolization of blood vessels.^{11, 12} This facilitates their extravasations at leaky capillaries, known as the “enhanced permeability and retention (EPR) effect” and

thereby leads to a passive accumulation in tumor tissues.^{13, 14} Also with the increase of the molecular weight of the polymers/PMs, the in vivo stability is enhanced and as a consequence, the accumulation tendency at the tumor site gradually increases.¹⁵⁻¹⁷ Most of these polymers contain poly(ethylene glycol) (PEG) as hydrophilic group, because PEGylation not only increases aqueous solubility and biocompatibility, but also it can prevent PMs from getting trapped by the reticuloendothelial system (RES), such as liver and spleen.¹⁷⁻²⁰

As drug delivery vehicle, block copolymers²¹ as well as dendrimers²² rather than the random copolymers have been investigated extensively due to their unique and excellent associating behavior with well-defined structures. But unlike block copolymers random copolymers can be synthesized very easily and their properties also can be regulated with an appropriate design of the copolymers.^{15, 23-26} We along with others have shown that amphiphilic random copolymers have a strong tendency to form thermodynamically stable micelles in water above a very low critical aggregation concentration (CAC).^{14, 27-31} The nature of aggregation (inter-polymer or intra-polymer) depends not only on the hydrophile-lipophile balance (HLB) but also on the linkage group (ester or amide), nature of the hydrophobe and molecular weight.^{23, 32} Therefore, exact choice and balance of the hydrophobe in the preparation of

random amphiphilic copolymers is crucial to maintain and regulate their properties. In addition, this kind of polymeric nano-carriers with sensitivity to external stimuli have actually broadened their potential applications ranging from drug delivery^{33, 34} to personal care products³⁵ to gene transfection.³⁶ Depending on the nature of stimulus (light,^{37, 38} pH,³⁹⁻⁴² temperature,^{39, 41} redox reaction¹⁴ etc.), the encapsulated guest molecule can be triggered to the specific action site when it is needed. In the body, different organs, cells or tissues have different pH. Therefore, pH can be a very attractive stimulus for drug delivery. Amphiphilic copolymers with a pH-sensitive/cleavable polyester backbone have been widely used for cancer chemotherapy, because after the release of the guest molecule the whole polymer can be degraded into small biodegradable fragments.

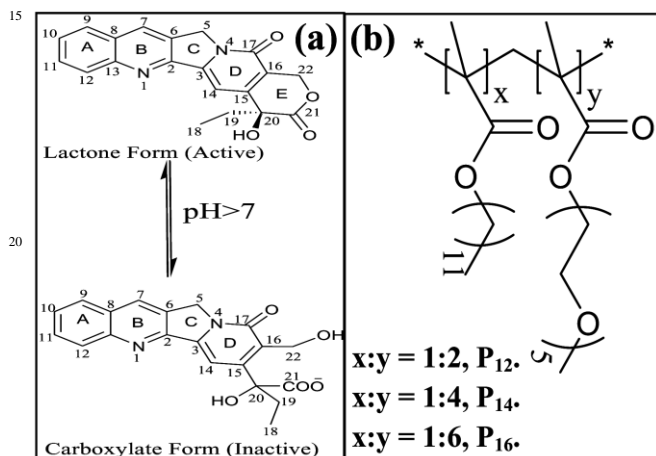


Figure 1. (a) Chemical structure of the two forms of CPT; (b) chemical structure of poly[DMA_x-co-mPEG_y] copolymers with x:y = 1:2 (P₁₂), 1:4 (P₁₄) and 1:6 (P₁₆).

The objective of this study is to prepare, characterize, and evaluate pH-sensitive PMs with mPEG chains forming hydrophilic shell as a part of surface modification technique. Since for injectable DDS, the PMs are highly effective,⁴³ we have made an attempt to improve the aqueous solubility of the hydrophobic anticancer drug camptothecin (CPT) by use of PMs of random copolymers as intravenous DDS. CPT is a potent anticancer drug, which generally exists in an active lactone form at pH below 5, but converts to the inactive carboxylate form at basic pH (see Figure 1(a)).^{44, 45} At physiological pH, most CPT molecules, though poorly water-soluble, exist in an inactive carboxylate form. The lactone form also shows higher diffusibility through the lipid bilayer than the carboxylate form.⁴⁶ In addition, CPT has a tendency to bind to human serum albumin (HSA) preferentially in the carboxylate form, resulting in the more rapid opening of the lactone ring.⁴⁷ In order to increase the bioavailability and antitumor effects of CPT, DDS must have the capability to protect the labile lactone ring as well as to increase the aqueous solubility. Keeping these facts in mind, we have designed and synthesized a new type of amphiphilic copolymer, poly(dodecyl methacrylate-co-polyethylene glycol methyl ether methacrylate), poly[DMA_x-co-mPEG_y] (Figure 1(b)) with three different feed ratios [x:y = 1:2 (P₁₂), 1:4 (P₁₄), and 1:6 (P₁₆)] of DMA and mPEG (M_n = 300). Although there are several PEG chains of various molecular weight with free -OH or -OMe group, we have chosen the mPEG of M_n = 300 to see the

properties of polymeric association when the length of core and corona-forming chains are similar. The choice of -OMe group over -OH was based on two reasons: first, with -OMe group there will be less chance of any non-specific interaction and second, due to the presence of -OMe group it would be easy to find peak in ¹H NMR spectra. The HLB was gradually changed in such a way that we can understand the effect of hydrophobe content on the various properties of the PMs. The PMs formed by these copolymers are expected to have hydrophobic core made of DMA within which CPT can be entrapped, and a hydrophilic shell of PEG chains that will reduce interaction with HSA. The self-assembly of these copolymers was studied using surface tension, fluorescence, dynamic light scattering (DLS) and transmission electron microscopic (TEM) techniques. We have evaluated their potential as DDS for CPT. We have also carried out in vitro evaluation of toxicity and blood compatibility of the copolymers. An attempt to demonstrate pH-triggered drug release has also been made.

2. Experimental section

2.1. Materials

Polyethylene glycol methyl ether methacrylate (mPEG; M_n = 300), dodecyl methacrylate (DMA), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole), chloroform-d, D₂O, DCI were purchased from Sigma-Aldrich (Bangalore, India) and were used without further purification. S-(+)-camptothecin (CPT) was purchased from Tokyo Chemical Industry, Japan. 2, 2'-Azo-bis-(isobutyronitrile) (AIBN), was purchased from sigma-Aldrich (Bangalore, India) and was used after recrystallization from acetone. All the fluorescent probes like pyrene (Py), 1, 6-diphenyl-1, 3, 5-hexatriene (DPH), N-phenyl-1-naphthyl amine (NPN) and coumarin 153 (C-153) were purchased from Sigma-Aldrich (Bangalore, India). Solvents like methanol (MeOH), dimethyl formamide (DMF), tetrahydrofuran (THF), ethyl acetate, petroleum ether, and chlorinated solvents were purchased from Merck and were distilled and purified before use. Milli Q (18.2MΩ) water was obtained from Millipore water purifier (Elix, Bangalore, India).

2.2. Synthesis of copolymers

The copolymers P₁₂, P₁₄ and P₁₆ were synthesized according to the Scheme S1 of "Electronic Supporting Information" (ESI) using different mole ratios of hydrophobic monomer DMA and hydrophilic monomer mPEG by reversible addition-fragmentation chain transfer (RAFT) polymerization technique. The chain transfer agent (CTA) 2-(2-cyanopropyl)-dithiobenzoate (CPDB, Figure S1) was employed as there are already many reports for successful utilization of CPDB for polymerization of acrylate based monomers.^{48, 49} The details of synthesis and chemical identification of CPDB are included under ESI. The reaction medium in dry DMF/THF mixture (4:1, v/v) was purged with N₂ gas for 30 minutes and after that CTA (1/500 mole ratio with respect to total reactant) and AIBN (1/5 mole ratio with respect to CTA) were added and then again N₂ gas was purged for 30 minutes and finally the reaction mass was heated at 70-75 °C for 46-48 h. The reaction was quenched by keeping the flask in chilled water. Then the solvents were evaporated under high vacuum to concentrate the reaction mass and water was added to it. The clear water part was taken for dialysis against distilled water for 4-5 days with intermittent change of water. Water was

then removed by lyophilization technique and the polymer was obtained as a viscous liquid. The chemical structures of the copolymers were confirmed by ^1H - and ^{13}C - NMR, and Fourier transform infrared (FT-IR) spectra. Representative ^1H -NMR spectra of the copolymer are presented in Figures S2-S4 of ESI.

2.3. Molecular weight determination

The average molecular weight (\bar{M}_w) and polydispersity index (PDI) of the copolymers were determined by gel permeation chromatography (GPC, Waters 2414, Refractive Index Detector, Waters 515 HPLC PUMP) using poly(methyl methacrylate) (PMMA) as molecular weight standard. DMF (HPLC grade) was employed as an eluent at a flow rate of 0.3 mL/min at 25 °C.

2.4. General instrumentation

^1H - and ^{13}C -NMR spectra were recorded on an AVANCE DAX-400 (Bruker, Sweden) 400 MHz NMR spectrometer using TMS as internal standard. The FTIR spectra were taken on a Perkin-Elmer (model 883 IR) spectrometer. Electronic spectra were recorded by use of a UV-vis spectrophotometer (Shimadzu, model UV-2450). For solid samples, KBr pellet was used as solvent. The solution pH was measured using a digital pH meter model 5652 (EC India Ltd., Calcutta) using a glass electrode.

2.5. Surface tension (ST) measurement

Du Nuöy ring detachment method was employed for surface tension (γ) measurements of the aqueous copolymer solutions at 25 °C using a semiautomatic surface tensiometer (model 3S, GBX, France). The platinum ring was used after proper cleaning with ethanol/HCl (1:1, v/v) solution and burning in oxidizing flame immediately before every use. For each polymer, the γ value of water was measured first and then an aliquot from stock of polymer solution was added to it gradually for the surface tension measurements at different polymer concentrations. Before measurement, each sample was equilibrated for 15-20 minutes. For each concentration, γ value was measured in triplicate and the mean value was noted.

2.6. Fluorescence measurements

Steady-state fluorescence. Microenvironment of the PMs in water was characterized by steady-state fluorescence spectroscopy using Py, DPH and NPN as fluorescent probes. A SPEX Fluorolog-3 spectrophotometer (450 WATT ILLUMINATOR, Model: FL-1039/40, HORIBA JOBIN YVON, EDISON, NJ, USA) was used for steady-state fluorescence measurements. CAC of the polymers in aqueous media was measured using Py probe. An aliquot of Py stock solution (1.0×10^{-3} M in MeOH) was taken into 5 mL volumetric flasks and the solvent was evaporated by a continuous stream of N_2 gas. Then polymer solutions in Milli-Q water in different concentrations were added to the volumetric flasks, making the final concentration of Py to 1.0×10^{-6} M. Before measurement, more than 12 h of incubation time was given to all the polymer solutions. The solutions containing Py probe were excited at 343 nm, and the emission spectra were recorded in the range of 350-600 nm. The excitation and emission slit widths were 5 and 2 nm, respectively.

The microviscosity and micropolarity of the PMs were estimated using DPH and NPN probes, respectively. Solutions containing NPN ($\text{ca. } 1 \times 10^{-6}$ M) were excited at 340 nm and

emission spectra were recorded in the wavelength range 350-550 nm on the SPEX Fluorolog-3 spectrophotometer. Both excitation and emission slit widths were fixed at 5 nm. DPH was used as a fluorescence anisotropy probe. Steady-state fluorescence anisotropy (r) of DPH was measured on a Perkin Elmer LS-55 spectrophotometer equipped with a thermostating cell holder and filter polarizer/analyzer assembly that used the L-format configuration. For anisotropy measurements excitation and emission slit widths were fixed at 2.5 nm and 5 nm, respectively. Each anisotropy measurement was repeated at least six times and the mean value of r was noted. The temperature of the samples was controlled using the water jacketed magnetically stirred cell holder in the spectrometer connected to Thermo Neslab RTE-7 circulating water bath that enabled temperature control within ± 0.1 °C.

Time-resolved fluorescence. Fluorescence lifetime of the DPH probe in polymer solution was measured by time-correlated single-photon counting (TCSPC) technique using a nano-second diode laser at $\lambda = 380$ nm (EasyLife) as light source. The fluorescence decay kinetics of DPH was recorded at the emission wavelength of 450 nm. The data analysis was performed using the software provided by the supplier. The decay curves were fit to double exponential decay function. The goodness of the fit was judged by the randomness of residual plot as well as by the χ^2 value (0.8-1.2).

2.7. Dynamic light scattering (DLS)

The hydrodynamic diameter (d_H) of the PMs in aqueous media was measured by conventional DLS technique using Malvern Nano ZS instrument employing a 4 mW He-Ne laser ($\lambda = 632.8$ nm). All the scattering photons were collected at a 173° scattering angle. The temperature was set to 25 °C and before every measurement each polymer solution was filtered through 0.45 μm (Millipore Millex syringe filter) filter paper. The software provided by the supplier calculated the hydrodynamic size using Stokes-Einstein equation.

2.8. Transmission electron microscopy (TEM)

A high resolution transmission electron microscope (HRTEM, TECNAI G2-20S TWIN, Japan) operating at an accelerating voltage of 200 kV at room temperature was employed to take micrographs of the polymers solutions at a known concentration above their CAC value. A 5 μL aqueous polymer solution was drop cast on a carbon-coated copper grid (400 mesh size) and it was kept in vacuum desiccators overnight for drying.

2.9. Solubilization study

Solvent evaporation method^{33, 50} was used to solubilize CPT. Exactly same amount of the drug with a different amount of polymers (required for various concentrations) were dissolved in 2 ml of CHCl_3 and sonicated for 5 min in a bath type sonicator (Bandelin, Sonorex, ultrasonic frequency 35 kHz). Then the CHCl_3 solution was added drop wise into 10 mL of Milli Q water in vigorously stirring condition. After complete evaporation of CHCl_3 from this heterogeneous mixture by simple stirring, the solution was left stirring for another 24 h in dark to ensure the maximum solubilization of CPT. The supernatant was then filtered using Millipore Millex filter (0.45 μm pore diameter) to remove the insoluble drug, if any. A measured volume of the filtrate was diluted with MeOH to measure the absorbance value

at a wavelength of 360 nm using the previously recorded calibration curve. The copolymer solutions without any drug at the same dilution were used as reference.

2.10. Hemolytic assay

Hemolytic assay was performed following the standard protocol.⁵¹ Briefly, red blood cells (RBC) were collected from human blood by centrifugation at 3000 rpm for 10 min at room temperature and were washed 4 times with 150 mM NaCl solution followed by a suspension in PBS (pH 7.4) to a final cell concentration of 5×10^8 RBC/mL. For making the 1 mL sample solution, different amount of polymer (in PBS, pH 7.4) was added into 200 μ L of the above RBC suspension and the final volume was made up by PBS. As positive and negative control, RBC cells were suspended in triton X-100 (1%, w/v) and PBS, respectively. All the samples were incubated for 60 min at 37 $^{\circ}$ C with an intermittent mixing followed by centrifugation at 12,000 rpm for 5 min. The absorbance of the supernatant solutions was taken at 541 nm by ELISA reader (Biorad, USA) using PBS as the blank. Each experiment was performed in triplicate.

2.11. Cell viability assay

The cell viability assay was performed to following standard protocol with some modifications.⁵⁰ HeLa cells (cervical cancer cell line) and L929 (normal cell line) were cultured in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% fetal bovine serum (FBS), antibiotics solution containing penicillin (100 units/mL), streptomycin (0.1 mg/mL), and amphotericin B (0.25 μ g/mL). The cells were maintained at 37 $^{\circ}$ C in T-25 flasks in a humidified incubator (5% CO₂) with a feeding cycle of 48 h. The confluent cell monolayer was trypsinized (0.25% Trypsin + 0.1% EDTA) and cells were harvested by centrifugation.

In an incomplete DMEM media (pH 7.4), the copolymers were dissolved and then filtered through 0.2 μ m polycarbonate filter after 12 h of incubation. Cell suspensions were seeded at 3×10^3 cells/well in 100 μ L of complete DMEM in a 96-well plate. The cells were allowed to adhere and were grown for 24 h at 37 $^{\circ}$ C in an incubator. The medium was aspirated and replaced with 100 μ L of fresh medium containing control and polymers with the desired concentrations. After 24 h of incubation with the polymers, the medium was removed and cells were washed thrice with sterile PBS. Cell viability was performed using a conventional MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) dye reduction assay. 100 μ L of MTT reagent (5 mg/mL in PBS) was added to each well and incubated for 3 h. MTT reagent mixture was gently removed and 200 μ L of DMSO was added into each well. The developed formazan dye was measured spectrophotometrically at 540 nm. This experiment was performed in triplicate. The cell viability for these polymers was expressed as percentage with respect to the untreated control cells. The following formula was applied to calculate cell viability

$$\text{Cell viability (\%)} = \left(\frac{\text{Mean of absorbance value of treated cells}}{\text{Mean of absorbance value of untreated control cells}} \right) \times 100.$$

2.12. Cellular uptake

Confocal fluorescence microscopic images were taken using C-153 as fluorescent probe (excitation at 422 nm and emission at 510 nm) to confirm whether these polymers can cross the cell

membrane.⁵² A stock solution (1.0×10^{-3} M) of C-153 was prepared in MeOH and an aliquot was added into 5-mL volumetric flasks so that final concentration of C-153 becomes ca. 1.0×10^{-6} M. Then MeOH was evaporated by a stream of N₂ gas and polymer solutions (in PBS, pH 7.4) of different concentrations were prepared in these volumetric flasks following usual method. After 12 h of incubation in dark at room temperature each sample was dialyzed against PBS solution (pH 7.4) for 2-3 h using biodialyser to completely remove the insoluble dye. HeLa (cervical cancer cells) cells of mid log phase growth were seeded in a 24 well flat bottom plate at a concentration of 3×10^3 /well and were left to grow for 24 h at 37 $^{\circ}$ C in a CO₂ environment. Then the culture media was replaced with polymers solutions containing C-153. After 3 h of incubation at 37 $^{\circ}$ C, sample solution was aspirated and wells were washed gently 3-4 times with PBS and were finally suspended in 200 μ L of PBS before taking the images by Olympus confocal microscope (FV1000, Olympus).

3. Results and Discussion

3.1. Molecular characterization of copolymers

Polymerization was confirmed by the disappearance of vinylic proton signals in the ¹H-NMR spectrum (Figure S2-4, ESI). The DMA/mPEG mole ratio in each copolymer was determined using respective ¹H-NMR spectrum. The average molecular weights (\bar{M}_w) of the polymers, P₁₂, P₁₄, and P₁₆ as obtained from GPC were 35279, 36728, and 45611, respectively. The relatively low PDI values of P₁₂ (1.26), P₁₄ (1.31), and P₁₆ (1.37) suggest a narrow molecular weight distribution for each copolymer. The chromatograph (Figure S5, ESI) clearly reveals that all three polymers have molecular weight in a very close range in spite of having different DMA/mPEG mole ratios. As observed, the molecular weight increased with the increase of overall mPEG content in the polymer backbone and with a gradual increase of PDI value. Thus the copolymer P₁₆ has the highest average molecular weight among all the polymers.

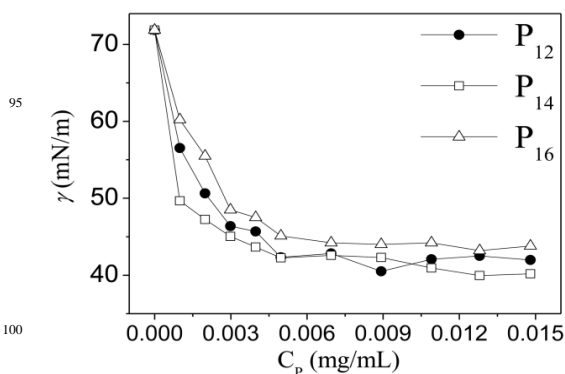


Figure 2. Variation of surface tension (γ) of water with the concentration (C_p , mg/mL) of copolymers, P₁₂, P₁₄ and P₁₆.

3.2. Surface activity

All three copolymers are expected to behave like surface-active agents as the polymer backbone contains both hydrophilic and hydrophobic groups. Therefore, we measured surface tension (γ) of water in the presence of various concentrations (C_p) of the copolymers at room temperature. The data are plotted in Figure 2. It is evident that all three polymers reduced the γ -value of water

to ca.45mN/m at a very low concentration, showing good surface activity. The data in Figure 2 suggest that the copolymer (P₁₆) having least hydrophobe (DMA) content is least surface active. Such a large reduction of surface tension of water proved amphiphilic nature of all the copolymers. The polymer concentration (C_p~7x10⁻³ mg/mL) corresponding to the minimum γ -value can be taken as the CAC value of the copolymer. Thus all the polymers have very low CAC value.

3.3. Self-assembly formation

All the copolymers are expected to self-assemble in water at a concentration greater than CAC (ca. 7x10⁻³ mg/mL) as they behave like amphiphilic molecules. The self-assembly formation of the copolymers was studied by steady-state fluorescence technique using NPN, pyrene, and DPH as probe molecules. These hydrophobic fluorescent probes are known to be solubilized within the hydrophobic core of (micellar) aggregates.^{53, 54} Both NPN and DPH are weakly fluorescent in water, but fluorescence intensity increases in non-polar medium or within hydrophobic core of the micelles.⁵⁴ The fluorescence spectrum of NPN in the presence of polymer exhibits a large blue shift (Figure S6) in addition to intensity rise (Figure 3(a)), indicating existence of hydrophobic domains, the formation of which is concentration dependent and therefore can be attributed as micelles.⁵⁴ For determination of CAC values of the copolymers, the relative fluorescence intensities (F/F₀), where F and F₀ are respectively the fluorescence intensities in the presence and absence of polymer) of NPN at different polymer concentrations were measured. The data are plotted against C_p in Figure 3(b). The plots show that for all three copolymers, F/F₀ is very low and is independent of C_p in dilute solutions, but they exhibit a sharp rise above a critical concentration. This observation leads to the conclusion that the aggregation started above this critical concentration through the inter-chain aggregation and no unimer micelles are formed. The C_p value corresponding to the onset of intensity rise was taken as the CAC value. It is observed that the CAC values of all three polymers are nearly equal to 5x10⁻³ mg/mL to that obtained from ST method.

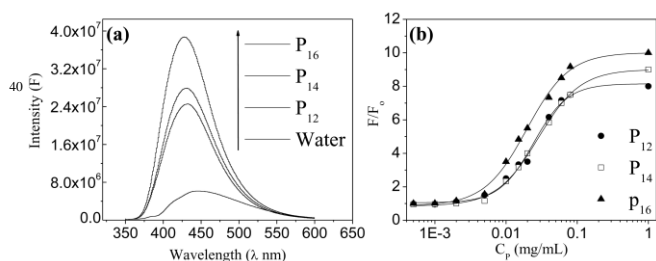


Figure 3. (a) Fluorescence spectra of NPN in pure water and in the presence of copolymers (C_p= 0.02 mg/mL) at 25 °C; (b) Plots of relative fluorescence intensity (F/F₀) of NPN versus polymer concentration (C_p) in water at 25 °C.

3.4. Hydrodynamic size of the PMs

Hydrodynamic size of the PMs is an important parameter for drug delivery application, because nano-sized micelles have a low tendency of rapid clearance from the circulation by phagocytosis in post intravenous administration than the microparticle and also nanoparticle shows efficient EPR effect and minimal renal excretion for passive tumor targeting.⁵⁵ As because blood

capillaries in the body are 5–6 μm in diameter, so the size of administered particles into the bloodstream must be significantly smaller than 5 μm without the possibility of embolism. A size less than 100 nm with a modified hydrophilic surface is crucial for achieving the reduction of opsonization reactions.⁵⁶ Therefore, the hydrodynamic diameters of the PMs in aqueous solutions (1.0 mg/mL) of the copolymers were measured using DLS technique. The size distribution histograms are shown in Figure 4(a-c). It is observed that both polymer P₁₂ and P₁₄ show broad distributions with mean hydrodynamic diameters of ca. 65–80 nm in comparison to P₁₆ the PMs of which has mean hydrodynamic diameter of ca. 100 nm. This is consistent with the higher molecular weight of P₁₆. We also performed DLS measurements at various concentrations of all the three copolymers. The results are presented in Figure S7 (a, b, c) of ESI. The data suggest that the size distribution profiles become sharper with the increase of polymer concentration. Thus the PMs formed by these amphiphilic polymers are in acceptable range for drug delivery applications.

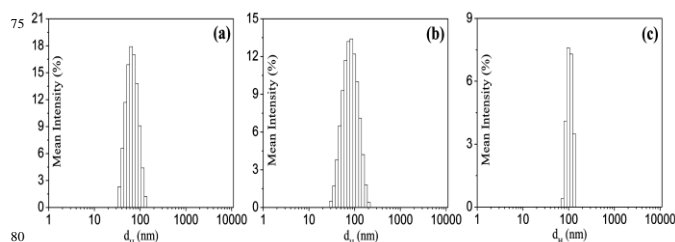


Figure 4. Hydrodynamic size distribution of the aggregates formed in aqueous solutions (1.0 mg/mL) of the copolymers (a) P₁₂, (b) P₁₄ and (c) P₁₆ at 25 °C.

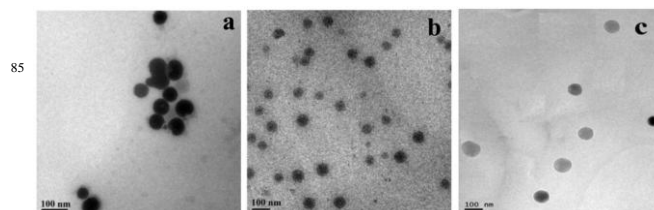


Figure 5. TEM images of the PMs in aqueous solution (1.0 mg/mL) of (a) P₁₂, (b) P₁₄ and (c) P₁₆ at 25 °C.

3.5. Shape of the PMs

The shape of the PMs was visualized by TEM. The micrographs (Figure 5(a-c)) of the copolymer solutions (1.0mg/mL) clearly reveal the presence of spherical particles (micelles) of diameter in the range of 50-100 nm with a reasonably narrow size distribution. These results closely similar to those obtained from DLS measurements.

3.6. Micropolarity of the PMs

In the Py emission spectrum among the five vibronic peaks, the intensity ratio of the first and the third vibronic peak (I₁/I₃) is known to be very sensitive to the polarity of medium and therefore is used as an index of solvent polarity.^{57, 58} The fluorescence spectra of Py were recorded in the presence of different concentrations of each of the polymers. The I₁/I₃ ratio measured at different polymer concentrations are plotted in Figure 6. Representative spectra are shown as inset of Figure 6. One can easily see that below a critical concentration (equal to

CAC), the I_1/I_3 ratio is close to the value in pure water (1.8), but it decreases nonlinearly above CAC with the increase in polymer concentration, reaching minimum at about 0.1 mg/mL. The CAC values (4×10^{-3} - 7×10^{-3} mg/mL) as obtained from the inflection point of the plots are similar to the values obtained from fluorescence titrations using NPN probe. Thus the accuracy of the determined CAC values of the polymeric amphiphiles in aqueous media is further confirmed. The initial concentration-independent plateau at low concentrations confirms inter-molecular association of the polymers. In general, there is always a tendency to form intra-polymer aggregates by this type of amphiphilic random copolymers.⁵⁹ But because of low PDI values (i.e., greater homogeneity of macromolecules) and probably not having proper \bar{M}_w and enough hydrophobe content in the polymer backbone, only inter-polymer association occurs.^{23, 59} It should also be noted that ester linkage always helps formation of inter-polymer aggregates.²³ The minimum value of I_1/I_3 ratio (~1.2) of Py, for all the polymers indicates that the polarity of the microenvironment is similar to THF-like organic solvents.⁵³

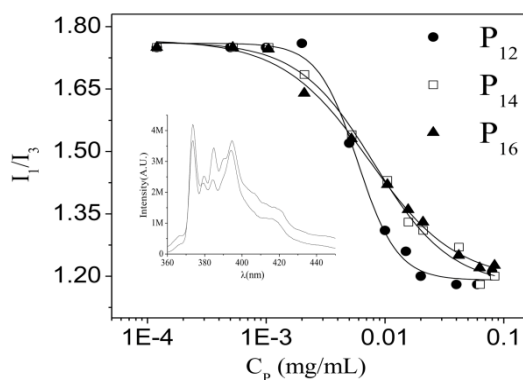


Figure 6. Plots of intensity ratio (I_1/I_3) of Py fluorescence spectrum versus copolymer concentration (C_p). Inset: Fluorescence emission spectra of Py showing I_1 and I_3 bands in water and in the presence of polymer.

3.7. Microviscosity of the PM

Steady-state fluorescence anisotropy (r) of DPH probe was measured in the presence of different concentrations of the polymers in order to determine rigidity of the microenvironment of the PMs. The plots of variation of r with the C_p are depicted in Figure S8 (ESI), which shows that the r -value for the polymeric micelles of the copolymers is quite high, indicating very rigid (i.e. viscous) microenvironment.^{60, 61} This is expected because the long fatty acid chains that are covalently linked to the polymer backbone, make the polymer less mobile. This may be the reason why the copolymer having comparatively higher hydrophobe content (P_{12}) showed higher r -value at the same polymer concentration. The microviscosity (η_m) of the PMs was determined using r -value and fluorescence lifetime data of DPH probe according to the Stokes-Einstein-Debye (SED) equation:⁶²

$$\eta_m = k_B T \tau_f / [v_h(r_o/r - 1)] \quad (1)$$

where τ_f is the fluorescence lifetime of DPH probe, v_h is the hydrodynamic volume of DPH (313 \AA^3) molecule, $r_o (= 0.362)$ and r are the fluorescence anisotropy values of DPH in a highly viscous medium and in the presence of polymer, respectively, k_B

is the Boltzmann constant, and T is absolute temperature. The data are collected in Table S1 (ESI). The η_m values for the copolymers P_{12} (175 mPa s), P_{14} (141 mPa s), and P_{16} (104 mPa s) were observed to be quite high in comparison to micelles of neutral surfactants, e.g. Triton-X100 (39.81 mPa s).⁶² The data also show that as the PEG-content increases the η_m value decreases. This is because of the lack of hydrophobic interaction among the DMA chains forming the core of the polymeric micelles.

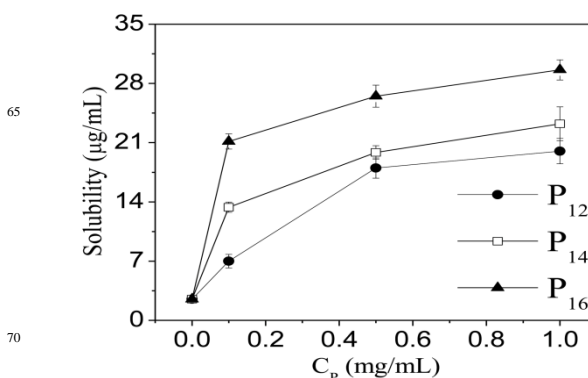


Figure 7. Plots of solubility ($\mu\text{g/mL}$) of CPT drug versus copolymer concentration (C_p) in water at 25°C .

3.8. Solubilization of hydrophobic drugs

The I_1/I_3 and η_m data of the PMs of P_{12} , P_{14} and P_{16} copolymers suggest that they are capable of solubilizing lipophilic molecules within the hydrophobic core. Therefore, we studied aqueous solubility of the poorly water-soluble drug CPT in the presence of different concentrations of the copolymers at room temperature. The results are presented in Figure 7. The plots in Figure 7 show that the solubility of CPT in water increases with the gradual increase of polymer concentration due to physical entrapment of the drug within the hydrophobic core of PMs. Though all the polymers can be used as a good sequestering agent for CPT, but P_{16} showed highest drug loading (29.6 mg/g or 2.96% w/w) in comparison to those of P_{14} (23.2 mg/g or ca. 2.32% w/w) and P_{12} (20 mg/g or 2.0% w/w). This is surprising because according to common belief PMs of polymers with higher hydrophobe content should have low micropolarity and consequently, they should solubilize hydrophobic drugs more. The lower solubility of CPT in the micelles of the P_{12} and P_{14} in comparison to the P_{16} is due to their low permeability. Also in comparison to P_{14} and P_{12} the molecular weight of P_{16} is quite high. However, the drug loading capacity and entrapment efficiency (Table S2, ESI) of the PMs of the copolymer are reasonably good. However, the drug loading capacity of these copolymers is better than those of polymer-drug conjugates. Indeed, the CPT loading by its conjugate to a PEG with a molecular weight of 40 kDa was only 0.86–1.72% (w/w).^{63, 64} So, these PMs can be used easily for a passive targeting of any kind of hydrophobic drug. It should also be noted that the fluorescence spectra of CPT molecule (Figure S9a-c, ESI) when solubilized within the PMs match with the fluorescence spectrum of its lactone form, suggesting solubilization of the active form of the drug molecule.⁶⁵ Further, it is observed that the intensity of fluorescence spectra of CPT-loaded PMs decreased with the increase of polymer concentration. This occurrence of concentration quenching of CPT fluorescence also strongly supports solubilization of more than one CPT molecule within each PMs. The fluorescence spectra of CPT at various pH and in the PMs of P_{12} , P_{14} and P_{16} are presented in

Figure S9a-d (ESI). Under basic condition (pH 12), the λ_{\max} of CPT fluorescence exhibit a red shift accompanied by the decrease of intensity in comparison to that at pH 7, indicating hydrolysis of the lactone ring.⁴⁵ At pH 7, in the presence of polymeric micelles, however, the spectrum remained unchanged over a long period of time showing protection of the hydrolytically labile lactone ring.

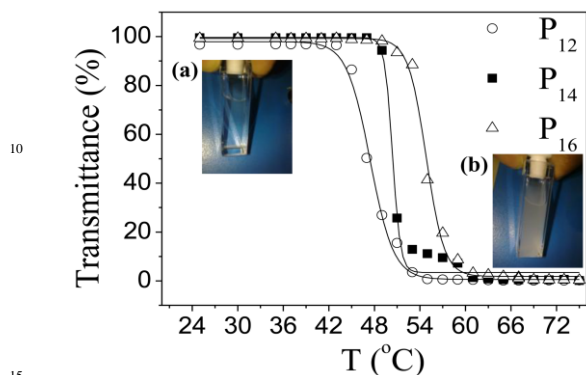


Figure 8. Plots of transmittance (%) as a function of temperature ($^{\circ}\text{C}$) for aqueous polymer solutions (1.0 mg/mL); Inset: photographs of the vials containing polymer solutions (a) below and (b) above the LCST, respectively.

3.9. Thermal stability of the PMs

The PEG-containing polymers are known to exhibit lower critical solution temperature (LCST) due to temperature-induced dehydration of the PEG chain (as a result of the rupture of the H-bonding between PEG and water).⁶⁶ Therefore we monitored percent transmittance (%T) of the aqueous solutions of the polymers with $C_p > \text{CAC}$ at different temperatures. The plot %T versus temperature for each of the polymer solutions with $C_p = 1.0 \text{ mg/mL}$ is shown in Figure 8. As can be seen, the transmittance falls sharply with the rise of temperature in all the cases. This is due to the appearance of turbidity which increases with the increase of temperature. The appearance of turbidity must be caused by the dehydration of PEG chain at elevated temperatures which is a common feature of many PEG-containing block copolymers.⁶⁶ The PMs with dehydrated PEG chains on the surface transform into nanosize oil droplets. The phenomenon was observed to be reversible that is the solution becomes clear when cooled back to room temperature. The phase transition temperature can be taken as the LCST. The LCST value was observed to increase in the order $P_{12} (46^{\circ}\text{C}) < P_{14} (50^{\circ}\text{C}) < P_{16} (55^{\circ}\text{C})$, i.e., in the order of increasing PEG content of the polymer. Similar experiments (Figure S10, ESI) using lower concentration ($C_p = 0.5 \text{ mg/mL}$) of the polymers, however, resulted in a slightly blue color dispersion. Interestingly, for any given polymer, the LCST value was observed to be higher than that of the corresponding value in 1.0 mg/mL polymer solution. However, LCST value of the 0.5 mg/mL polymer solutions increased in the same order ($P_{12} (55^{\circ}\text{C}) < P_{14} (60^{\circ}\text{C}) < P_{16} (66^{\circ}\text{C})$). The appearance of turbidity at higher temperatures occurs as a result of coalescence of the nanosize oil droplets to form larger droplets leading to the formation of a stable O/W emulsion. The formation of larger size particles on heating polymer solution was confirmed by the results of DLS measurements. As can be seen in Figure S11 (a, b, c) (ESI), with all the three polymers, the size distribution histograms become broader showing a gradual

increase of hydrodynamic diameter with the increase of temperature.

It is important to note that even at a higher concentration, the LCST values of the polymers are well above the physiological temperature (37°C). Since the LCST values of all three copolymer solutions are well above 37°C at this high concentration ($C_p = 1.0 \text{ mg/mL}$), the clouding of the solution will not occur at the body temperature. Consequently, premature release of any encapsulated pharmaceutically active agent at the body temperature can be ruled out.

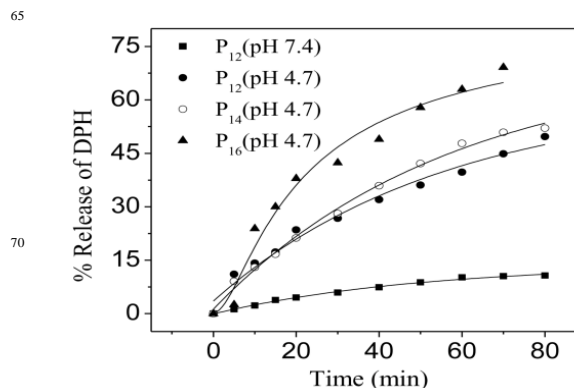


Figure 9. Cumulative (%) release of DPH as a function of time (min) in pH 4.7 at 37°C .

3.10. pH-Stability of the PMs

Since the hydrophobes (DMA) and hydrophiles (mPEG) are linked to the polymer backbone through ester linkage, the PMs can get easily hydrolyzed in acidic or alkaline medium. Thus depending upon the pH change, the encapsulated guest within the microenvironments of PMs can be released. Since the tumor cells have pH in the range 4.5-6.5,^{33, 39-41} the stability the PMs in acidic pH (pH 4.7) was measured by using intensity change of DPH fluorescence at the physiological temperature (37°C). For these measurements, all the polymers were employed at a C_p value equal to 1.0 mg/mL, as encapsulation efficiency of the PMs was observed to be highest at this concentration. These results are presented in Figure 9. Due to the hydrolysis of the ester bonds in the polymer branches, the fluorescence intensity of DPH probe decreases, indicating a gradual release of DPH molecules from the PMs. In almost 2 h period, 50% of the encapsulated guest molecule is released following a first order decay rate for P_{12} and P_{14} , whereas about 70% release was observed with P_{16} . The higher amount of release of DPH in the case of P_{16} is consistent with its high mPEG content which makes the PMs more permeable compared to those of P_{12} , P_{14} polymers. As a control experiment the release study at pH 7.4 was also performed, but no significant release of DPH could be observed within the period of measurement. This clearly suggests that the release of DPH is due to the hydrolysis of the ester bonds connecting DMA and PEG chains to the polymer backbone. To further confirm hydrolytic cleavage of the DMA and PEG chains, DLS measurements (Figures S12 (a, b, c), ESI) of the aqueous solution of P_{12} , P_{14} and P_{16} in pH 4.7 and 1.0 were performed after $\sim 14 \text{ h}$ of incubation at 37°C . The size distribution profiles clearly show formation of smaller size PMs along with larger size particles due to the polymethacrylic acid, indicating degradation of the mPEG as well as DMA chains from the polymeric backbone.

3.11 Hemocompatibility of the copolymers

Hemocompatibility for the polymers is a necessary requirement for intravenous (i.v.) drug delivery system. Hemocompatibility assay was performed by studying interaction of collected human RBC with the copolymers. Percentage of hemolysis of RBCs at various concentrations (0.15 to 2.0 mg/mL) of copolymers is plotted in Figure 10(a) and compared with the negative (RBC suspended in PBS) as well as positive (RBC suspended in 1% triton X-100) controls. Interestingly, all the polymers were found to be hemocompatible at a moderate to high concentrations. However, at elevated concentrations (2.0 mg/mL), P₁₂ clearly shows highest hemolysis (though still very low) among all the polymers due to its highest hydrophobe content.⁵⁰ Other two polymers, however, have shown more or less same hemocompatibility at all concentrations employed. The results indicate that perhaps P₁₄ and P₁₆ polymers have potential uses as intravenous DDS.

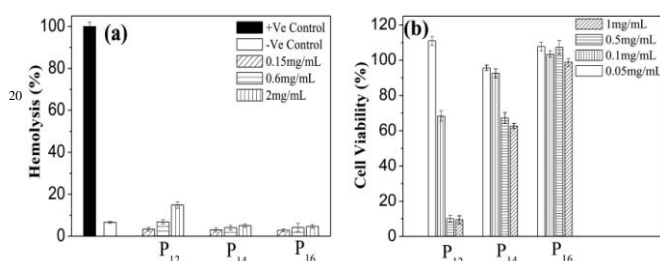


Figure 10. (a) Hemolysis assay of copolymers (0.15 to 2.0 mg/mL) at physiological pH (7.4); (b) cytotoxicity effects of the copolymers (0.05 to 1.0 mg/mL) on HeLa cells based on MTT assay after 24 h of incubation with respect to untreated control cells. Cell viability without polymer (Control) was considered as 100%. The data were presented as the mean \pm SD.

3.12. In vitro cytotoxicity assay

For the cell viability assay of the polymers, both cancer (HeLa) and normal (L929) cells were cultured and treated with different concentrations (0.05–1.0 mg/mL) of the polymers followed by the detection of the cytotoxicity using MTT assay. As observed from Figure 10(b), for P₁₆, cell viability remains very high even at very high concentrations (0.5 and 1.0 mg/mL). Though P₁₄ is still compatible at this concentration, P₁₂ is clearly cytotoxic. The results clearly suggest that with the increase of mPEG content in the polymer backbone the cell viability gradually increases and as a result P₁₆ is the least toxic among the three polymers. The same trend of hydrophilic effect on cell viability can also be observed with the normal cells (L929) (Figure S13, ESI).

3.13. In vitro cellular uptake of PMs

The confocal microscopy images showed a bright blue fluorescence (Figure 11a) when the HeLa cells were treated with C-153 encapsulated PMs of the different polymers (C_p 0.25 mg/mL). This blue fluorescence indicated that the cancer (HeLa) cells can easily uptake these PMs in their cellular compartment within a short incubation time of 3 h at 37 °C. However, the cellular compartmentalization was found to be independent of the mPEG content of the copolymers. Confocal micrographs of cell sections (Figure 11b) are also helpful to understand the intracellular localization of the probe.

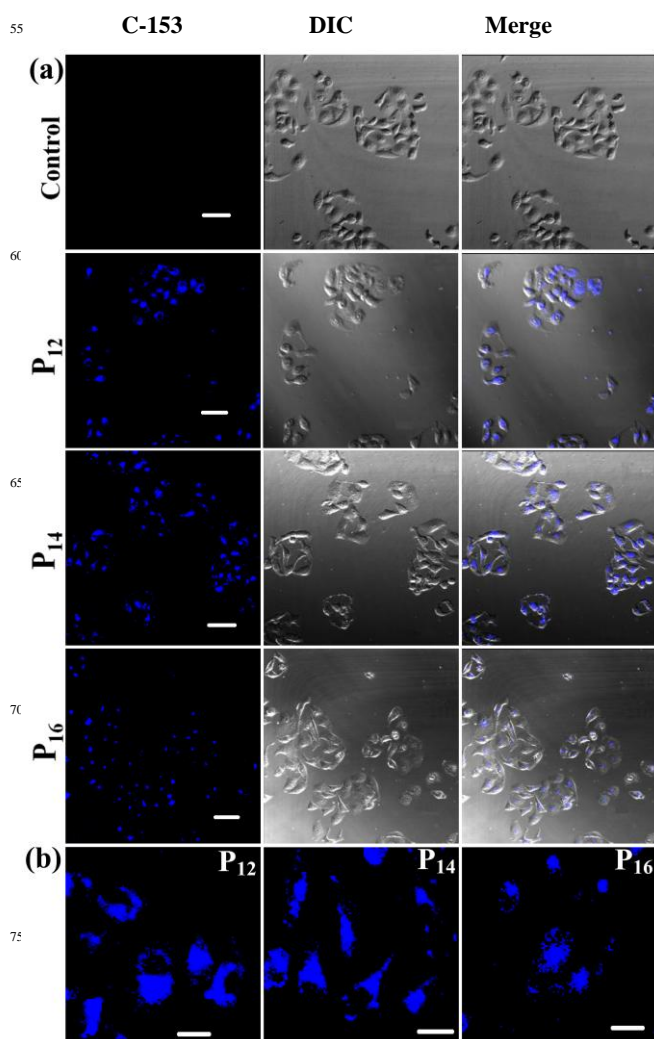


Figure 11. (a) Confocal micrographs of HeLa cells incubated with C-153 loaded PMs of copolymers. Untreated cells were taken as control; Scale bar is 60 μ m. (b) Confocal micrographs of cell sections showing internalization of different polymers. Scale bar is 20 μ m.

4. Conclusions

In summary, we have synthesized three mPEG based neutral amphiphilic random copolymers (P₁₂, P₁₄ and P₁₆) with different hydrophobe (DMA)/hydrophile (mPEG) mole ratios using RAFT copolymerization technique. All the copolymers were observed to form micelles in water above a very low critical concentration through inter-chain association. The polymeric micelles (PMs) thus formed were found to have similar spherical morphology. The PMs have mean hydrodynamic size in the range of 70–100 nm and have less polar and relatively rigid microenvironment compared to nonionic surfactant micelles. As a result, the PMs of the copolymers were observed to solubilize and protect poorly water-soluble anticancer drug, such as CPT within the less polar microenvironment. The aqueous solubility of CPT increased 8–12 times in the presence of 1.0 mg/mL polymer. However, the solubilization capacity of these copolymer micelles was observed to be less than those of corresponding cholesterol-containing copolymers.⁶⁷ Like cholesterol-containing copolymers, all three

copolymers also exhibit LCST and the LCST value increased with the increase of mPEG content as well as with dilution. Above LCST the PMs are transformed into partially dehydrated oil droplets thus producing stable O/W emulsion. However, all three copolymers were observed to be quite stable and remain water-soluble at a concentration as high as 1.0 mg/mL at the physiological temperature and pH. On the other hand, in acidic pH, the polymers undergo hydrolytic cleavage disrupting the PMs. This triggered release of encapsulated DPH, a hydrophobic model drug. In vitro biological studies have shown that all three copolymer micelles can enter into the cellular compartment and except P₁₂, they do not exhibit any significant cytotoxicity at C_p ≤ 1.0 mg/mL. The cell viability study suggested that the copolymers with higher hydrophobe content are more toxic. However, cytotoxicity of these copolymer micelles was observed to be comparable to those of corresponding cholesterol-containing copolymers.⁶⁷ These copolymers were also found to be hemocompatible up to a relatively high concentration (2 mg/mL). Based on these results it can be concluded that the copolymers P₁₄ and P₁₆ can be used as intravenous delivery systems in the viable concentration range.

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Notes and references

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† Electronic Supplementary Information (ESI) available: [Details of synthesis and chemical identification of polymers by 1H-NMR spectroscopy, surface tension plots, fluorescence spectra and lifetime data, and hydrodynamic size distributions are given]. See DOI: 10.1039/b000000x/

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