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New folate-functionalized biocompatible block copolymer micelles as potential anti-cancer drug delivery systems

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Abstract

The main objective of this study was to synthesize novel folic acid-functionalized diblock copolymer micelles and evaluate their solubilization of two poorly water-soluble anti-tumor drugs, tamoxifen and paclitaxel, which suffer from low water solubility and/or poor hydrolytic stability. The diblock copolymer consisted of a permanently hydrophilic block comprising 2-(methacryloyloxy)ethyl phosphorylcholine (MPC) residues and a pH-sensitive hydrophobic block comprising 2-(diisopropylamino)ethyl methacrylate (DPA) residues. Folic acid (FA) was conjugated to the end of the MPC block so that this group was located on the micelle periphery. Tamoxifen- and paclitaxel-loaded micelles were prepared from FA–MPC–DPA copolymers prepared with two different block compositions that were designed to produce optimal solubilization of each drug. Their drug-loading capacities and aqueous stabilities were determined by high performance liquid chromatography. The hydrodynamic diameters of tamoxifen- and paclitaxel-loaded FA–MPC–DPA micelles ranged from 30 to 60 nm, as judged by dynamic light scattering (DLS) and transmission electron microscopy (TEM) studies. Finally, tamoxifen and paclitaxel release profiles were evaluated in phosphate buffer solution at pH 7.4 and 5. These studies demonstrated that FA–MPC–DPA micelles acted as useful drug carriers, leading to relatively slow release of both tamoxifen and paclitaxel into aqueous solution over a period of 7 days. In addition, rapid release can be triggered by lowering the solution pH to 5, which leads to protonation of the DPA block and hence rapid micellar dissociation.
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1. Introduction

Over the last decade there has been increasing interest in the potential use of block copolymer micelles as tumor-selective drug delivery vehicles [1,2]. Such systems have the potential to provide enhanced circulation times and also to reduce the problem of rapid phagocytosis and renal clearance [3]. In principle, the hydrophobic micelle core can solubilize poorly water-soluble anti-tumor drugs, while the solvated micelle corona confers good colloidal stability in aqueous solution and controls the distribution of the drug-loaded micelles within the body [4,5]. However, the ability to achieve high targeting efficiency at the tumor site and associated cells remains a significant challenge for the development of micelle-mediated drug delivery systems.

Although nanosized micelles can accumulate spontaneously in tumors with leaky vasculature by the well known enhanced permeability and retention (EPR) effect [6], they can also accumulate at reticuloendothelial sites such as the liver, spleen and kidney as a consequence of their colloidal properties (for example, their surface charge) [7]. Consequently, insufficient uptake at tumor sites will decrease the therapeutic benefit of the administered drug dose, and non-specific association with healthy tissues can lead to toxic side effects, limiting the maximum dosage that can be safely applied. This limitation prevents drug-loaded micelles from achieving the potential therapeutic effects they might otherwise attain.

One strategy to achieve cancer-targeted drug delivery is the utilization of unique molecular markers that are specifically overexpressed within the cancerous tissues. It is well know that many malignant tissues, especially the ovary, nasopharyngeal, cervical and chorion carcinomas, consistently express high

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levels of folate receptors (FR-α), which is accessible via the bloodstream [8]. Moreover, FR-α is also expressed in certain normal tissues, such as the placenta, kidney (proximal tubules), fallopian tube and choroids plexus. However, this expression is restricted to the luminal surface of epithelial cells, where it is inaccessible to blood circulation [9]. Folic acid is a water-soluble B vitamin, which is essential for de novo nucleotide synthesis and one-electron transfer reactions [10]. It retains a high affinity for the FR even after derivatization via its γ-carboxylic acid group, thus making it a potentially useful tumor-targeting ligand. FR-targeting has been evaluated for enhancing tumor cell-selective delivery of a wide variety of therapeutic agents. These include: radiopharmaceuticals [11]; chemotherapeutics [12]; antisense oligodeoxyribonucleotides [13]; prodrug-converting enzymes [14]; antibodies [15]; gene transfer vectors [16] nanoparticles [17] and liposomal drug carriers [18]. For example, ^{111}In-diethylenetriamine pentaacetic acid (DTPA)-folate has been evaluated clinically as an imaging agent for detecting ovarian carcinomas. Preliminary results indicated a sensitivity of 85% and a specificity of 82% for identifying malignant tumors [19]. These findings demonstrated that FR-specific tumor uptake of a folate conjugate can occur despite the presence of physiological levels of folate and FR in the bloodstream. Moreover, they suggest that targeting the FR in ovarian cancer is potentially feasible in the clinic.

Folate-directed delivery of hydrophobic drugs with polymeric micelles has been reported in only a few cases [20,21]. Herein we synthesized new biocompatible block copolymer micelles that have been designed to allow the selective delivery of proven hydrophobic anti-cancer drugs, such as tamoxifen [22] and paclitaxel [23], to various tumor cells that are known to over-express FRs [24,25]. To this end, we conjugated folic acid to the primary amine terminus of an AB diblock copolymer comprising 2-methacryloyloxyethyl phosphorylcholine (MPC) units in the A block and 2-(diisopropylamino)ethyl methacrylate (DPA) units in the B block. These copolymers have been recently synthesized using atom transfer radical polymerization (ATRP) and are known to undergo pH-modulated micellar self-assembly [26]. It is well known that phosphorylcholine-based copolymers offer an effective means of reducing protein adsorption and cell attachment by mimicking the surface of natural phospholipid membrane bilayers: this is due to the highly hydrophilic nature of the phosphorylcholine head-groups, which inhibits surface biofouling [27]. Thus these MPC-based copolymers should be an ideal component for the formulation of long-circulating micelles.

These diblock copolymers dissolve molecularly in acidic solution due to protonation of the DPA block (pK_a 6.2), but undergo micellar self-assembly at around neutral pH to form micelles comprising dehydrated DPA-based cores and MPC-based coronas [28–30]. Thus MPC-based diblock copolymer nanoparticles may be designed to entrap a hydrophobic drug within the hydrophobic micelle cores at physiological pH and to deliver triggered release of this drug at a lower (local) pH.

In principle, the presence of folic acid groups on the micelle periphery should allow specific targeting of these micelles to tumors that express folate receptors. The expected local decrease in pH in the vicinity of the tumor should then stimulate drug release. This approach should significantly reduce the systemic toxic side-effects that are often observed with traditional methods of parenteral administration. This paper provides the first report of the physicochemical characterization and solubilization properties of new biocompatible diblock copolymer micelles loaded with two proven anti-cancer drugs, namely tamoxifen and paclitaxel. To date, the successful employment of polymeric micelles as carrier systems for these two hydrophobic anti-cancer drugs has been the subject of only a few reports [31–34]. Clearly the development of a carrier system that allows optimization of drug loading/solubility, drug stability under physiological conditions and specific tumor-targeting remain important challenges. The FA–MPC–DPA diblock polymer micelles examined in the present study seem to be particularly promising in this regard.

2. Experimental

2.1. Materials

MPC monomer (99.5% purity) was obtained from Biocompatibles UK Ltd. The Cu(I)Br, 2,2′-bipyridine (bpy), 9-fluorenylmethyl chloroformate (Fmoc), 5-amino-1-pentanol, 2-bromoisobutyryl bromide, 1,8-diazabicyclo(5.4.0)undecen-7-ene (DBU), *N*-hydroxysuccinimide (NHS), 1,3-dicyclohexylcarbodiimide (DCC), folic acid (98% purity), methanol, 2-propanol, paclitaxel and tamoxifen free base, were all purchased from Aldrich and were used as received. *N*-(3-Dimethylaminopropyl)-*N*′-ethyl-carbodiimide hydrochloride (EDC·HCl), was purchased from Fluka. The silica used for removal of the ATRP copper catalyst was column chromatography grade silica gel 60 (0.063–0.200 mm) purchased from E. Merck (Darmstadt, Germany). 2-(Diisopropylamino)ethyl methacrylate (DPA) was purchased from Scientific Polymer Products. The water used in all experiments was deionized and doubly distilled prior to use.

2.2. Synthesis of FA–MPC–DPA diblock copolymers

Diblock copolymers of 2-methacryloyloxyethyl phosphorylcholine (MPC) with 2-(diisopropylamino)ethyl methacrylate (DPA) were prepared by ATRP using an Fmoc-protected initiator, a Cu(I)Br catalyst and a 2,2′-bipyridine (bpy) ligand using the protocol described previously [26].

MPC was polymerized first (2.00 g, 6.78 mmol) in a 3:2 methanol/isopropanol solvent mixture (5 mL) using [MPC]:[Fmoc-protected initiator]:[CuBr]:[bpy] = 30:1:1:2 under a nitrogen atmosphere for approximately 2 h at 20 °C. This protocol led to very high conversion (>95%) of the MPC monomer. The required amount of the DPA monomer was then added to this reaction solution and ^{1}H NMR was used to monitor the reaction until monomer consumption was complete (disappearance of vinyl signals at 5.5–6.0 ppm).

The copolymer solutions were then treated with silica gel to remove the spent ATRP catalyst. After solvent evaporation, the solid copolymers were washed with excess *n*-hexane to remove any traces of residual tertiary amine monomer and 2,2′-bipyridine ligand. The resulting Fmoc–MPC–DPA diblock copolymers (1.00 g) were dissolved in methanol (5 mL) and DBU (0.5 mL, 3.34 mmol, 20–50 equiv.) was added to the stirred solution. After 3 h, the resulting primary amine-functionalized copolymer (H_2N–MPC–DPA) was precipitated into *n*-hexane and washed with further *n*-hexane. The dried copolymer was dissolved in water, purified by exhaustive dialysis against water (using cellulose membrane dialysis tubing (Sigma) with a 12,400 MW cut-off) and finally freeze–dried from water overnight. The successful removal of the Fmoc protecting group was confirmed by the positive reaction of the amine-functionalized copolymers with a 2 wt% ethanol solution of ninhydrin. Finally, folic acid was conjugated to H_2N–MPC–DPA. A 5.0 mL solution of a 3:2 water/DMSO mixture containing folic acid (40.0 mg, 0.0693 mmol, 1.5 equiv.), EDC.HCl (35.0 mg, 0.138 mmol, 3.0 equiv.) and NHS (16.5 mg, 0.138 mmol, 3 equiv.) was added to H_2N–MPC–DPA (1.00 g, 0.046 mmol) dissolved in a 3:2 water/DMSO mixture (5 mL). After stirring this reaction solution for 20 h at 30 °C, the pH was lowered to 3 using aqueous HCl and the precipitated free folic acid was removed by filtration. The remaining solution was purified by exhaustive dialysis against water, using cellulose membrane dialysis tubing (Sigma) with a 12,400 MW cut-off and finally freeze–dried from water overnight (yield ~90%). The amount of free folic acid was negligible (<0.01%) as determined by gel permeation chromatography using a UV detector operating at 360 nm. The amount of conjugated folic acid was determined by UV spectroscopy by comparing the absorbance of the FA-functionalized copolymer conjugates at 365 nm in distilled water at pH 7 with a previously constructed folic acid calibration curve.

2.3. Characterization of FA–MPC–DPA block copolymers

^{1}H NMR spectra were recorded on a 300 MHz Bruker Avance DPX300 spectrometer in either deuterated methanol (CD_3OD) or chloroform ($CDCl_3$) at room temperature.

The molecular weights and molecular weight distributions of FA–MPC–DPA diblock copolymers were determined by aqueous gel permeation chromatography (GPC) as described previously [26]. The standard GPC protocol involved using two ViscoGel (G5000 PWXL and G2500 PWXL) columns connected to a Polymer Labs ERC-7517A refractive index detector. The eluent was an aqueous solution comprising 0.50 M acetic acid and 0.30 M Na_2SO_4 at pH 2; a series of near-monodisperse poly(2-vinylpyridine) standards (PSS, Germany) were used for calibration.

A Perkin–Elmer UV/visible Lambda 2S spectrophotometer was used to determine the amount of FA that was conjugated to the FA–MPC–DPA block copolymers. Serially diluted concentrations of folic acid in distilled water at pH 7 were used to construct the calibration curve. The folic acid content was expressed either as the percentage of conjugated folic acid per unit mass of copolymer or as the percentage molar ratio. This analysis also indicated a molar extinction coefficient at 365 nm (ε_{365}) of 7600 ± 50 mol^{-1} dm^3 cm^{-1} for folic acid.

2.4. Preparation of tamoxifen and paclitaxel FA–MPC–DPA loaded micelles

Drug-loaded micelles were prepared by intimately mixing a known amount of tamoxifen or paclitaxel (10 mg) with 100 mg of FA–MPC_{30}–DPA_{50} (sample A), or FA–MPC_{30}–DPA_{80} (sample B) block copolymers, respectively, in presence of ethanol (100 μL), in such a way to obtain a homogeneous pulp. Aliquots of 500 μL of distilled water at pH 7–8 (adjusted with 0.1 M NaOH) were added to each mixture with continuous mixing obtaining a final total water volume of 10 mL. The resulting suspensions were sonicated for 10 min and freeze-dried. The dried copolymer/drug mixtures were then redissolved in 10 mL water at pH 8, centrifuged at 10,000 rpm for 7 min, filtered through a 0.45 μm (Whatman) cellulose membrane and freeze–dried once more. No further drug incorporation was obtained when increasing the above copolymer/drug weight ratio, while lowering this ratio led to a reduction in the extent of drug incorporation.

2.5. Determination of the tamoxifen and paclitaxel loading within the FA–MPC–DPA micelles

Two HPLC methods were developed to determine the tamoxifen or paclitaxel loading capacity of the FA–MPC–DPA micelles (samples A and B). A reversed-phase C_{18} column (μBondpack, 5 μm, 250×46 mm^2 i.d., Waters) was used as the stationary phase, while CH_3OH/0.1 M K_2HPO_4 (pH 8.7) (90/10 v/v) was used as the mobile phase for tamoxifen determination at a flow rate of 1.5 mL min^{-1} and the eluent was monitored at a wavelength of 250 nm. CH_3CN/35 mM $AcO^-NH_4^+$ (pH 5) (45/55 v/v) was used as the mobile phase for paclitaxel determination at a flow rate of 0.8 mL min^{-1} and the eluent was monitored at a wavelength of 230 nm. Typically, 5 mg of freeze–dried copolymer micelles were solubilized in 5 mL of distilled water and the aqueous solutions were analyzed by HPLC after being filtered through 0.45 μm cellulose membrane filters. Drug loading capacities were expressed as weight percent of drug per 100 mg of dried material.

2.6. Dynamic light scattering (DLS) and zeta potential measurements

Dynamic light scattering studies (DLS) and aqueous electrophoresis measurements were performed at 25 °C using a Malvern Zetasizer NanoZS instrument, fitted with a 532 nm laser at a fixed scattering angle of 90°. Aqueous micellar solutions prepared using copolymers A and B were prepared using distilled water at different concentrations and these solutions were adjusted to pH 7–8. The micelle solutions were filtered through a 0.45 μm cellulose

membrane filter before analysis. The intensity-average hydrodynamic diameter and polydispersity index (PDI) were obtained by cumulants analysis of the correlation function. The zeta potential (millivolt) was calculated from the electrophoretic mobility using the Smoluchowsky relationship and assuming that $ka \gg 1$ (where k and a are the Debye–Hückel parameter and particle radius, respectively).

2.7. Transmission electron microscopy (TEM) studies

TEM images were obtained using a Philips CM100 electron microscope operating at 100 kV and equipped with a LaB6 gun and a Gatan 1K×1K digital camera. To prepare the TEM samples, 5 μL of an aqueous solution of block copolymer micelles was dropped onto a carbon-coated copper grid and the water droplet was allowed to evaporate slowly in air.

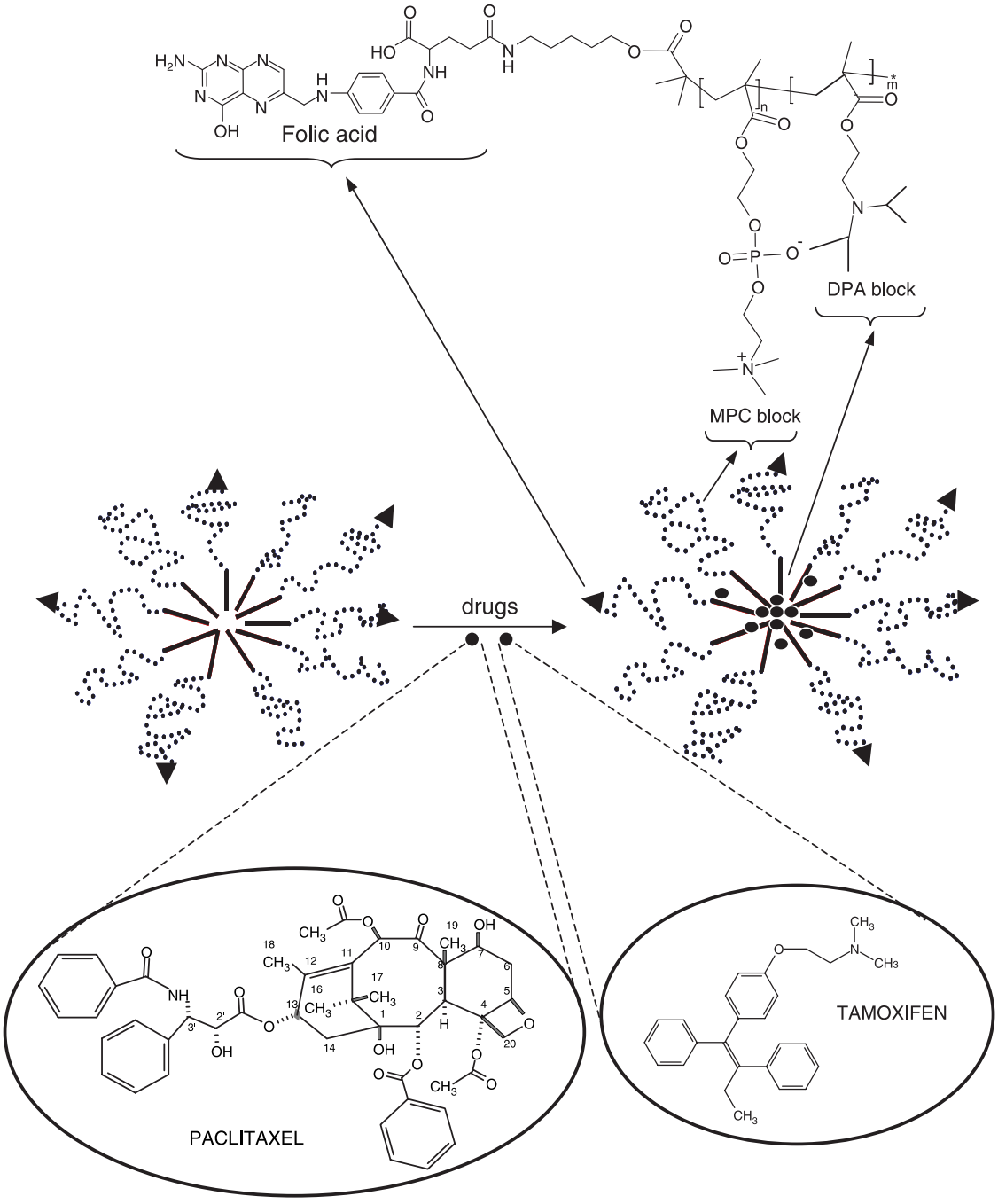

Fig. 1. Encapsulation of either tamoxifen or paclitaxel within folic acid-functionalized MPC–DPA diblock copolymer micelles at pH 7.4. In principle, the folic acid groups allow cell-targeting strategies to be explored, while the micelles increase the aqueous solubility and bioavailability of the anti-cancer drug and at the same time enhance its resistance towards hydrolytic degradation.

2.8. In vitro release of tamoxifen and paclitaxel from FA–MPC–DPA copolymer micelles at pH 7.4 and 5.0

Freeze–dried FA–MPC_{30}–DPA_{50}/tamoxifen and FA–MPC_{30}–DPA_{80}/paclitaxel loaded-micelles (samples A and B, respectively, 6.3 mg each) were resuspended in 0.02 M phosphate +0.15 M NaCl (PBS) buffer solutions at either pH 7.4 or pH 5.0 and transferred into a 3.5 mL cellulose ester dialysis tubing (M_w cut-off: 10,000 Da, supplied by Spectra/Por, USA). The tubing was placed into 13 mL PBS buffer at pH 7.4 or acetate buffer at pH 5.0. Release studies were conducted at 37 °C using a ThermoForma Orbital Shaker (USA). At selected time intervals, 1 mL of buffered solution outside the dialysis bag was removed, freeze–dried, and replaced with 1 mL of fresh buffer solution. The freeze–dried micelles were resuspended in CH_3CN (100 mL), filtered through a 0.45 μm cellulose membrane and the tamoxifen (or paclitaxel) content of the supernatant solution was estimated by HPLC as described above. All experiments were carried out in triplicate.

2.9. Tamoxifen and paclitaxel stability at pH 7.4 and 5.0

The stabilities of the respective drugs in tamoxifen- and paclitaxel-loaded FA–MPC–DPA micelles (samples A and B) were investigated in PBS solution, buffered at pH 7.4 and 5.0, respectively. Known amounts of each type of micelle (10 mg) were incubated at 37±0.1 °C in PBS solutions (10 mL, at either pH 7.4 or 5.0). At scheduled time intervals, samples were withdrawn and assayed by HPLC using the above protocol in order to evaluate the concentrations of non-degraded tamoxifen and paclitaxel. Aqueous solutions of tamoxifen and paclitaxel prepared in the absence of any copolymer micelles were also subjected to the same experiment in order to evaluate the stability of these drugs in aqueous solution at pH 7.4 and 5.0. Experiments were carried out for 30 days and repeated in triplicate.

2.10. Tamoxifen and paclitaxel stability in human plasma

Copolymer micelles (7 mg of either sample A or B) were suspended in 1 mL of PBS solution at pH 7.4; 100 μL aliquots of each solution were added to 1000 μL of preheated human plasma and maintained at 37±0.1 °C with continuous stirring in an orbital shaker incubator. At scheduled times CH_3CN (1 mL) was added to each solution, followed by centrifugation at 10,000 rpm for 10 min, filtered through a 0.45 μm cellulose membrane and analyzed by HPLC. To verify the hydrolytic stability of tamoxifen and paclitaxel in human plasma, saturated solutions of these micelle-free drugs were prepared in PBS buffer at pH 7.4. Aliquots (100 μL) of these solutions were added to preheated plasma solutions (500 μL) and incubated at 37±0.1 °C. At scheduled time intervals, CH_3CN (500 μL) was added and solutions were centrifuged at 10,000 rpm for 10 min, filtered through a 0.45 μm cellulose filter and assayed by HPLC. Preliminary experiments confirmed that there were no interfering peaks in the blank plasma chromatograms and that this protocol allowed the recovery of up to 98% w/w drug from the plasma. Each experiment was carried out in triplicate.

3. Results and discussion

The FA–MPC–DPA diblock copolymers synthesized for this study undergo pH-induced self-assembly to form well-defined micelles that are potentially suitable for the encapsulation of hydrophobic antitumor drugs, such as tamoxifen and paclitaxel.

Fig. 1 shows the encapsulation of either tamoxifen or paclitaxel within folic acid-functionalized MPC–DPA diblock copolymer micelles at pH 7.4.

In this work the drug-loading capacities of these micelles and their drug release profiles were studied under physiologically relevant conditions. Previously, we reported a protocol for the conjugation of FA to the primary amine terminus of MPC–DPA diblock copolymer precursors [26]. Although very high degrees of functionalization were obtained for FA–MPC–DMA diblock copolymers (where DMA is 2-(dimethylamino)ethyl methacrylate), only relatively low degrees of functionalization (around 30 mol%) were obtained for FA–MPC–DPA diblock copolymers. However, we subsequently increased the conjugation efficiency for the latter copolymer up to 60 mol% by adjusting the reaction temperature to 30 °C (Table 1).

3.1. Dependence of drug loading on copolymer composition

Two FA–MPC–DPA diblock copolymers with different block compositions (fixed MPC block length, variable DPA block lengths) were evaluated for the encapsulation of tamoxifen and paclitaxel. Surprisingly, the drug loading capacity appears to depend on the block composition. Thus FA–MPC_{30}–DPA_{50} micelles preferentially encapsulated tamoxifen rather than paclitaxel, whereas higher amounts of paclitaxel were encapsulated when the FA–MPC_{30}–DPA_{80} block copolymer was employed. However, it should be noted that these apparent differences are within our estimated experimental error. Table 1 summarizes the maximum drug loading capacities in relation to the block copolymer compositions.

It is known that physical entrapment of hydrophobic drugs in block copolymer micelles is driven by drug solubilization within the hydrophobic micelle cores. Paclitaxel (FW = 853.9) is a somewhat larger molecule than tamoxifen (FW = 371.5), thus it is

Table 1
The degrees of folic acid conjugation and maximum drug loadings achieved using the two biocompatible diblock copolymers used in this study

Block copolymer composition	Conjugated FA amount		Tamoxifen loading content (wt%)	Paclitaxel loading content (wt%)
	wt%	mol%		
FA–MPC_{30}–DPA_{50}	1.54	58.5	5.0±0.2	3.5±0.3
FA–MPC_{30}–DPA_{80}	1.62	61.4	4.5±0.4	5.1±0.3

not unreasonable that the former drug may require a longer DPA block to become entrapped. It is well documented that increasing the length of the core-forming block invariably leads to larger micelles with higher micelle aggregation numbers [34,35].

FA–MPC_{30}–DPA_{80} micelles allowed higher paclitaxel loadings than FA–MPC_{30}–DPA_{50} micelles, whereas the latter micelles were marginally preferable for tamoxifen solubilization. In each case the maximum amount of loaded drug was around 5% w/w and the water solubility of tamoxifen- and paclitaxel-loaded micelles was up to 50 mg mL^{-1}, which is more than three orders of magnitude greater than the aqueous solubilities of the corresponding free drug (the water solubilities of tamoxifen and paclitaxel are 0.4 and 0.1 μg mL^{-1}, respectively) [31,32]. Cavallaro et al. also reported that the aqueous solubility of amphiphilic poly(hydroxyethylaspartamide) (PHEA–PEG–C_{16}) based micelles containing tamoxifen [31] or paclitaxel [32] was around 50 mg mL^{-1}. Nevertheless, the maximum drug loading capacity was less than 5% w/w in both cases (4.1% w/w for tamoxifen and only 1% w/w for paclitaxel). Thus, the FA–MPC–DPA based copolymer micelles employed in this study allow significantly higher drug solubilities to be achieved than those previously reported [31–34].

We developed a new protocol to incorporate these two hydrophobic drugs within the copolymer micelles, based on the formation of a homogeneous drug/copolymer mixture prior to dilution with water. This solid-state technique allows the drug to be incorporated during micelle formation, rather than adding drug into preformed micelles (direct dissolution protocol), or using organic co-solvents (which requires either dialysis or emulsification) [36]. Thus, any drug degradation that may occur due to prolonged contact with solvent was minimized and the amount of encapsulated drug was increased. Analysis of tamoxifen and paclitaxel solubilized in aqueous copolymer micelle solutions by reverse-phase HPLC indicated that this new drug-loading protocol did not cause drug degradation.

3.2. Physical properties of the micelles

As shown in Fig. 2, the mean hydrodynamic micelle diameter obtained by dynamic light scattering (DLS) depends on both the block copolymer composition and also the drug-loading. Micelle diameters were typically less than 100 nm and different micelle sizes were observed for drug-free (empty) and drug-loaded micelles.

Prior to drug loading, the FA–MPC_{30}–DPA_{80} micelles had an intensity-average diameter of 80 nm while the FA–MPC_{30}–DPA_{50} micelles had an intensity-average diameter of 54 nm (Table 2). This difference was anticipated, since the longer hydrophobic DPA block should lead to a higher micelle aggregation number [34,35].

However, unexpected effects were observed for the drug-loaded micelles. In particular, the intensity-average diameter of the paclitaxel-loaded FA–MPC_{30}–DPA_{80} micelles was reduced from 80 to 58 nm, whereas the tamoxifen-loaded

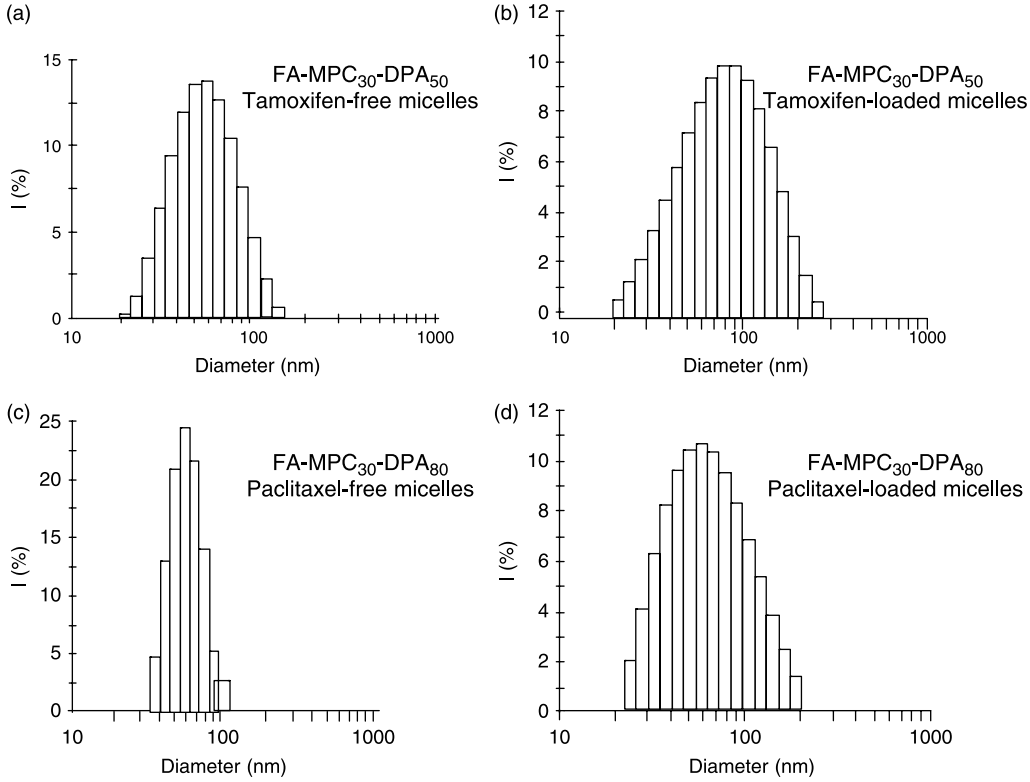

Fig. 2. DLS particle size distributions of tamoxifen-free (a) and tamoxifen-loaded (b) FA–MPC_{30}–DPA_{50} micelles, paclitaxel-free (c) and paclitaxel-loaded (d) FA–MPC_{30}–DPA_{80} micelles.

Table 2
Physical properties of drug-free and drug-loaded FA–MPC–DPA micelles

Micelle type	Micelle diameter (nm)[a]	Polydispersity[a]	Zeta potential (mV)
FA–MPC_{30}–DPA_{50} empty micelles	54	0.20	+40.4
Tamoxifen-loaded FA–MPC_{30}–DPA_{50}	68	0.21	+3.5
FA-MPC_{30}–DPA_{80} empty micelles	80	0.19	+37.9
Paclitaxel-loaded FA–MPC_{30}–DPA_{80}	58	0.21	+5.0

The zeta potential and DLS studies were conducted at pH 7.4.
[a] Determined by DLS (intensity-average).

FA–MPC_{30}–DPA_{50} micelles increased from 54 nm (empty micelles) to 69 nm (Table 2).

However, an increase in the polydispersity for the drug-loaded micelles was observed compared to that for the empty micelles, which obviously affects the respective intensity-average diameters. Thus the observed intensity-average diameters do not necessarily correspond to the maxima indicated in the particle size distribution. This is particularly true for the FA–MPC_{30}–DPA_{80} micelles, whose maximum diameters before and after drug loading are almost unchanged (see the size distributions in Fig. 2(c) and (d)). It is also possible that solubilization of a hydrophobic drug such as paclitaxel could make the drug-loaded micelle core less compact than the cores of empty micelles and consequently reduce the micelle aggregation number.

The spherical morphology and nanoscale dimensions of the drug-loaded micelles are confirmed by transmission electron microscopy (TEM) studies of paclitaxel-loaded FA–MPC_{30}–DPA_{80} micelles, see Fig. 3.

The paclitaxel-loaded micelles have mean diameters of less than 50 nm by TEM. Since, TEM reports a number-average diameter, this result is reasonably consistent with the intensity-average diameter of 58 nm obtained by DLS.

Aqueous electrophoresis studies of drug-free and drug-loaded micelles indicated positive zeta potentials in each case, with significantly higher zeta potentials obtained for the drug-free micelles, see Table 2. The lower zeta potentials of the drug-loaded micelles suggest that drug molecules may be solubilized not only within the micelle cores but also close to the micelle periphery. This tentative explanation is in agreement with the relatively rapid drug release observed in the first hour of the drug release experiments. However, we cannot exclude the possibility that a change in the aggregation number of the copolymer micelles due to drug solubilization may influence the zeta potential, but we do not have any experimental data to test this hypothesis. However, we speculate that the localization of hydrophobic drugs such as tamoxifen or paclitaxel within the micelle corona may shield the surface charge by shifting the shear plane further from the micelle surface, leading to a reduced zeta potential [37].

3.3. In vitro tamoxifen and paclitaxel release studies

The in vitro release behavior of tamoxifen-loaded FA–MPC_{30}–DPA_{50} and paclitaxel-loaded FA–MPC_{30}–DPA_{80} micelles in two buffer solutions (pH 7.4 and 5.0) were studied. The freeze–dried micelles (50.0 mg) were resuspended in CH_3CN (100 mL), filtered through a 0.45 μm cellulose membrane and the tamoxifen (or paclitaxel) content of the supernatant solution was estimated by HPLC as described above. The results of release experiments are depicted in Fig. 4. At pH 7.4, a typical two-phase release profile was observed.

Relatively rapid release was observed within the first 3 h, followed by slow, sustained release over a 4-day period. Nevertheless, the release rates of these two drugs are significantly different, even if the initial drug loadings are similar (5% w/w). In fact, tamoxifen release from FA–MPC_{30}–DPA_{50} micelles (sample A) is essentially complete after 30 h incubation at pH 7.4, whereas paclitaxel release from FA–MPC_{30}–DPA_{80} micelles (sample B) remains incomplete even after 50 h incubation in the same medium. At pH 5.0, the release rates for tamoxifen and paclitaxel are much faster. This is due to micellar dissociation, since the DPA blocks become protonated (and hence hydrophilic) below around pH 5.5–6.0, as previously demonstrated by ^{1}H NMR and fluorescence

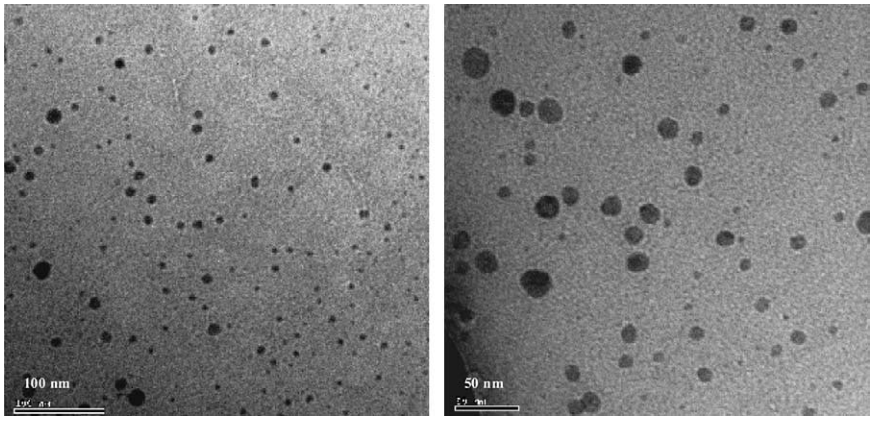

Fig. 3. Transmission electron microscopy images of FA–MPC_{30}–DPA_{80} micelles loaded with 5.1% paclitaxel at two different magnifications.

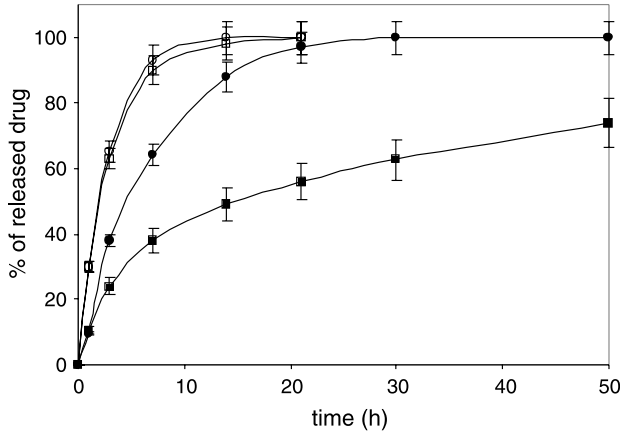

Fig. 4. In vitro tamoxifen (circles) and paclitaxel (squares) release profiles from FA–MPC_{30}–DPA_{50} and FA–MPC_{30}–DPA_{80} micelle formulations rexpectively at pH 7.4 (●, ■) and pH 5.0 (○, □), at 37 °C.

studies [26,30]. This pH-triggered release is of particular interest in the context of FR-based targeting of tumor cells. Based on the above results, it is anticipated that the majority of both tamoxifen and paclitaxel will remain encapsulated within these micelles in plasma under physiological conditions for sufficiently long time scales to allow micelle accumulation in the vicinity of the tumor. Fast, triggered release should then occur in situ due to the relatively low local pH surrounding the tumor site compared to normal tissues [38]. In addition, micellar particles interacting with FRs will be most likely internalized within the cells by endocytosis [8]. Therefore, further accelerated release may well occur inside the endosome or lysosome of the tumor cells due to the relatively low intracellular pH (pH 5.5).

3.4. Increased physical and hydrolytic stability of encapsulated tamoxifen and paclitaxel under physiological conditions

It is well known that paclitaxel is susceptible to hydrolytic degradation on long-term storage in aqueous solution, even at ambient temperature [39]. Thus it was of particular interest to investigate whether the copolymer micelles were effective in preventing, or at least minimizing, drug degradation. In contrast, tamoxifen is generally considered to be chemically stable with respect to long-term storage in aqueous solution over a broad pH range [32], but it is prone to aggregation phenomena, which causes its precipitation. Accordingly, the physical stability of tamoxifen and the hydrolytic stability of paclitaxel within FA–MPC–DPA micelles were investigated in PBS buffer at pH 7.4 and human plasma.

Figs. 5 and 6 show the stability profiles of tamoxifen-loaded FA–MPC_{30}–DPA_{50} micelles and paclitaxel-loaded FA–MPC_{30}–DPA_{80} micelles under physiological conditions (pH 7.4 and human plasma) as monitored by HPLC analysis. The stabilities of the encapsulated tamoxifen and paclitaxel were monitored for up to 1 month at pH 7.4 and for 24 h in human plasma and compared to the stabilities of the free drugs under the same conditions.

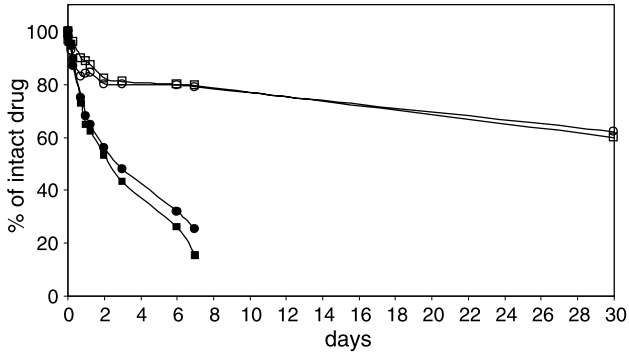

Fig. 5. Effect of ageing time on the in vitro hydrolytic stabilities of tamoxifen (circles) and paclitaxel (squares) using FA–MPC_{30}–DPA_{50} and FA–MPC_{30}–DPA_{80} copolymer micelle formulations rexpectively at pH 7.4 (□, ○) compared with free tamoxifen (●) and free paclitaxel (■), at 37 °C.

The stabilities of tamoxifen and paclitaxel at pH 7.4 were significantly enhanced by encapsulation within FA–MPC–DPA micelles (Fig. 5). The concentration of active drug decreased by only around 20% after 1 week's incubation, with the subsequent reduction most likely being related to precipitation caused by micelle destabilization. These results are particularly interesting for paclitaxel, since the poor hydrolytic stability of this drug constitutes a genuine barrier to its effective administration in vivo [39]. The same protection effect is also evident for the drug-loaded micelles in human plasma. As shown in Fig. 6, tamoxifen and paclitaxel are both much more stable to hydrolytic degradation when encapsulated within micelles and no significant differences between the stability profiles of the two drugs were observed in this medium. As expected, no tamoxifen degradation products were observed in the HPLC chromatograms. This result most likely reflects the high binding affinity between tamoxifen and plasma proteins [40]. Thus the biocompatible MPC coronas of the micelles appear to prevent, or at least suppress, unwanted interactions between tamoxifen (or paclitaxel) and plasma

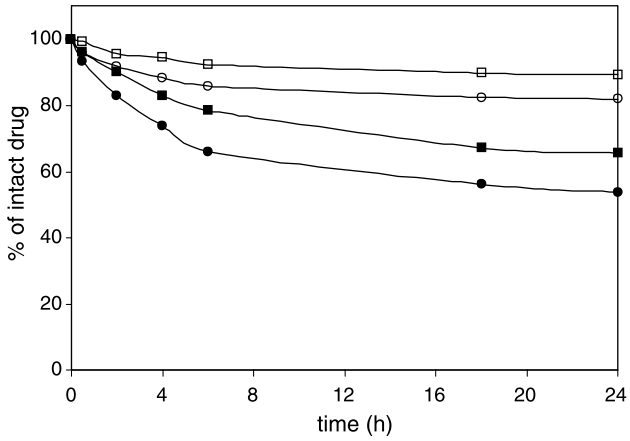

Fig. 6. In vitro tamoxifen (circles) and paclitaxel (squares) hydrolytic stability profiles encapsulated in FA–MPC_{30}–DPA_{50} and FA–MPC_{30}–DPA_{80} micelle formulations rexpectively in human plasma (□, ○) compared with free tamoxifen (●) and paclitaxel (■), at 37 °C.

proteins, thus maintaining a relatively high drug concentration after intravenous administration.

4. Conclusions

Relatively high degrees of FA functionalization (around 60 mol%) were obtained for two well-defined FA–MPC–DPA diblock copolymers of differing DPA block lengths. These FA–MPC–DPA diblock copolymers both underwent pH-induced micellar self-assembly in aqueous solution, as expected, and were able to encapsulate two poorly water-soluble hydrophobic anti-cancer drugs, namely paclitaxel and tamoxifen. FA–$PMPC_{30}$–$PDPA_{50}$ micelles seem to preferentially encapsulate tamoxifen, while paclitaxel appears to be taken up more efficiently by FA–$PMPC_{30}$–$PDPA_{80}$, but the apparent differences in drug loading ability may well be negligible within the estimated experimental error. In both cases the maximum drug loading that could be achieved was around 5% w/w. DLS and TEM studies of tamoxifen-loaded FA–MPC_{30}–DPA_{50} micelles and paclitaxel-loaded FA–MPC_{30}–DPA_{80} micelles indicated the formation of well-defined nanoparticles at physiological pH. Drug release studies on drug-loaded micelles at pH 7.4 indicated relatively rapid release within the first 3 h followed by slow, sustained release over several days. On lowering the solution pH to 5, the triggered release of both tamoxifen and paclitaxel was demonstrated due to the rapid dissociation of the micelles at this pH. In addition, the hydrolytic and physical stabilities of both drugs were dramatically improved at both pH 7.4 and also in human plasma when encapsulated within the copolymer micelles. In conclusion, these new FA–MPC–DPA micelles offer considerable potential for employment as tumor-selective nano-sized carriers for the efficient delivery of anti-cancer drugs.

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