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Research Article

Poly(ester amide)-Poly(ethylene oxide) Graft Copolymers: Towards Micellar Drug Delivery Vehicles

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Micelles formed from amphiphilic copolymers are promising materials for the delivery of drug molecules, potentially leading to enhanced biological properties and efficacy. In this work, new poly(ester amide)-poly(ethylene oxide) (PEA-PEO) graft copolymers were synthesized and their assembly into micelles in aqueous solution was investigated. It was possible to tune the sizes of the micelles by varying the PEO content of the polymers and the method of micelle preparation. Under optimized conditions, it was possible to obtain micelles with diameters less than 100 nm as measured by dynamic light scattering and transmission electron microscopy. These micelles were demonstrated to encapsulate and release a model drug, Nile Red, and were nontoxic to HeLa cells as measured by an MTT assay. Overall, the properties of these micelles suggest that they are promising new materials for drug delivery systems.

1. Introduction

While many advances have been made in the development of therapeutics to treat human diseases over the past several decades, many drugs and drug candidates still possess undesirable properties. For example, the low aqueous solubilities of hydrophobic drugs such as paclitaxel present major hurdles for their administration as the use of excipients including Cremophor EL or ethanol for drug solubilization can result in undesirable side effects upon injection [1]. In addition, many anti-cancer drugs undergo rapid elimination from circulation and lack specificity for tumor cells, leading to decreased efficacy and severe side effects [2, 3]. Over the past couple of decades there has been significant interest in the development of drug delivery vehicles based on polymer assemblies such as spherical micelles [4–6], worm-like micelles [7], and vesicles [8–10]. These materials can enhance the solubilities of hydrophobic drugs through encapsulation. Furthermore, their nanoscale sizes can lead to significantly increased *in vivo* drug circulation times and tumor targeting via the enhanced permeability and retention effect [11]. Micelles have been one of the most widely investigated classes of polymer assemblies [4–6]. Thus far, a wide variety of amphiphilic copolymers such as poly(ethylene oxide)(PEO)-poly(ε-caprolactone) [12], PEO-poly(propylene oxide) [13], and PEO-poly(aspartic acid) [14] have been used for the preparation of drug delivery micelles, and some of these have been demonstrated to provide enhanced therapeutic efficacy *in vitro* and *in vivo*.

Poly(ester amide)s (PEAs) are a class of polymers comprising both ester and amide linkages in their backbones. The presence of ester moieties introduces the possibility for hydrolytic degradation and enzymatic degradation by mechanisms similar to those observed for polyesters, while the amide linkages provide opportunities for enzymatic degradation and also impart some of the desirable thermal and mechanical properties that are commonly derived from polyamides [15]. By tuning the chemical structures of

the monomers, their properties can be readily tuned [16–24]. Furthermore, their monomers can be selected from simple metabolic intermediates such as amino acids and dicarboxylic acids such that their degradation should result in nontoxic products. Another advantage is that by using amino acid monomers with functional handles, PEAs with pendant functional groups can be prepared [16, 25–32]. These pendant functional handles have potential utility for the conjugation of drug molecules in delivery systems, cell signaling molecules in tissue engineering scaffolds, or simply for tuning the properties of the polymers.

In recent work, PEAs have shown promise in several biomedical applications. For example, PEAs have been used to formulate drug-loaded microparticles [33, 34], coatings [35–37], and hydrogels [38–40]. They have also been investigated as gene carriers [41] and as tissue engineering scaffolds [26, 42–44]. However, to the best of our knowledge, there are no examples of micellar drug delivery systems based on PEAs. While PEAs are generally hydrophobic and water insoluble, as shown in Figure 1, it was envisaged that using our previously reported PEAs that contain reactive pendant groups [16, 25, 26], it would be possible to graft hydrophilic chains onto the PEA backbone, resulting in an amphiphilic graft copolymer. It was anticipated that the resulting amphiphilic copolymers might assemble into biodegradable micelles for drug delivery applications. While such amphiphilic polymers based on PEAs have not been previously reported, other amphiphilic graft copolymers have been demonstrated to form micelles in aqueous solution [45–49].

Described here are the synthesis and characterization of PEA-PEO graft copolymers, an investigation of their micellization, and initial work towards their application as drug carriers. A PEA backbone based on phenylalanine, lysine, 1,4-butanediol, and sebacic acid was selected based on its ease of synthesis by an interfacial method [26] and the presence of amine functional handles for conjugation of the hydrophilic blocks. PEO was chosen as the hydrophilic block for grafting due to its high water solubility, known biocompatibility in drug delivery applications, and its stealthy properties *in vivo* [50–53]. Several different loadings of PEO were explored, and the effects on the micelle sizes were investigated. The encapsulation and release of a model drug, Nile Red, were demonstrated, and experiments were performed to investigate the toxicity of the micelles.

2. Experimental

2.1. General Procedure and Methods. Polymer **1** was prepared as previously reported [26]. Unless noted otherwise, all other chemicals were purchased from commercial suppliers and used as received. Anhydrous CH_2Cl_2 was obtained by distillation over CaH_2. Infrared (IR) spectra were obtained using a Bruker Tensor 27 instrument as films from CH_2Cl_2 on NaCl plates. ^{1}H NMR spectra were obtained at 400 MHz on a Varian Mercury 400 Spectrometer. Chemical shifts are reported in ppm and are calibrated against residual solvent signals of $CDCl_3$ (δ 7.27). All coupling constants (J) are reported in Hz. Size exclusion chromatography (SEC) data were obtained using a Waters 2695 separations module equipped with a Waters 2414 refractive index detector (Waters Limited, Mississauga, ON, Canda) and two PLgel 5 μm mixed-D (300 mm × 7.5 mm) columns connected in series (Varian, Canada). Samples (5 mg/mL) dissolved in the eluent, which comprised 10 mM LiBr and 1 vol% triethylamine in *N*,*N*-dimethylformamide (DMF) at 85°C, were injected (100 μL) at a flow rate of 1 mL/min. Calibrations were performed using either polystyrene or PEO standards. Molecular weights are reported in grams/mol (g/mol). Preparative SEC was performed at flow rate of 3 mL/min using a system comprising a Waters 515 pump, a PLgel Prep (25 × 25 mm) guard column, a PLgel 10 μm 100 Å (600 × 25 mm) column, a PLgel 10 μm 500 Å (600 × 25 mm) column, and a Wyatt Optilab Rex Refractive Index detector. The eluent was composed of HPLC-grade DMF with 1 vol% triethylamine. Dynamic light scattering was performed on a ZetaSizer Nano instrument from Malvern. Dialysis was performed using Spectra/Por 6 regenerated cellulose membranes from Spectrum Laboratories with a molecular weight cut-off (MWCO) of either 12000–14000 g/mol or 25000 g/mol (Rancho Dominguez, CA, USA).

2.2. Synthesis of 4-Nitrophenyl-Carbonate-Activated PEO ***5***. PEO **2** (4.0 g, 2.0 mmol, 1.0 equiv.) and 4-nitrophenyl chloroformate (0.81 g, 4.0 mmol, 2.0 equiv.) were dissolved in CH_2Cl_2 (5 mL). To this solution, pyridine (0.90 mL, 8.0 mmol, 4.0 equiv.) was added dropwise, and the reaction was stirred overnight. The reaction mixture was then precipitated in cold diethyl ether (250 mL). The precipitate was recovered, dried *in vacuo*, dissolved in CH_2Cl_2, and washed twice in 1 M HCl. Yield: 75%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.29 (d, 2H, J = 9.0, Ar-*H* ortho to NO_2), 7.40 (d, 2H, J = 9.0, Ar-*H* meta to NO_2), 4.45–4.43 (–CH_2–O–C(O)–O–), 3.62 (br s, 449H, –O–CH_2–CH_2–O–), 3.36 (s, 3H,–O–CH_3). IR (cm^{-1}): 2883 (sp^3 C–H stretch), 1769 (C=O stretch), 1526 (CH_2 bend, C=C ring stretch), 1468 (CH_3 bend, C=C ring stretch), 1360 (symmetric Ar-NO_2 stretch), 1280 (Ar-O stretch), 1115 (asymmetric C–O–C stretch), 843 (out of plane C–H on Ar bending). SEC (relative to PEO standards): M_n = 1700, M_w = 1800, PDI = 1.06.

2.3. Synthesis of 4-Nitrophenyl-Carbonate-Activated PEO ***6***. The same procedure described above for the preparation of **5** was used except that PEO **3** having a MW of 5,000 g/mol (10 g, 2.0 mmol) was used as the starting material. Yield: 92%.^{1}H NMR (400 MHz, $CDCl_3$): δ 8.28 (d, 2H, J = 9.0, Ar-*H* ortho to NO_2), 7.39 (d, 2H, J = 9.0, Ar-*H* meta to NO_2), 4.46–4.44 (–CH_2–O–C(O)–O–), 3.65 (br s, 449 H, –O–CH_2–CH_2–O–), 3.39 (s, 3H,–O–CH_3). IR (cm^{-1}): 2880 (sp^3 C–H stretch), 1765 (C=O stretch), 1526 (CH_2 bend, C=C ring stretch), 1462 (CH_3 bend, C=C ring stretch), 1380 (symmetric Ar-NO_2 stretch), 1259 (Ar-O stretch), 1111 (asymmetric C–O–C stretch), 847 (out of plane C–H on Ar bending). SEC (relative to PEO standards): M_n = 4500, M_w = 4600, PDI = 1.08.

2.4. Synthesis of PEA-PEO Graft Copolymer ***7***. Polymer **1** (53 mg, 19 μmol of pendant amine, 1.0 equiv.), activated

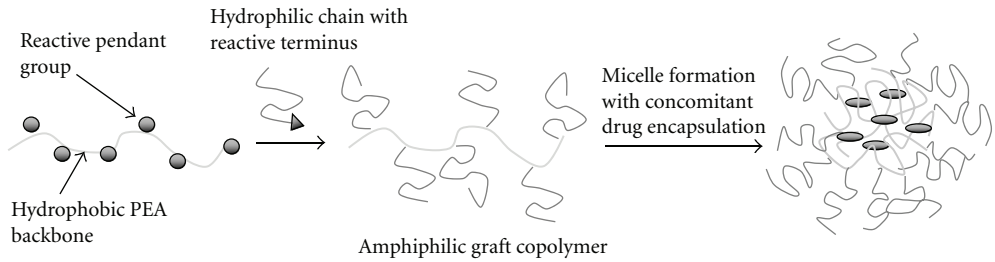


Figure 1: Schematic for the preparation of amphiphilic PEA graft copolymers and their assembly into micelles for drug delivery.

PEO **5** (44 mg, 22 μmol, 1.2 equiv.), and 4-(dimethylamino)pyridine (DMAP) (570 μg, 3.8 μmol, 0.20 equiv.) were added to a flame-dried flask under an argon atmosphere. CH_2Cl_2 (4 mL) was added to dissolve the solids. Upon dissolution, *N*,*N*-diisopropylethylamine (DIPEA) (6.7 μL, 38 μmol, 2.0 equiv.) was added dropwise. The reaction mixture was stirred overnight, and then the solvent was removed *in vacuo*. To remove small molecule byproducts, the product was dialyzed against DMF using a 25000 g/mol MWCO membrane. The solvent was removed *in vacuo*, and the off-white solid was separated from any uncoupled PEO through preparative SEC. The solvent was removed *in vacuo* yielding polymer **7**. Yield: 28%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.28–7.10 (m, 9H, Ph), 6.15–6.05 (m, 0.2H, –C(O)–NH–C_αH–$(CH_2)_4$–NH–C(O)–O–) 6.01 (d, 1.8H, J = 8.2, –C(O)–NH–C_αH–CH_2–Ph), 4.88–4.85 (m, 1.8, –C_αH–CH_2–Ph), 4.58–4.51 (m, 0.2H, –NH–C(O)–O–), 4.12–4.01 (m, 4H, –C(O)O–CH_2–), 3.65 (br s, 19H, –O–CH_2–CH_2–O–), 3.38 (s, 0.3H, –O–CH_3), 3.15–3.05 (m, 3.8H, –C_αH–CH_2–Ph), 2.21–2.12 (m, 4H, –NH–C(O)–CH_2–), 1.57–1.55 (m, 8H, –C(O)O–CH_2–CH_2–, –NH–C(O)–CH_2–CH_2–), 1.26–1.22 (m, 8.4H, –NH–C(O)–CH_2–CH_2–$(CH_2)_4$). IR (cm^{-1}): 3099 (N–H stretch), 3022 (sp^2 C–H stretch), 2880 (sp^3 C–H stretch), 1749 (C=O etser stretch), 1693 (C=O amide stretch), 1556 (N–H bending), 1527 (CH_2 bend, C=C ring stretch), 1468 (CH_3 bend, C=C ring stretch), 1429 (C–N stretch), 1107 (asymmetric C–O–C stretch), 992 (C–O stretch), 851 (symmetric C–O–C stretching), 832 (out of plane C–H on Ar bending), 758 (monosubstituted Ar C–H bending) 687 (monosubstituted Ar C–H bending). SEC (relative to PEO standards): M_n = 23300, M_w = 34700, PDI = 1.49.

*2.5. Synthesis of PEA-PEO Graft Copolymer **8**.* This polymer was prepared by the same procedure described above for the preparation of copolymer **7** except that 0.85 equivalents (relative to the number of pendant amines on polymer **1**) of PEO **6** were used. Yield: 25%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.26–7.05 (m, 9H, Ph), 5.95 (d, 1.8H, J = 7.4, –C(O)–NH–C_αH–CH_2–Ph), 4.86–4.79 (m, 1.8, –C_αH–CH_2–Ph), 4.51–4.42 (m, 0.2H, –NH–C(O)–O–), 4.12–3.97 (m, 4H, –C(O)O–CH_2–), 3.65 (br s, 26H,–O–CH_2–CH_2–O–), 3.37 (s, 0.2H, –O–CH_3), 3.09–3.00 (m, 3.8H, –C_αH–CH_2–Ph), 2.13–2.09 (m, 4H, –NH–C(O)–CH_2–), 1.55–1.50 (m, 8H, –C(O)O–CH_2–CH_2–, –NH–C(O)–CH_2–CH_2–), 1.23–1.19 (m, 8H, –NH–C(O)–CH_2–CH_2–$(CH_2)_4$). IR (cm^{-1}): 3100 (N–H stretch), 3030 (sp^2 C–H stretch), 2883 (sp^3 C–H stretch), 1745 (C=O etser stretch), 1690 (C=O amide stretch), 1550 (N–H bending), 1526 (CH_2 bend, C=C ring stretch), 1468 (CH_3 bend, C=C ring stretch), 1400 (C–N stretch), 1115 (asymmetric C–O–C stretch), 999 (C–O stretch), 850 (symmetric C–O–C stretching), 843 (out of plane C–H on Ar bending), 750 (monosubstituted Ar C–H bending), 690 (monosubstituted Ar C–H bending). SEC (relative to PEO standards): M_n = 29900, M_w = 46900, PDI = 1.57.

*2.6. Synthesis of PEA-PEO Graft Copolymer **9**.* This polymer was prepared by the same procedure described above for the preparation of copolymer **7** except that 1.2 equivalents (relative to the number of pendant amines on polymer **1**) of PEO **6** were used. Yield: 27%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.28–7.09 (m, 9H, Ph), 6.34–6.24 (br m, 0.2H, –C(O)–NH–C_αH–$(CH_2)_4$–NH–C(O)–O–) 6.00 (d, 1.8H, J = 7.6, –C(O)–NH–C_αH–CH_2–Ph), 4.88–4.84 (m, 1.8, –C_αH–CH_2–Ph), 4.59–4.48 (m, 0.2H, –NH–C(O)–O–), 4.12–4.01 (m, 4H, –C(O)O–CH_2–), 3.65 (br s, 45H, –O–CH_2–CH_2–O–), 3.37 (s, 0.3H, –O–CH_3), 3.12–3.03 (m, 3.8H, –C_αH–CH_2–Ph), 2.18–2.12 (m, 4H, –NH–C(O)–CH_2–), 1.57–1.52 (m, 8H, –C(O)O–CH_2–CH_2–, –NH–C(O)–CH_2–CH_2–), 1.28–1.22 (m, 8H, –NH–C(O)–CH_2–CH_2–$(CH_2)_4$). IR (cm^{-1}): 3110 (N–H stretch), 3032 (sp^2 C–H stretch), 2886 (sp^3 C–H stretch), 1755 (C=O ester stretch), 1696 (C=O amide stretch), 1551 (N–H bending), 1528 (CH_2 bend, C=C ring stretch), 1468 (CH_3 bend, C=C ring stetch), 1405 (C–N stretch), 1114 (assymetric C–O–C stretch), 979 (C–O stretch), 853 (symmetric C–O–C stretching), 843 (out of plane C–H on Ar bending), 737 (monosubstituted Ar C–H bending) 692 (monosubstituted Ar C–H bending). SEC (relative to PEO standards): M_n = 30500, M_w = 49700, PDI = 1.63.

2.7. Procedure for Micelle Formation. The PEA-PEO graft copolymer (2.0 mg) was dissolved in either 0.05, 0.6, or 0.8 mL of THF. The solution was stirred rapidly while distilled water was rapidly added to provide a final volume of 2 mL. THF was then removed by dialysis against distilled water using a Spectra/Por regenerated cellulose membrane with a MWCO of 12000–14000 g/mol.

*2.8. Determination of the Critical Aggregation Concentration for Copolymer **9**.* Micelles were prepared as described above

from copolymer **9**. Nile Red (0.94 mg, 3.0 μmol) was dissolved in 9 mL of CH_2Cl_2, and 0.1 mL of this solution was added to a series of 12 vials. The CH_2Cl_2 was removed under a stream of nitrogen. A series of concentrations of the micelle suspension ranging from 0.5 μg/mL to 1 mg/mL was prepared by dilution with pH 7.4, 100 mM phosphate buffer. The micelle suspensions were added to the vials containing Nile Red, and were allowed to equilibrate with stirring for 40 hours. The fluorescence spectra were obtained on a QM-4 SE spectrometer from Photon Technology International (PTI), equipped with double excitation and emission monochromators. An excitation wavelength of 550 nm was used for Nile Red and the emission spectra were recorded from 565 to 700 nm. The maximum emission intensity was recorded for each micelle concentration.

2.9. Transmission Electron Microscopy. The micelle suspension (prepared as described above, then diluted to 0.2 mg/mL) was placed on a Formvar/Carbon grid and was left to stand for 5 min. The excess solution was then blotted off using a piece of filter paper. The resulting sample was dried in air overnight before imaging. Imaging was performed using a Phillips CM10 microscope operating at 80 kV with a 40 μm aperture.

2.10. Encapsulation and Release of Nile Red. Micelles formed from copolymer **9** were prepared as described above except that Nile Red (0.5 mg, 1.6 μmol) was dissolved in the THF solution. After removal of the THF by dialysis, centrifugation (6000 rpm for 30 min) was used to remove any precipitated Nile Red. The micelle suspension was placed in a Slide-A-Lyzer dialysis cassette and kept at 37°C in either pH 7.4, 100 mM phosphate buffer or pH 5.0, 100 mM citric acid/phosphate buffer. The fluorescence spectrum of the micelle suspension from the cassette was obtained every hour (QM-4 SE spectrometer from Photon Technology International (PTI) as above). An excitation wavelength of 550 nm was used, and emission spectra were recorded from 565 to 700 nm. To correct for fluctuations in the fluorometer lamp intensity, the measurement at each time point was compared to that of a standard of Nile Red in THF that was covered in aluminum foil and kept in fridge.

2.11. MTT Assay. HeLa cells were cultured at 37°C and 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (Invitrogen). The cells were seeded into 88 wells of a 96-well plate (Nunclon TC treated) at a density of 2×10^3 cells per well in a final volume of 100 μL of DMEM containing 10% (v/v) serum and antibiotics (penicillin and streptomycin, 100 units/mL each). Cells were allowed to adhere for 24 hours at 37°C in a humidified incubator with 5% CO_2. After 24 hours the growth media were aspirated. Control cells were then grown in growth media alone while those subjected to the micelle suspension were incubated in two-fold decreasing concentrations from 2 mg/ml to 0.0039 mg/mL in growth media at each concentration. 8 replicates per concentration were performed. After 48 hours, the media were aspirated and then 100 μL of fresh media and 10 μL of MTT solution (5 mg/mL) were added to each well and incubated for another 4 hours. The media were aspirated and the formazan product was solubilized by addition of 50 μL of DMSO to each well. The absorbance of each well was measured at 540 nm using a plate reader (Tecan Safire), and after subtraction of the blank, the result was compared to that of the control cells that were not exposed to micelles in order to calculate the relative cell viability.

3. Results and Discussion

3.1. Polymer Synthesis and Characterization. PEA **1** (Figure 2) was prepared by an interfacial polycondensation method as previously reported [26]. This polymer was composed of sebacic acid, 1,4-butanediol, and an approximately 9 : 1 ratio of phenylalanine : lysine randomly incorporated. The resulting material had an weight average molecular weight (M_w) of 89600 g/mol and a polydispersity index (PDI) of 1.83, as measured by size exclusion chromatography (SEC) in DMF relative to polystyrene standards and a M_w of 32100 and PDI of 1.71 relative to PEO standards. It should be noted that the discrepancy in molecular weights (MWS) obtained by these different calibration methods can be attributed to the large difference in hydrodynamic volumes of PEO and polystyrene in DMF.

PEO with a methyl ether group at one terminus, an alcohol at the other terminus, and a MW of either 2000 g/mol (**2**) or 5000 g/mol (**3**) was activated by reaction with 4-nitrophenyl chloroformate (**4**) to form the 4-nitrophenyl-carbonate-activated polymers **5** and **6** as shown in Figure 2. Subsequently, these activated PEOs were reacted with PEA **1** in CH_2Cl_2 in the presence of 4-dimethylaminopyridine (DMAP) as a catalyst and *N*,*N*-diisopropylethylamine (DIPEA) as a base. In the case of the activated PEO **5**, 1.2 equivalents were used in the reaction relative to the number of pendant amines in the PEA, to provide copolymer **7**. For PEO **6** either 0.85 or 1.2 equivalents of PEO were used in the reaction, to provide copolymers **8** and **9**. This was done to obtain PEA-PEO copolymers with different PEO content and to determine the effect of the number of equivalents of PEO on the conjugation yield. Preliminary work indicated that increasing the number of PEO equivalents beyond 1.2 did not lead to significant increases in conjugation yield, so higher quantities of activated PEO were not investigated further.

Following the reaction, various purification methods were explored in order to remove the uncoupled PEO as well as other reaction byproducts such as 4-nitrophenol, DMAP, and DIPEA. Surprisingly, while successful in removing the low MW molecules, CH_2Cl_2/H_2O extractions or dialysis in water using molecular weight cut-offs as high as 50000 g/mol were unsuccessful in removing the free PEO, as a peak assigned to free PEO was still observed in the SEC trace of the product (Figure 3(a)). This can likely be attributed to dimerization of the unreacted PEO upon the breakdown of some 4-nitrophenyl carbonates, and the resulting difficultly in removing higher MW PEO by dialysis. A comprehensive study of the dialysis membrane cut-offs for the removal of

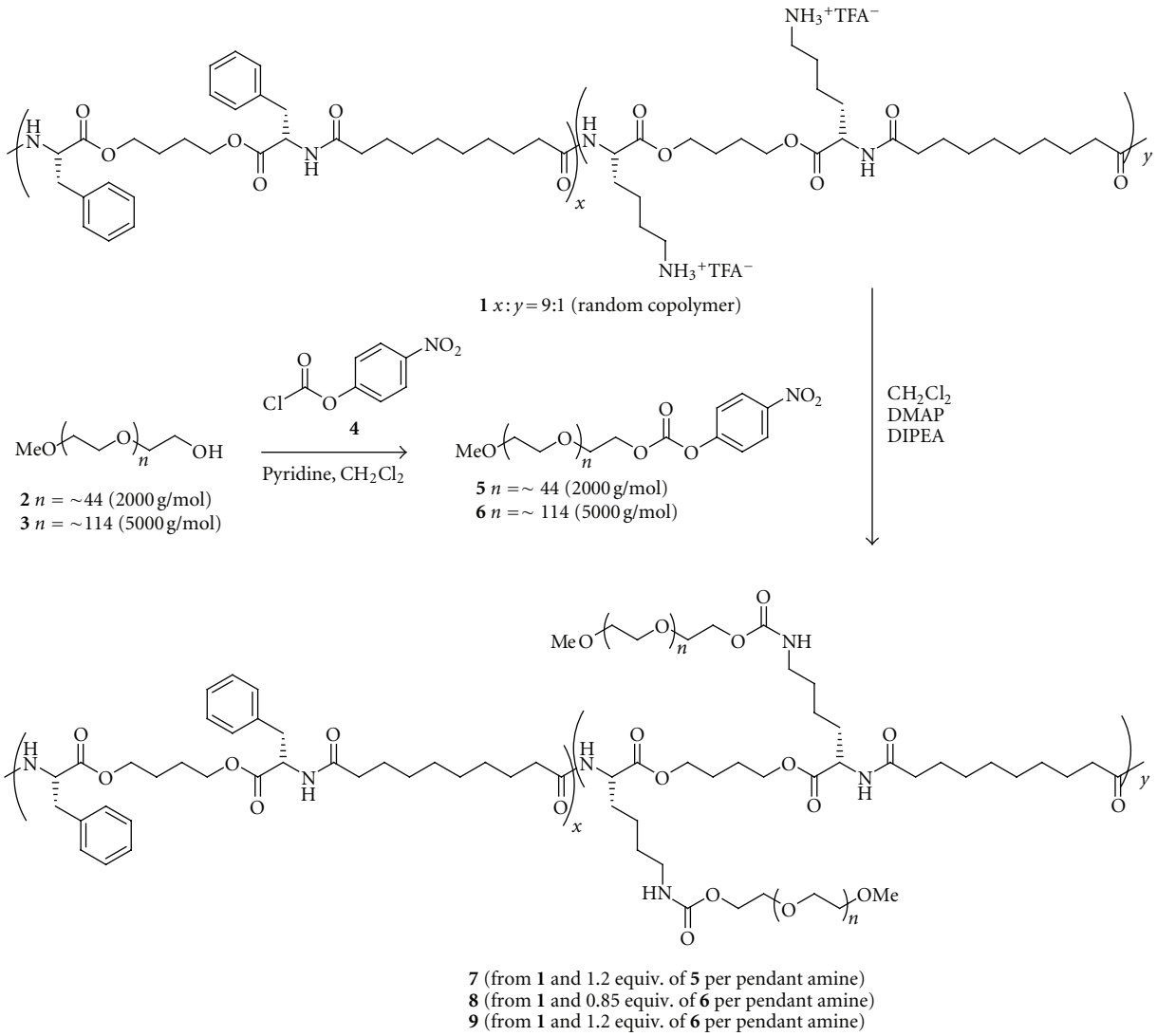


Figure 2: Synthesis of PEA-PEO graft copolymers **7–9.**

different MWs of PEOs is currently underway in our lab. However, in the meantime, preparative SEC was successful in removing the free PEO as shown in Figure 3(b).

Following the removal of free PEO, the degree of lysine amine functionalization was quantified by NMR spectroscopy. As shown in Table 1, using 1.2 equivalents of activated PEO, the degree of conjugation was approximately 50% for both the 2000 g/mol and 5000 g/mol PEO. Using only 0.85 equivalents of the 5000 g/mol PEO led to a somewhat lower degree of functionalization of only 29%. As expected, the resulting M_w did increase with the degree of coupling and with the MW of the PEO. It should be noted that these data are expected to be underestimates of the MW due to the branched nature of these polymers [54].

3.2. Preparation and Characterization of Micelles. Several different methods of micelle formation were investigated in preliminary work. Nanoprecipitation, solvent exchange, thin film hydration, and chloroform emulsion evaporation were all investigated for their abilities to form nanosized micelles with low polydispersities as measured by dynamic light scattering (DLS). It was found that nanoprecipitation best met these criteria and was therefore chosen as the method for subsequent work. In short, the copolymer was dissolved in tetrahydrofuran (THF), and then water was added with rapid stirring. Finally, THF was removed by dialysis against water.

The z-average micelle diameters and polydispersity indices are shown in Table 2, and representative DLS traces are shown in Figure 4. Initially, water was added to the THF solution of polymer such that the resulting solution contained 1 mg/mL of polymer in a 2.5 volume % solution of THF in water prior to dialysis. This led to micelles with z-average diameters of 123 nm for **7**, 114 nm for **8**, and 60 nm for **9**. This decrease in micelle size was expected as the PEO content increases from copolymers **7–9** because increased

Table 1: Characterization data for PEA-PEO graft copolymers **7–9**.

Polymer	Equivalents of activated PEO[a]	PEO MW (g/mol)	Degree of lysine functionalization[b]	M_w (g/mol)[c]	PDI[c]
7	1.2	2000	53%	34700	1.49
8	0.85	5000	29%	46900	1.57
9	1.2	5000	50%	49700	1.63

[a]During synthesis, relative to the number of pendant amine groups on PEA **1**.
[b]Based on ^{1}H NMR spectroscopy.
[c]Based on SEC.

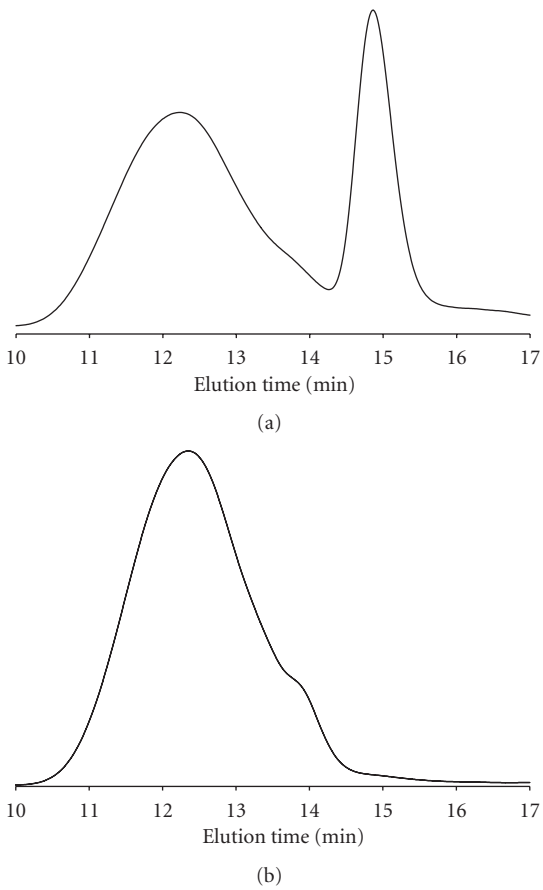


Figure 3: SEC chromatograms of copolymer **9** (a) prior to and (b) following purification by preparative SEC.

PEO content would be expected to reduce the intermolecular aggregation between the polymer chains, leading to smaller micelles that are composed of fewer polymer chains. To test this hypothesis, the critical aggregation concentration (CAC) of copolymer **9** was measured by Nile Red (Figure 5(a)) encapsulation. As shown in Figure 5(b), no CAC was detected and a linear relationship was observed between Nile Red fluorescence and copolymer concentration at concentrations down to 0.5 μg/mL, suggesting that the copolymer is capable of forming unimolecular micelles.

The micelle size was also found to be dependent on the method of micelle preparation. For example, the addition of water to the THF solution of polymer such that the content of the solution was 30 vol% THF prior to dialysis, while maintaining a copolymer concentration of 1 mg/mL led to a z-average micelle size of 252 nm for copolymer **7** and 143 nm for copolymer **9**. A 40 vol% THF solution led to a z-average size of 187 nm for copolymer **9**. This can likely be explained as follows. In THF both the PEA and PEO blocks are soluble, so the polymers exist in solution as individual chains. As water is added, the hydrophobic PEA block becomes insoluble and collapses to form the micelle core. If a sufficient amount of water is added rapidly by the initial injection prior to dialysis, the individual molecules or small aggregates are trapped as small micelles. On the other hand, if sufficient THF is present in the solution prior to dialysis, the micelles are not trapped and the slow removal of THF by dialysis allows time for the PEA blocks of multiple polymer chains to aggregate, forming larger multimolecular micelles. Thus the micelle size can be tuned by the amount of water added to the THF prior to dialysis. This could be advantageous if different micelle sizes are desired for different applications. Overall, it should also be noted that in all cases, the micelles had relatively low PDIs and very good batch-to-batch reproducibility, as indicated by the low standard deviations between batches (Table 2).

Based on the fact that materials with diameters <100 nm are generally considered ideal for *in vivo* applications as they can circulate in the blood without rapid removal by the reticuloendothelial system [55], copolymer **9** was selected from the 3 copolymers for subsequent work. To verify the micelle sizes that were measured by DLS, TEM measurements were also performed. As shown in Figure 6, micelles prepared using the 2.5% THF solution followed by dialysis had sizes on the order of 30 nm. This size reduction with respect to the DLS measurements is likely a result of the difference between the hydrated micelles measured in solution versus the micelles in the dry state measured by TEM. While these small micelles are of most interest for biomedical applications, those prepared from copolymer **9** from 40% THF solution were also imaged had sizes on the order of 100–200 nm. These results confirm that the sizes measured by DLS did reflect the relative sizes of the different micelle samples.

*3.3. Investigation of Micelles Formed from Copolymer **9**: Model Drug Encapsulation and Toxicity Evaluation.* In order to demonstrate that micelles formed from copolymer **9** have potential utility as drug delivery vehicles, the encapsulation and release of a model drug, Nile Red, was investigated. This is a hydrophobic dye molecule that exhibits significant fluorescence when incorporated into hydrophobic environments

Table 2: Characterization of micelles formed by copolymers **7–9** by DLS. z-average micelle diameters represent the means of the measurement data for 3 different batches of micelles.

Polymer	THF content of micelle suspension prior to dialysis (vol%)	z-average micelle diameter (nm)	Micelle PDI
7	2.5	123 ± 2	0.15 ± 0.001
7	30	252 ± 1	0.04 ± 0.03
8	2.5	114 ± 1	0.13 ± 0.01
9	2.5	60 ± 0.4	0.29 ± 0.01
9	30	143 ± 1	0.16 ± 0.02
9	40	187 ± 0.5	0.15 ± 0.01

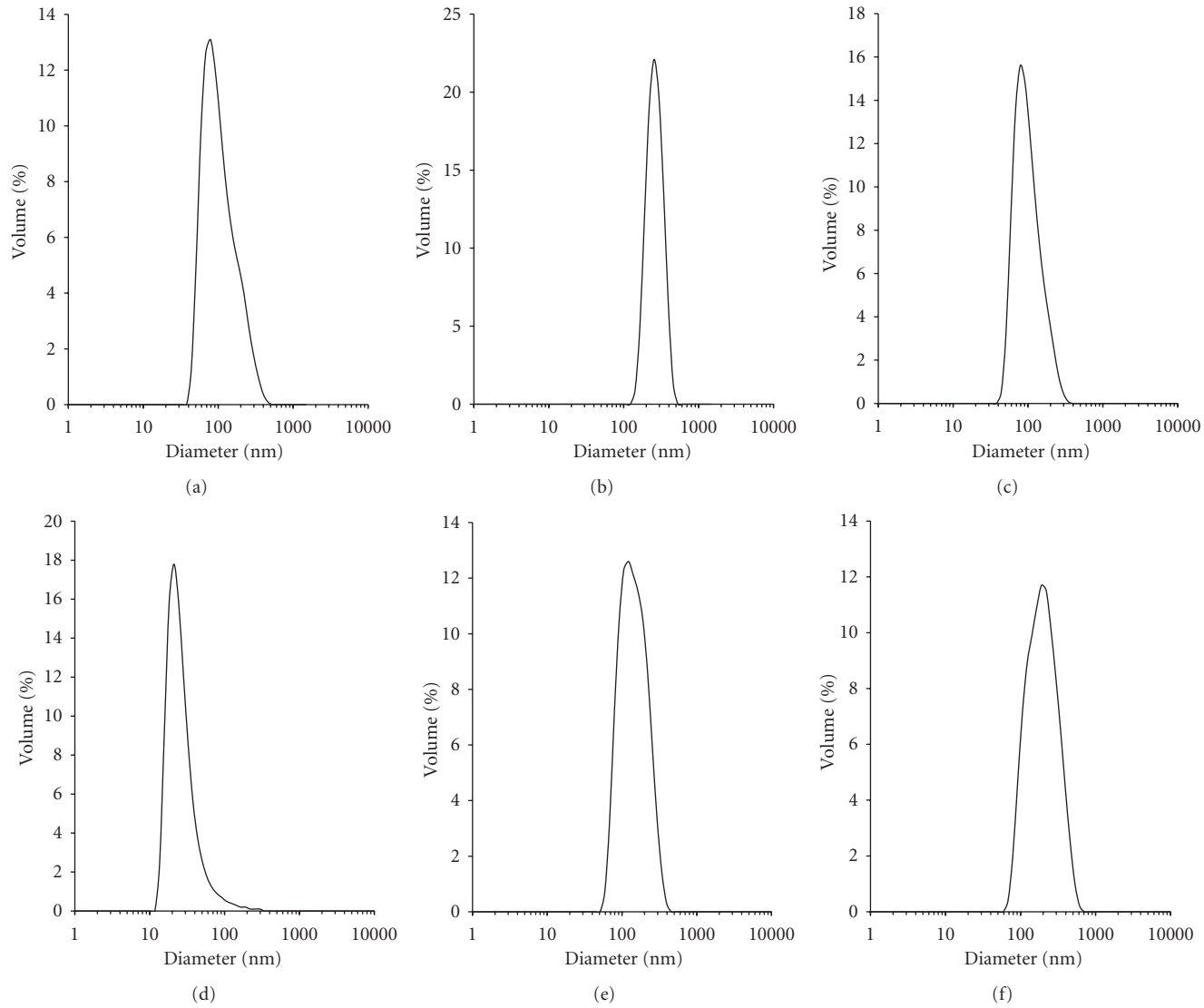
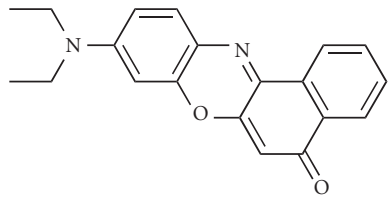
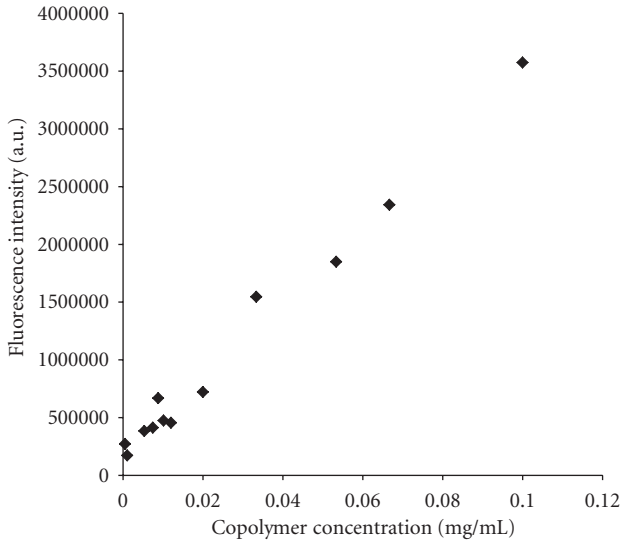


Figure 4: Representative DLS traces for micelles formed from copolymers **7–9** by dialyzing (against water) THF/water suspensions of the micelles containing varying THF content: (a) copolymer **7** from 2.5 vol% THF; (b) copolymer **7** from 30 vol% THF; (c) copolymer **8** from 2.5 vol% THF; (d) copolymer **9** from 2.5 vol% THF; (e) copolymer **9** from 30 vol% THF; (f) copolymer **9** from 40 vol% THF.

such as the cores of micelles, but negligible fluorescence in aqueous solutions due to its very low solubility [56, 57]. It was encapsulated into the micelles by dissolving it in the THF solution along with the copolymer during the micelle preparation. Following the addition of water and removal of THF by dialysis, any precipitated unencapsulated Nile Red was removed by centrifugation. The release of Nile Red was then monitored by fluorescence spectroscopy. As shown in Figure 7, at pH 7.4 Nile Red was completely released from the micelles over a period of approximately 15 hours. At pH

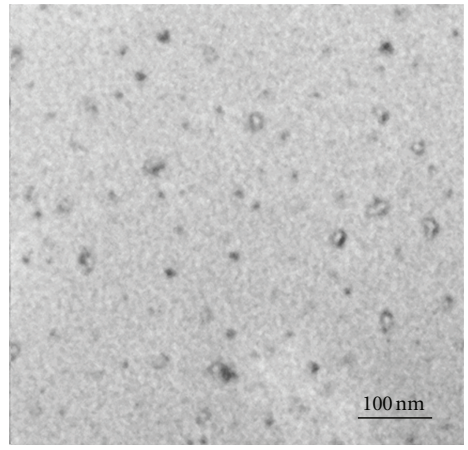


(a)

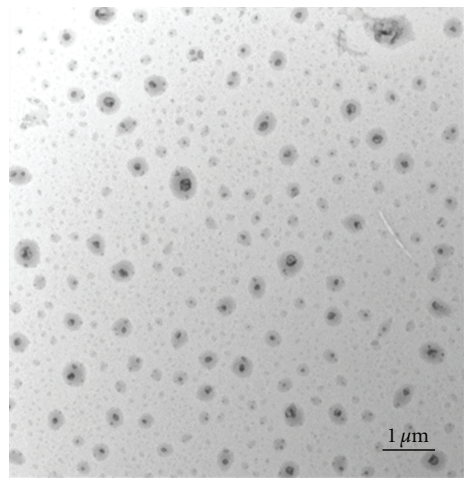


(b)

Figure 5: (a) Chemical structure of Nile Red; (b) fluorescence intensity of Nile Red as a function of copolymer concentration, illustrating a relatively linear relationship between concentration and fluorescence intensity over the concentration range from 0.5 to 100 μg/mL.



(a)



(b)

Figure 6: TEM images of micelles formed from copolymer **9** by dialyzing (against water) THF/water suspensions of the micelles containing (a) 2.5 vol% THF and (b) 40 vol% THF.

5.0, the release was slightly faster, reaching completion over a period of 12 hours. This can be attributed to two possible causes. First, protonation of residual pendant amine groups on the PEA backbone would increase the hydrophilicity of the micelle core, accelerating drug release. Secondly, partial protonation of the aniline nitrogen of Nile Red at pH 5.0 would make the model drug molecule more hydrophilic, favoring its release into the aqueous environment. Overall, these release rates are in a range that would be reasonable for drug delivery applications.

Finally, the toxicity of micelles composed of copolymer **9** was also investigated. Varying concentrations of micelles ranging from 4 μg/mL to 2 mg/mL were added to HeLa cells. This upper limit was based on the maximum micelle concentration of approximately 20 mg/mL that could readily be prepared in pure water and then diluted 10-fold into cell culture media. After incubation for 48 hours, an MTT assay [58] was performed to assess cell viability. As shown in Figure 8, toxicity (as defined by a cell viability <70% of the blank) [59] was not detected at any of these concentrations. While further studies must be performed to assess the toxicity of the micelles *in vivo*, the lack of toxicity of the materials even at the high 2 mg/mL concentration *in vitro* suggests that they should be well tolerated.

4. Conclusions

Amphiphilic PEA-PEO graft copolymers were prepared for the first time by the reaction of 4-nitrophenyl-carbonate-activated PEO with the pendant amine groups in a PEA containing lysine residues. Varying PEO content was achieved by varying the MW of the PEO chains and by using different equivalents of PEO. Nanoprecipitation using THF and water was found to be the most effective method for micelle formation, and it was demonstrated that the micelle sizes could be tuned by the manner in which the water was added. Micelles with diameters of less than 100 nm were obtained from copolymer **9**, as measured by DLS and TEM. This diameter should be ideal for circulation *in vivo*. It was demonstrated that these micelles were capable of encapsulating the model hydrophobic drug Nile Red and releasing it over a period of 12 to 15 hours, depending on the pH of the solution. Furthermore, the micelles were found to be nontoxic to HeLa cells *in vitro*. Overall, these results suggest that micelles comprising PEA-PEO graft copolymers

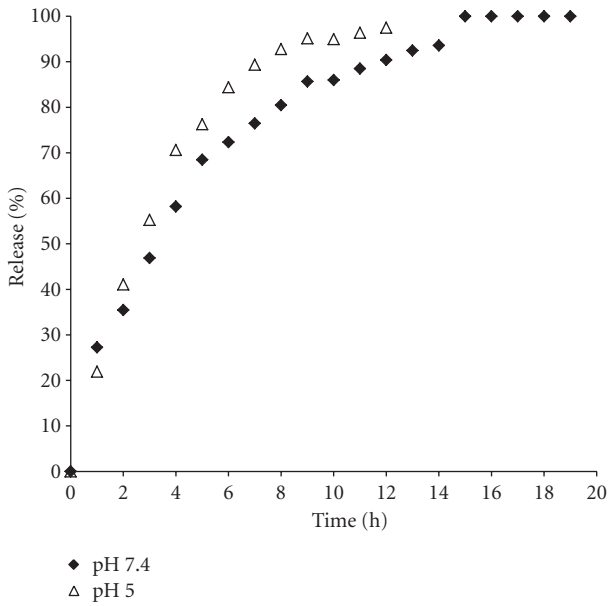


Figure 7: Release rate at pHs 7.4 and 5.0 of the hydrophobic dye Nile Red from micelles formed from copolymer **9**.

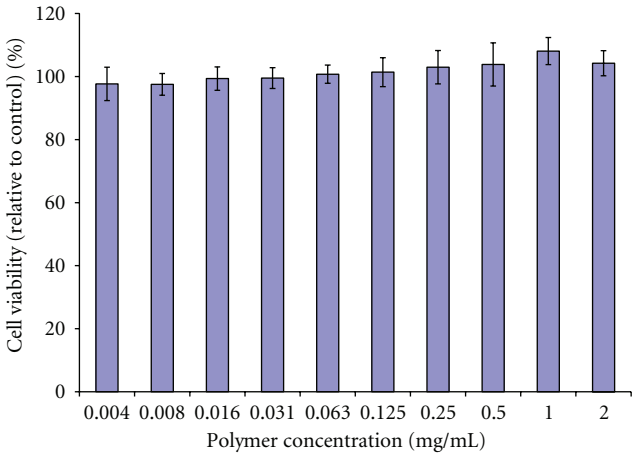


Figure 8: Assessment of the *in vitro* toxicity of micelles formed from copolymer **9**. An MTT assay was performed following 48 hours of incubation of varying concentrations of micelles with HeLa cells.

are promising new carriers for drug delivery applications. Future work will focus on studying the biodegradability of the micelles, the encapsulation of drug molecules and further assessment of the micelles both *in vitro* and *in vivo*.

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