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Polylactide-poly(ethylene Glycol) Micellar-like Particles as Potential Drug Carriers: Production, Colloidal Properties and Biological Performance

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The micellar-like particle systems produced from poly-D,L-lactide–poly(ethylene glycol) (PLA–PEG) copolymers have been assessed using a range of physicochemical characterisation methods, followed by *in vivo* studies of their biodistribution after intravenous administration to the rat. The size of the PEG chain was kept constant at 5 or 2 kDa, while the PLA size increased within a series from 2 to 25 kDa. The results obtained reveal, that in an aqueous medium the copolymers assembled into micellar-like structures, with the PLA segments forming the core and the PEG segments the surrounding corona. The size of the PLA segments dominated the process of assembly of the molecules and the characteristics of the resultant micellar-like particles. The PLA–PEG micellar particles were found to be less dynamic than those obtained from conventional surfactants. Particles formed from the lower molecular weight PLA polymers allowed a level of chain mobility while the cores of the micellar particles formed from higher molecular weight PLA appeared to be solid-like in nature. The size of the micellar particles was dependent on the copolymer molecular weight and the *z*-average diameter increased from 25 to 76 nm as the molecular weight of the PLA moiety increased. This provides an ability to control the particle size by adjusting the molecular weight of the PLA moiety. Following intravenous administration to the rat model, micellar-like particles smaller than approximately 70 nm accumulated in the liver, despite the fact that the PEG corona provided an effective steric stabilization effect. Micellar-like particles with a diameter of more than approximately 70 nm exhibited prolonged systemic circulation and reduced liver uptake, although the steric stabilisation of these particles was shown to be less effective. These findings agree with recent observations from other research groups; that indicate a possibility that very small particulates can pass through the sinusoidal fenestrations in the liver and gain access to the parenchymal cells of the liver.

Keywords: PLA–PEG copolymers; Micelles; Biodistribution; Self-assembly; Drug delivery; Poly(ethylene glycol); Steric stabilisation; Liver uptake

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INTRODUCTION

It is now well established, that it is possible to avoid, at least to some extent, the sequestration of intravenously injected particulate drugs carriers by the Kupffer cells of the liver or by other macrophages belonging to the reticuloendothelial system. This can be achieved by sterically stabilising such particles with a hydrophilic surface layer of for example poly(ethylene glycol) (PEG) (Gref *et al.*, 1994; Stolnik *et al.*, 1995; Peracchia *et al.*, 1997, 1998; de Jaeghere *et al.*, 2000). Copolymers composed of poly(ethylene glycol) (as the hydrophilic segment) and for example polymers of lactic acid, glycolic acid, caprolactone, organophosphazene or combinations of these (as the hydrophobic segment) have been shown to have advantageous properties for production of such sterically stabilised nanoparticulate drug delivery systems, for achieving long circulation times or the site specific delivery of drugs (Gref *et al.*, 1994; Vandorpe *et al.*, 1997; Peracchia *et al.*, 1997, 1998; Riley *et al.*, 1999; Stolnik *et al.*, 1997). In aqueous media, these copolymers form micellar type constructs, similar to conventional amphiphilic surfactants (Riley *et al.*, 1999). The core of these constructs is formed by an association of the relatively hydrophobic polymeric moieties. A hydrophilic corona is formed from the PEG chains protruding into the aqueous environment. The hydrophobic core matrix of such structures offers the possibility of incorporation of drugs with hydrophobic characters by a process of simple physical entrapment. Recent publications have demonstrated the possibility of a high level of incorporation of anticancer drugs, such as taxol (Zhang *et al.*, 1996), lidocaine (Gref *et al.*, 1994) or adriamycin (Kwon *et al.*, 1994), by micellar-like systems made from low molecular weight poly-D,L-lactide-poly(ethylene glycol) (PLA–PEG), poly(lactide–*co*-glycolide)–poly(ethylene glycol) (PLGA–PEG) or poly(ethylene oxide-aspartate) copolymers.

The PEG corona surrounding the micellar particle structure provides a mean for achieving a prolonged circulation of the particles in the systemic vasculature and the possibility for site specific targeting of drugs, as has previously been shown for submicron colloidal particles with a surface coverage of PEG chains (either adsorbed or grafted) (Illum *et al.*, 1987; Stolnik *et al.*, 1994) or for PEG-modified liposomes (Woodle *et al.*, 1994; Kostarelos *et al.*, 1999). Furthermore, recent experimental evidence has demonstrated that the stability of the PEG layer to desorption/displacement under *in vivo* conditions is essential for the long circulation effect (Labarre *et al.*, 1994; Neal *et al.*, 1998) and that the micellar type systems based on amphiphilic PEG copolymers appear to be an elegant way to eliminate this problem. The small sizes achievable for the polymeric micellar-like particle systems, with diameters of the smallest systems being in the range of tens of nanometers, should allow these to penetrate the small fenestrae of the bood vessels in certain body tissues (liver, inflamed tissue areas or tumours) and thereby reach the target cells (Wisse, 1970; Kataoka, 1994; Bazile *et al.*, 1995).

Various copolymers of PEG and poly(D,L-lactide) (PLA–PEG) or poly(lactide-*co*-glycolide) PLGA–PEG) have been synthesised. These have had different molecular weights and ratios of polymers in their composition and have been exploited to provide a physically adsorbed surface coating layer of PEG on preformed PLGA nanoparticles, thereby providing an alternative to the use of poly(ethylene glycol)–poly(propylene oxide)–poly(ethylene glycol) (PEG–PPO–PEG) copolymers, such as the poloxamers and poloxamines (Stolnik *et al.*, 1994). Hence, two water soluble PLA–PEG copolymers, with a 2 or 5 kDa PEG chain and a 2 kDa PLA moiety, were adsorbed physically onto PLGA nanoparticles. This created a sterically stabilising layer similar to that obtained using a poloxamer and poloxamer system (Stolnik *et al.*, 1994). Other groups have used mixtures of PLA (or PLGA)–PEG copolymers and a matrix forming PLGA material for the preparation of nanoparticles with PEG on the surface (Gref *et al.*, 1994; Bazile *et al.*, 1995). Both approaches have resulted in systems with particle diameters in the range between 100–200 nm and which have exhibited prolonged systemic circulation times in the rat model.

In recent years, nanoparticles formed directly from PLA–PEG or PLGA–PEG have been produced and shown to have favourable physicochemical properties for achieving a decreased interaction with macrophages and a longer blood circulation time (Bazile *et al.*, 1995; Peracchia *et al.*, 1997; Riley *et al.*, 1999; de Jaeghere *et al.*, 2000). de Jaeghere *et al.* (2000) recently studied the *in vitro* characteristics of nanoparticles produced from PLA–PEO copolymers with a PLA of fixed molecular weight (about 25 kDa) and PEO chains of 2, 5, 6 and 10 kDa and nanoparticles produced from PLA–PEO–PLA type copolymers. The particle diameters were in the order of 300 nm. They found that both types of nanoparticles where the PEG was in a "brush" and "loop" conformation resisted to some degree uptake by human monocytes *in vitro*. Similarly, Mosqueira *et al.*, 1999 have investigated the cellular uptake of naked and surface modified PLA nanoparticles by a macrophage cell line. The PLA–PEG copolymers used in their experiments were in the PLA–PEG molecular ratios of 2:5, 45:5 and 45:20. Coated particles were also produced from mixtures of the copolymers. They found that for similar PEG surface densities the PEG chain length was important for the prevention of cell interaction and uptake.

In the present work, we have employed a range of PLA–PEG block copolymers with a constant molecular weight of the PEG chain (2 or 5 kDa) and increasing molecular weight of the PLA chain from 2 to 25 kDa, in order to conduct a systematical evaluation of the factors affecting the characteristics and performance of the particle systems. The following assumptions were made: (i) due to the AB block structure of the PLA–PEG copolymers they would form micellar-like entities, as recently shown for a low molecular weight PLA–PEG system (Tanodekaew *et al.*, 1997); (ii) the molecular composition of the copolymers (the PLA–PEG ratio) would affect the association properties; (iii) the size of the formed micellar-like systems should be dependent on the size of the copolymer PLA portion and (iv) the dynamic properties of the micelles in an aqueous media should depend on the composition of the copolymer, i.e. the PEG–PLA ratio, whereby the copolymers with higher molecular weight PLA polymers should form less dynamic structures.

TABLE I Composition and molecular weight of PLA–PEG copolymers under investigation

Copolymer	*NMR*	*GPC*		
PLA–PEG	*PLA:PEG ratio*	M_w	M_n	*Polydispersity* (M_w/M_n)
2:5*	1.87:5	6680	5890	1.1
3:5	2.78:5	8130	6650	1.2
4:5	4.14:5	8360	6780	1.2
6:5	5.92:5	9380	6350	1.5
10:5	9.78:5	15600	11000	1.4
13:5	12.74:5	16500	9830	1.7
25:5	25.34:5	22100	9730	2.3

* PLA–PEG acronym.

Previously, we have studied the physicochemical properties of particles formed from a range of PLA–PEG copolymer where the molecular weight of the PLA moiety ranged from 3 to 110 kDa and the PEG chain was of constant 5 kDa molecular weight (Riley *et al.*, 1999). We found that the particle size of the micellar-like particles formed varied with the size of the PLA moiety and that the larger particles (formed from PLA in the range 30–110 kDa) were less physically stable as compared to the smaller particles. In the present study, we have concentrated the investigations on a range of PLA–PEG copolymers with PLA moieties from 2 to 25 kDa and PEG molecular weights of 2 or 5 kDa. The association behaviour of these PLA–PEG copolymers, the effects of the copolymer molecular composition on their self-assembly, the dynamics of the structures formed and the colloidal properties of the systems have been studied. Finally, selected systems that were characterized *in vitro* were radiolabeled and tested for their biological performances in the rat. By this approach we provide a comprehensive and systematic investigation into the mechanism of self-assembly of these copolymers, and the colloidal and surface properties of the micellar-like particles. Furthermore, their *in vivo* performances provides information as to their possible use as drug carriers for parenteral administration.

MATERIALS AND METHODS

Materials

The PLA–PEG copolymers were synthesised by Zeneca Pharmaceuticals plc, Macclesfield, Cheshire, U.K and were used without further purification. The copolymers were synthesised by ring opening polymerisation of D,L-lactide in the presence of either the 5 or 2 kDa methoxypolyethylene glycol (Sigma, UK), using stannous octoate as a catalyst (Churchil and Hutchinson, 1986). ^{1}H NMR spectroscopy in deuterated acetone or chloroform (Sigma, UK) was used to determine the ratio between the PLA and PEG blocks (Table I). The values obtained were used in the text as an acronym for the copolymers. The average weight and number molecular weight and molecular weight distribution of the copolymers were determined by gel permeation chromatography in dimethyl formamide and the relevant data are listed in Table I. D,L-lactide homopolymers (uncapped) were purchased from Boehringer Ingelheim as Resomer R 206 with an average molecular weight of 137 kDa and Resomer R 203 of 28 kDa. A PLA homopolymer of 129.7 kDa (uncapped) was purchased from BPI-Birmingham Polymers Inc., UK. The In-111-oxine complex used for radiolabeling the PLA–PEG micellar type particles was obtained from Amersham International plc, Buckinghamshire, UK.

Methods

Production of PLA–PEG Micellar Type Particles

The PLA–PEG series studied consisted of copolymers with a constant size of the PEG chain and an increasing size of the PLA moiety, providing PLA/PEG ratios between 2:5 and 25:5. Apart from the 2:5 copolymer, all other members of the series were insoluble in water but soluble in common organic solvents such as acetone, acetonitrile, chloroform, dichloromethane, ethyl acetate, etc. The method of production of micellar particles was based on the phase separation of the copolymers from an organic solvent by the addition of a non-solvent for PLA–PEG (Fessi *et al.*, 1986). The organic solvent typically used was acetone, and the separation was achieved by the introduction of water. Two protocols for the production of the micellar-like particles were adopted: (i) the addition of the acetone solution containing the copolymer into the water phase (Preparation by "addition into water") and (ii) the addition of water into the acetone solution containing the copolymer (Preparation by "addition of water").

To assess the effect of the copolymer concentration in the organic phase on the particle size of the product, 1.0, 2.0, 3.0 and 4.0% w/v copolymer solutions were used. PLA homopolymers of different molecular weights were used for particle production as comparisons.

Morphological Characterisation

The micellar particle dispersions were placed on a stub and stained with phosphotungstic acid. The dried sample was examined by a transmission electron microscope (JEOL 1200 EX12, Japan).

Determination of Particle Size

Photon correlation spectroscopy (PCS), using a Malvern 4700 instrument (Malvern Instruments, U.K.) was employed to determine the hydrodynamic diameters of the micellar-like particles. Data analysis was performed using the CONTIN program (Malvern Instruments, U.K.). The width of the size distribution is expressed as a polydispersity, which is calculated by the cummulant method and can be interpreted as the variance of a supposed log-normal distribution. For each particle system two samples were taken and diluted with filtered deionised water (0.2 μm membrane filter). Ten measurements were performed on each sample. The mean value and standard deviation were calculated for the particle size and polydispersity.

NMR Analysis

NMR experiments were performed on the PLA–PEG copolymers dissolved in d_6-acetone and on the PLA–PEG micellar-like particles in D_2O. The aim of the NMR experiments conducted in D_2O was to determine the structure of the core and the PEG corona. The micellar-like particles were prepared by an addition of the acetone solution containing the polymer to the water phase, as described above; the protocol was modified such that water was replaced by D_2O. The reaction vessel was sealed and purged by blowing a steady stream of nitrogen gas over the solvent mixture, so that as little water vapour as possible reached the deuterated solvent. The vessel was left overnight upon which all the acetone was evaporated off. The NMR analysis was run on a Bruker AC250 instrument operating at 250 MHz for proton spectra.

A control NMR experiment was performed on PLGA (M_w of 15 kDa, synthesized by Zeneca Pharmaceuticals plc, Macclesfield, Cheshire, U.K) nanoparticles produced in a surfactant free manner by a precipitation-solvent evaporation method, as reported previously (Fessi *et al.*, 1986; Stolnik *et al.*, 1995a,b). NMR spectra for Triton X-100 (a polyoxyethylene ether) (Sigma, UK) solutions in $CDCl_3$ and D_2O were used to compare the dynamic properties of the PLA–PEG micellar-like particles with the micelles found in a solution of conventional amphiphilic PEG-based non-ionic surfactant.

Colloidal Stability

The stability of the particle dispersions was monitored by measuring their turbidity as a function of electrolyte concentration. Sodium sulphate solutions (2.0 ml of 0.55, 0.575, 0.60, 0.625, 0.65, 0.675, 0.70, 0.725, 0.75 mol/l) were added to the PLA–PEG particle dispersions (0.250 ml, 0.33 w/v) in test tubes placed on a horizontal shaker (Ika-Vibrax, Ika-Labortechnik, Germany) and the turbidity of the dispersions was measured after 15 min (Uvikon 860 spectrophotometer, Kontron Instruments, U.K.). From a plot of the dispersion turbidity versus electrolyte concentration the critical flocculation concentration was determined.

Biological Properties

For the *in vivo* study, the PLA–PEG micellar type particles were prepared by the "addition into water" method, as described above. The systems were radiolabelled by the incorporation of a hydrophobic gamma-emitter (In-111-oxine complex) during the preparation. The copolymer was dissolved in acetone (10 mg/ml, 5 ml) containing the indium-111-oxine complex (50 μl, 37 MBq/ml) and the solution added to the water phase (15 ml) stirred by a magnetic stirrer. Stirring was continued at ambient temperature until complete evaporation of the organic solvent had taken place. The micellar-like particles were separated from unincorporated label by gel permeation chromatography (Sepharose 4B CL gel). The PLA–PEG 2:5 particles were not included in the *in vivo* study since the loading capacity was too low to allow sensible activity measurements. It was shown from *in vitro* release studies that some In-111-oxine was released from the nanoparticles on incubation with rat serum. However, after 3 h incubation between 77% and 83% of the label was still associated with the PLA–PEG micellar particles. Furthermore, *in vivo* studies demonstrated that free In-111-oxine remained in the blood only at moderate levels after 3 h and showed no liver accumulation. Therefore, the radiolabel could be confidently used to follow the biodistribution of the different PLA–PEG micellar-like particle systems and effectively demonstrate the *in vivo* fate of a well incorporated model drug.

For each PLA–PEG micellar-like particle system a group of three Wistar rats (150 ± 10 g) was injected intravenously via the lateral tail vein with the particle dispersion; each rat received 1 mg of solid material. Blood samples (20 μl) were taken from the contralateral tail vein at the following time intervals: 0, 5, 15, 30, 60, 120 and 180 min. The rats were killed after 3 h by intravenous injection of pentobarbitone solution and the liver,

FIGURE 1 Transmission electron micrograph of PLA–PEG micellar like particles.

spleen, lungs, kidney and one femur (left hind leg) were removed. The organ and blood associated activity was counted using a gamma counter (LKB 182 Compugamma CS, LKB Wallac, Finland). The carcass associated activity was measured using a 75 mm well counter (John Count Scientific, Oxon, U.K.). A total blood volume per rat of 7.5% of body weight was assumed (Ritschel, 1987). The results for blood and organ associated radioactivity are expressed as a percentage of the injected radioactivity and are mean values for three rats ±standard deviation. A control group of rats was injected with 10 kBq of unincorporated (free) In-111-oxine complex.

RESULTS AND DISCUSSION

Production of PLA–PEG Micellar-like Particles

Fig. 1 is a transmission electron micrograph of the PLA–PEG micellar-like particles, and is typical of all the images from the series of copolymer systems. The micrographs confirm that the systems formed consisted of small, discrete and spherical particles. Data from the particle size analysis of the PLA–PEG micellar-like particles, produced by both preparation procedures ("addition into water" or "addition of water"), are summarized in Table II. Considering first the results for PLA–PEG systems produced by the addition of a solution of the copolymer in acetone to the water phase, it can be seen that the hydrodynamic diameters are between 24.8 and 76.4 nm. This shows that the particle size is dependent on the composition of the copolymers and that in general the size increases as the molecular weight of the PLA moiety increases. This is consistent with the results obtained previously for a range of PLA–PEG copolymers with the same 5 kDa PEG chain length and PLA segments varying from 3 to 110 kDa (Riley *et al.*, 1999).

The values for polydispersity index, which lie between 0.187 and 0.393, indicate that the distributions of the particle sizes follow a monomodal normal distribution for all the systems. From this it may be inferred that subpopulations with different degrees of association (such as unimers, dimers, or multimicellar particles) are not present in the samples (Tuzar *et al.*, 1988).

For comparison, micellar particle dispersions were prepared from PLA–PEG copolymers with the shorter PEG chain of 2 kDa (Table II). An effect of the PEG molecular weight on the phase separation of the PLA–PEG copolymers, formation and the size of the micellar particles might have been expected, since the formation of micelles in a selective solvent is believed to be a balance between attractive interactions of non-soluble PLA moieties and repulsive interactions of soluble PEG segments (Riley *et al.*, 1999). However, the micellar-like particles produced using PLA–PEG copolymers containing a 5 kDa PEG moiety had very similar hydrodynamic diameters to the particles produced from the corresponding PLA–PEG copolymers containing a shorter 2 kDa PEG moiety. This indicates that the determining factor in the micellar assembly of the PLA–PEG copolymers is mainly the characteristics of the PLA moiety.

Dispersions of PLA–PEG micellar-like particles were also prepared using different concentrations of the copolymer in the organic solvent. The particle size data are shown in Table III. It can be seen that for the PLA–PEG systems (3:5, 13:5 and 25:5) there is no significant effect of the polymer concentration on the resultant particle size.

Our previous work on the production of surfactant free nanoparticles by phase separation of poly(malic acid-*co*-benzyl malate) and PLGA polymers (not containing PEG) demonstrated a dependence of the particle size of the resultant particles and the concentration of the copolymer in the acetone solution (Stolnik *et al.*, 1995). The use of polymer concentrations (above 1.5%) resulted in an uncontrolled precipitation of the PLGA polymer with large amounts of non-spherical material deposited. It would therefore appear, that the PEG chain is able to moderate the association of the PLA–PEG molecules by preventing the agglomeration of the PLA chains. For the core of micellar-like particles produced from PLA–PEG with higher molecular weight PLA segments (>30 kDa), the PEG chain (5 kDa) was too small in relation to the size of the PLA chain, to influence the degree of agglomeration. Hence, for these PLA–PEG copolymers the resultant particle size was found to increase with increased initial polymer concentration (Riley *et al.*, 1999) by a process similar to that occurring in the more concentrated solutions of the PLGA copolymers.

The effect of different molecular compositions of the PLA and PEG copolymers on the phase separation production process, was assessed by determining the composition of the acetone/water mixture at which the phase separation occurred. This was done experimentally by measuring changes in light scattering intensity of the copolymer-acetone solution as water was gradually added (Fig. 2). Two distinctive regions in the light scattering profiles can be defined. Namely, an initial region of low scattering intensity, which is

TABLE II Hydrodynamic diameters for the PLA–PEG systems produced by two methods: addition of acetone into the water phase or addition of water into the acetone solution (PI, polydispersity index)

Copolymer PLA–PEG	*Acetone to the water phase*		*Water to acetone solution*	
	Diameter ± SD (nm)	*PI*	*Diameter ± SD* (nm)	PI
2:5	24.8 ± 0.5	0.187	50.5 ± 0.2	0.487
3:5	26.6 ± 0.4	0.186	59.4 ± 0.6; 67.7 ± 0.5*	0.517; 0.454
4:5	27.4 ± 0.3	0.181	132.7 ± 0.9; 95.3 ± 0.6	0.383; 0.457
6:5	30.3 ± 0.7	0.238	32.5 ± 0.8; 54.7 ± 1.4	0.231; 0.508
10:5	42.7 ± 1.9	0.393	42.2 ± 0.4	0.286
13:5	76.4 ± 0.2	0.150	388.9 ± 3.6; 161.6 ± 1.5	0.391; 0.338
25:5	71.2 ± 0.5	0.151	131.0 ± 0.7; 153.4 ± 1.1	0.301; 0.338
3:2	26.3 ± 0.4	0.282	43.4 ± 0.4	0.525
4:2	28.7 ± 0.2	0.257	38.8 ± 0.3	0.435
6:2	35.1 ± 0.3	0.201	39.1 ± 0.9	0.310

* Results for separate batches.

characteristic of unassociated chains in solution (the PLA–PEGs are dissolved as unimer molecules), followed by a region where the scattering intensity increases sharply, which is characteristic of the formation of micelles. The composition of the water/acetone mixture at which this intensity change occurred falls in a relatively narrow range of water/acetone ratios between 0.13 and 0.16 for all copolymers (apart from the PLA–PEG 2:5) (Fig. 2(b)). For the PLA–PEG 2:5 copolymer, the increase in light scattering was found to be gradual, occurring between water/acetone ratios of 0.2–0.3. This may be explained by the fact that, contrary to the other PLA–PEG copolymers, the 2:5 copolymer is soluble in both the water and the acetone solvents.

The sharp increase in the scattering intensity seen for the PLA–PEG copolymers with higher molecular weight PLA segments (Fig. 2(b)), suggests that the self-assembly process is instantaneous (Elias, 1973). This suggestion is also supported by the particle size analysis data. As indicated above, the unimer to micelle transition for the PLA–PEG copolymers with a lactide segment between 3 and 25 kDa occurs in a relatively narrow range of non-solvent to solvent composition (0.13–0.16 ratio of water/acetone). A comparison can be made with the separation properties of a range of D,L-PLA homopolymers under the same experimental conditions. For the homopolymers with the molecular weights of 85, 129 and 137 kDa the changes in scattering intensity occurred at a water/acetone ratio of 0.13, while for a lower molecular weight homopolymer of 28 kDa the separation point was at a water/acetone ratio of 0.16. The close agreement between the values obtained for the PLA homopolymers and the PLA–PEG copolymers further demonstrates the dominant effect of the PLA block on the solubility and phase separation behaviour of the PLA–PEG copolymers.

The characteristics of the PLA–PEG copolymer assembly mechanism and the formation of micellar-like particles have implications for drug delivery. Due to the fact that the micellar-like particles have a solid-

TABLE III .Particle size and polydispersity index for the PLA–PEG micelles. The effect of the concentration of the copolymer in the organic phase (PI, polydispersity index)

Copolymer	1%w/v		2% w/v		3% w/v		4% w/v	
PLA–PEG	*Size ± SD* (nm)	*PI*	*Size ± SD* (nm)	*PI*	*Size ± SD* (nm)	*PI*	*Size ± SD* (nm)	*PI*
3:5	33.3 ± 0.9	0.265	29.8 ± 0.5	0.244	26.5 ± 0.6	0.169	25.2 ± 0.3	0.165
13:5	89.8 ± 0.5	0.121	104.5 ± 0.5	0.126	89.3 ± 0.8	0.134	99.6 ± 1.4	0.136
25:5	82.3 ± 1.5	0.123	79.7 ± 0.7	0.123	84.3 ± 0.7	0.143	91.5 ± 2.2	0.180

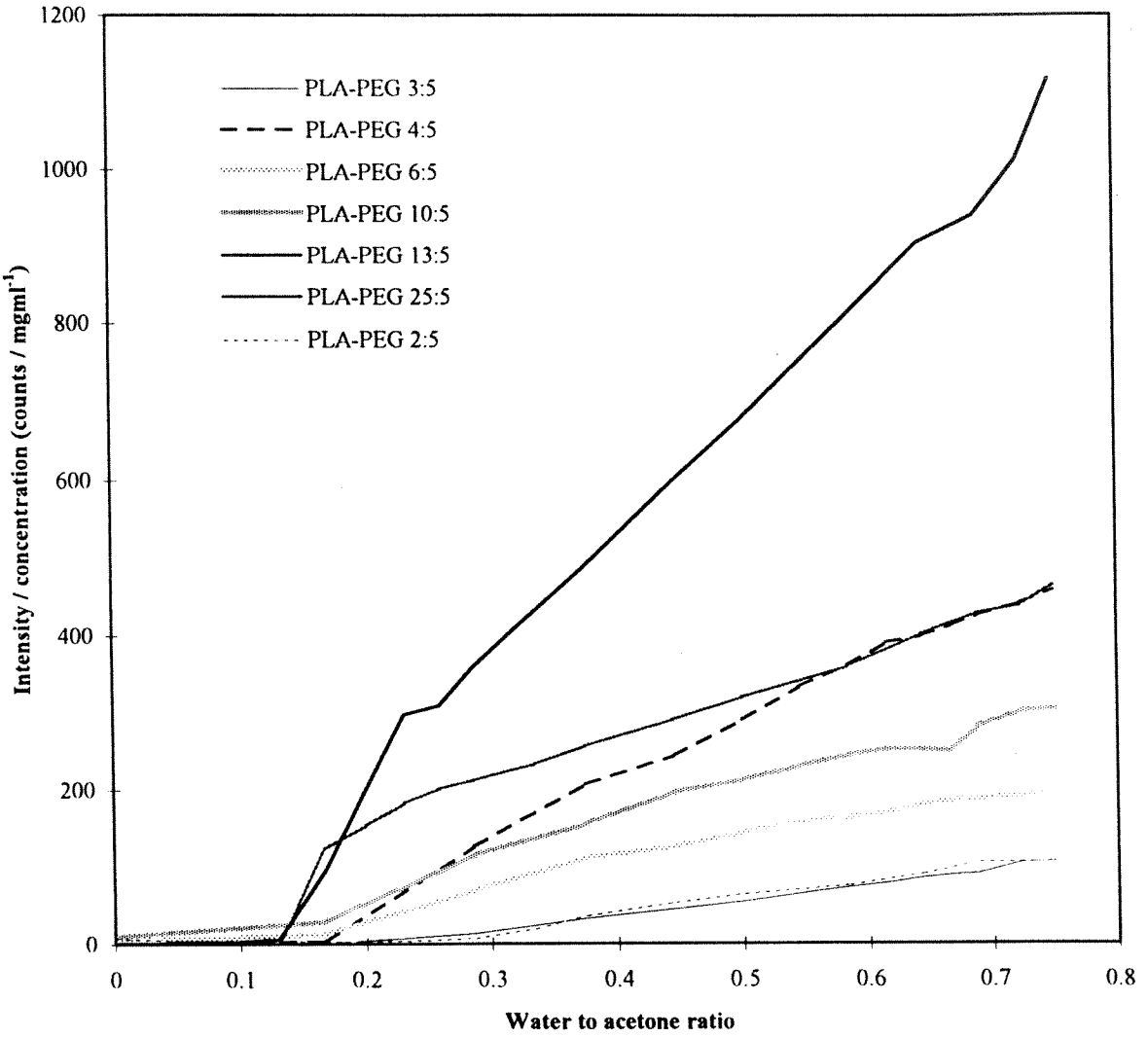

FIGURE 2 (*caption overleaf*)

like nature the incorporation of drugs is not likely to follow a phase partition equilibrium. In order for a drug to be incorporated into the PLA–PEG micellar-like particles using the phase separation production method, the drug will need to separate from the organic phase slightly earlier or simultaneously with the assembly of the PLA–PEG micelle. Variations in the composition of the PLA–PEG copolymers, could be considered as a possible option to coordinate these two events by tailoring a solubility/phase separation for the copolymer so as to meet the solubility requirements of the drug. However, our results suggest that such an approach would be difficult to achieve, at least for the PLA–PEG copolymers with the molecular weights and compositions investigated here.

Surprisingly, the light scattering intensity curve at higher ratios of water (Fig. 2) does not reflect the particle sizes of these systems as listed in Table II (first column). For instance, the scattering was higher

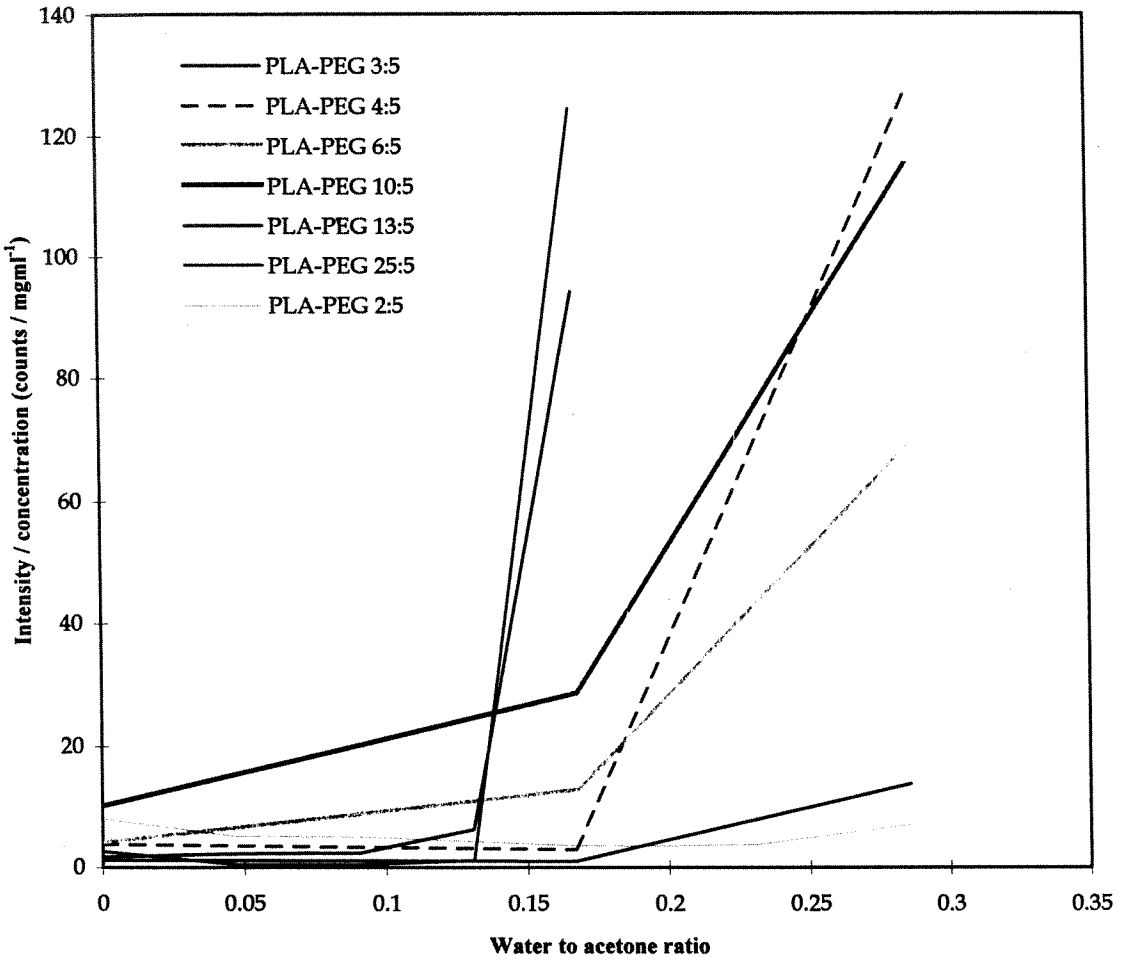

FIGURE 2 The light scattering intensity for the PLA–PEG copolymers as the function of the composition of the solvent (water to acetone ratio).

for the 4:5 PLA–PEG system than for 10:5 PLA–PEG system, although the particles obtained for the latter system were larger. To investigate this phenomenon further the particle sizes for the PLA–PEG micellar systems produced by the two different methods, "addition into water" and "addition of water" were compared (Table II, first and second column). It was assumed that the gradual addition of a non-solvent during the "addition of water" method might provide better control over the assembly of the micelles. However, "lumps" were observed during the production and the batch to batch particle size was not reproducible (Table II, second column). It can further be seen that the particle size of the resultant product was not dependent on the molecular weight of the PLA moiety. These results indicate that an uncontrolled agglomeration of the PLA–PEG molecules occurred rather than their association into micellar particles. A possible reason may be an inability of the PEG moiety to protect the copolymer chains from agglomeration during the separation process. As shown above, the association of the PLA–PEG copolymers takes place at water/acetone mixtures with a high content of acetone. Interestingly, the 5 kDa PEG homopolymer cannot be dissolved in acetone at room temperature, and it may therefore be that in a

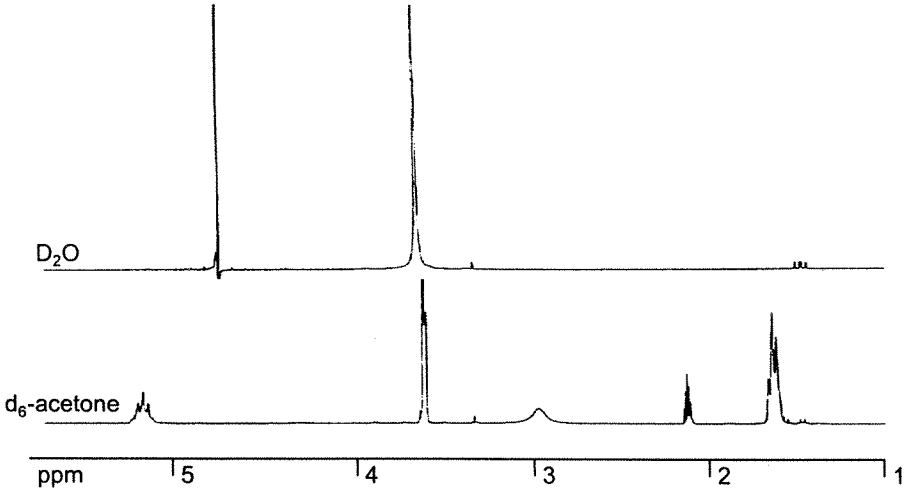

FIGURE 3 The NMR spectra of PLA–PEG10:5 in d_6-acetone and D_2O.

mixture with high acetone content a 5 kDa PEG moiety cannot provide adequate steric stabilisation of formed molecular associates, resulting in their uncontrolled agglomeration. In contrast, during the production of the PLA–PEG micellar particles by the "addition into water" method, the composition of the solvent mixture is always such that the water/acetone ratio is never lower than 0.75, such that the PEG moiety is in a "better" solvent and thus able to sterically stabilise the system.

NMR Analysis

NMR analysis was applied in order to determine the composition of the copolymers when dissolved in a deuterated organic solvent (d_6-acetone) and to assess the structure of the PLA–PEG micellar like particles in deuterated water.

Fig. 3 shows a typical NMR spectrum (for the PLA–PEG 10:5 system) recorded in d_6-acetone and D_2O. It can be seen that in d_6-acetone complete resolution of the PLA and PEG blocks is obtained. This indicates that the copolymer was dissolved in the form of unimer molecules, as also suggested by the light scattering experiments described above. The spectrum for the PLA–PEG micellar like particles in D_2O differs from that in d_6-acetone. First, the methine (CH) peak from the PLA, that was recorded in d_6-acetone at 5.23 ppm has "disappeared" (apart from that in the micellar system obtained with PLA–PEG 2:5 copolymers, data not shown). Then, the integral ratio of the signals characteristic of the PEG moiety (CH_2CH_2) and PLA moiety (CH_3) suggests that not all of the methyl protons (CH_3) from the PLA block were detected. Finally, the form of the peaks has changed, in that the methyl peak (CH_3) of the PLA, which was in a form of a multiplet signal in d_6-acetone (1.59 ppm), has changed into a double doublet signal in D_2O (1.45 ppm), and the PEG ethylene oxide (CH_2CH_2) peak has shifted slightly downfield to 3.68 ppm. These shifts can be attributed to the methyl groups being located in the vicinity of a solid–liquid interface between the PLA and PEG blocks. Here the PLA molecules are in a more liquid-like state and consequently detectable by NMR (Heald *et al.*, 2001).

The lack of the methine (CH) peak at 5.23 in the spectrum of the PLA–PEG micellar systems in D_20 reveals that the PLA moiety is in a solid-like environment that cannot be resolved by the liquid NMR. Regarding the methyl protons from the PLA portion, the signal for the proton detected in D_2O was compared to the signal in d_6-acetone. A comparison of the signals in the two different solvents are shown in Table IV. (It was assumed that all the methyl protons were observed in the d_6-acetone spectra.) Values

between 14 and 35% were obtained for D_2O. It can therefore be concluded that for the PLA–PEG micellar like particles in D_2O, many of the methyl protons (CH_3) were not detected in the liquid state NMR experiment.

A comparison between these two sets of NMR data clearly demonstrates a difference in the arrangement of the copolymer molecules in the two solvents. In acetone the PLA–PEG copolymers are dissolved as individual molecules. In contrast, in an aqueous environment, the PEG segments are dissolved while the PLA portions appears to be "hidden". It is tempting to conclude from these results that the surface is completely covered with PEG, which hides the PLA core. However, the fact that most of the methyl protons from the PLA cannot be detected could also be due to the solid-like nature of their environment. To investigate this further, a control experiment was performed in which a PLGA copolymer was either dissolved in $CDCl_3$ or employed as colloidal particles suspended in D_2O. (These were produced surfactant free by the precipitation method (Fessi *et al.*, 1986; Stolnik *et al.*, 1995a,b)). Complete resolution of the PLGA structure was obtained when the polymer was dissolved in $CDCl_3$, whereas in the case of the dispersion of PLGA particles in D_2O, signals for the PLGA structures were not obtained in the NMR spectrum (Table V). This suggests that the PLGA molecules in the nanoparticles matrix were in a solid-like environment and therefore the structure was not resolved by the liquid state NMR experiment. Hence, the lack of a characteristic PLA signal in the NMR spectrum obtained for the PLA–PEG micelles suspended in D_2O, indicates that the micellar core also had a relatively solid-like nature and that unimer PLA–PEG molecules were not present dissolved in D_2O.

In order to obtain information on the dynamic properties of PLA–PEG micellar-like particles, these systems were compared with a micellar solution of a conventional non-ionic surfactant, Triton X-100. The NMR spectra obtained for Triton X-100 dissolved in d_6-acetone and in D_2O were compared. Both spectra contained characteristic attributes of the Triton X-100 structure, reflecting the dynamic nature of the Triton X-100 micelles in aqueous media; the only difference being that the resolution of the signals was better in d_6-acetone as compared to D_2O where the signals were much broader. Similar results have been reported previously for non-ionic micelles in a series of organic solvents (Podo *et al.*, 1973). This comparison between Triton X-100 and the PLA–PEG copolymer spectra in D_2O lends further support to our hypothesis concerning the solid-like nature of the core of PLA–PEG micellar systems.

It should be noted, that two forms of the methyl (CH_3) signal were seen for the PLA–PEG 2:5, 3:5 and 4:5 micellar particles (Table IV), indicating that the methyl protons detected were located in two different environments. These can be attributed firstly to the protons at the PLA and PEG interface, as mentioned earlier, seen in the spectrum at 1.45 ppm for all the micellar systems. The second type of methyl protons are recorded in the spectra of these systems as a multiplet at 1.55 ppm. The fact that these methyl protons show a different chemical shift from those at the PLA/PEG interface (1.45 ppm) suggests that they belonged to a portion of the PLA chain that was in different chemical environment. Since these protons appeared only in the spectra of the micellar particles produced from the PLA–PEG copolymers containing low molecular weight PLA moieties it is possible that the chains/dynamics of these micellar like particles had a higher mobility.

With regard to the PEG corona, the results in Table IV indicate that the behaviour of the PEG portion were similar in all the micellar systems (i.e. all CH_2CH_2 have a similar chemical shift of ~3.67 ppm) and all had a corona layer that extended out from the central core into the aqueous environment of the D_2O solvent. The NMR peak shows that the PEG protons were all in a similar NMR environment. However, the broadening of the signal at the bottom of the peak, as compared to the PEG protons in the d_6-acetone environment, indicates an enhanced mobility of some of the PEG protons, probably those protruding furthest away from the central PLA core. Further NMR experiments conducted to study these structures in more detail (namely temperature, solid-state, T_1

TABLE IV Table of the NMR chemical shifts for the series of the PLA–PEG copolymers in d_6-acetone and in D_2O (dd, double doublet; s, singlet; m, multiplet)

Copolymer PLA–PEG	*d_6-acetone*			*D_2O*			
	δ (CH)	δ (CH_2CH_2)	δ (CH_3)	δ (CH)	δ (CH_2CH_2)	δ (CH_3)	% CH_3
2:5	5.23 m	3.62 s	1.59 m	5.21 m	3.67 s	1.56 m; 1.41 dd	33
3:5	5.23 m	3.62 s	1.53 m	–	3.68 s	1.55 m; 1.45 dd	18
4:5	5.22 m	3.65 s	1.58 m	–	3.68 s	1.54 m; 1.47 dd	35
6:5	5.25 m	3.63 s	1.61 m	–	3.67 s	1.42 dd	13
10:5	5.26 m	3.64 s	1.60 m	–	3.68 s	1.44 dd	17
13:5	5.23 m	3.64 s	1.63 m	–	3.67 s	1.45 dd	14
25:5	5.24 m	3.66 s	1.61 m	–	3.67 s	1.45 dd	21

and validation of the PEG corona using an external reference) are reported elsewhere (Riley *et al.*, 2001).

Colloidal Stability

A study of the colloidal stability of the PLA–PEG micellar systems was performed in order to probe differences in the ability of the PEG corona to provide steric stabilisation of the particles. Fig. 4 shows the effect of the addition of an electrolyte (sodium sulphate) on the stability of the micellar-like particles. An increase in turbidity was taken as a measure of particle flocculation. The results clearly indicate that the presence of the PEG corona provided an effective steric stabilisation barrier up to high concentrations of sodium sulphate (0.65–0.73 mol/l), apart from the case of the PLA–PEG 25:5 micellar particles. A decreasing colloidal stability was seen as the molecular weight of the copolymer and thereby the particle size increased. This effect can be correlated with higher attractive forces between particles that may be expected as their particle size increases (Israelchivili, 1985). The inability of the 5 kDa PEG moiety to stabilise the PLA–PEG 25:5 micellar system was unexpected since previous work had shown decreasing stability for PLA–PEG micellar particles only when the PLA segment was larger than 30 kDa (Riley *et al.*, 1999). Interestingly, previous work from our group has demonstrated that 5 kDa PEG chains in the block copolymer poloxamine 908 (tetrafunctional poly(ethylene oxide)–poly(propylene oxide) copolymer) (M_W 25 kDa) adsorbed on the surface of a 156 nm diameter polystyrene colloid could provide an effective steric stabilisation against flocculation for sodium sulphate concentrations up to 0.5 M (Stolnik *et al.*, 1994). Adsorption data for this system (adsorption isotherm and hydrodynamic layer thickness) demonstrated that PEG molecules covered the surface completely and that the PEG chains in the surface layer were in a form of random coils extended perpendicular to the surface (Stolnik *et al.*, 1997). The lower colloidal stability of the PLA–PEG 13:5 and 25:5 micellar-like particles could indicate that the surface coverage with PEG is lower than for the smaller more stable particles produced from PLA–PEG copolymers with lower PLA molecular weights. This suggestion is supported by a modelling study using a self-consistent field modelling technique and a range of PLA–PEG copolymers (2:5–15:50) (Heald *et al.*, 2001). Here it was shown that the surface area available for each PEG chain was larger for the 15:5 PLA–PEG system than for the rest of the series studied. This indicates that the density of the PEG on the surface should be lower for the larger particles. This could have an important effect on *in vivo* behaviour.

TABLE V Table of NMR chemical shifts for PLGA in $CDCl_3$ and in D_2O (m, multiplet; d, doublet)

	NMR peaks
PLGA ($CDCl_3$)	CH_3 1.92 m CH_2 5.21 d CH 5.58 m
PLGA (D_2O)	no peaks present

TABLE VI Organ deposition for a series of PLA:PEG micellar systems in rat three hours post intravenous injection

Organ	*PLAPEG 3:5*	*PLA:PEG 6:5*	*PLA:PEG 10:5*	*PLA:PEG 13:5*	*PLA:PEG 25:5*	*In-oxine**
Liver	82.3 ± 8.9	72.7 ± 4.4	72.2 ± 1.9	53.5 ± 0.7	57.5 ± 1.3	8.5 ± 0.6
Spleen	2.1 ± 0.2	2.0 ± 0.3	2.2 ± 0.4	4.1 ± 0.2	5.5 ± 0.5	1.7 ± 0.6
Lungs	0. 4 ± 0.1	0.1 ± 0.0	0.2 ± 0.1	0.9 ± 0.5	0.9 ± 0.7	0.6 ± 0.1
Kidney	0.2 ± 0.1	0.1 ± 0.0	0.3 ± 0.1	0.8 ± 0.1	0.61 ± 0.1	1.03 ± 0.1
Carcass	5.0 ± 1.7	3.1 ± 0.2	7.9 ± 1.2	13.5 ± 0.6	10.1 ± 0.5	14.1 ± 1.0
Blood	3.2 ± 0.9	0.5 ± 0.1	2.9 ± 0.1	28.9 ± 0.6	24.1 ± 0.6	15.4 ± 1.2
Recovery	89.9 ± 7.7	77.9 ± 4.9	82.9 ± 2.8	72.9 ± 0.4	74.6 ± 2.1	25.9 ± 1.3

* Organ deposition of free In-111-oxine.

Biological Performances

Fig. 5 and Table VI summarise the results of the *in vivo* experiments on the biodistribution of PLA–PEG micellar-like particles loaded with the model drug, In-111-oxine complex. The biodistribution of the complex incorporated into the micellar particles was very different to that found for the free label. Hence, since the label can be considered as a model drug/diagnostic agent, the results demonstrate the opportunity to change the biodistribution/pharmacokinetics of a drug by means of the PLA–PEG micellar carrier systems.

The fate of the model drug in the various PLA–PEG micellar-like systems can be defined, in terms of its clearance from the blood and organ deposition. Both were highly dependent upon the molecular weight of the PLA moiety and hence the particle size of the particles. The particles made from the lower molecular weight PLA–PEG copolymers (3:5, 6:5 and 10:5) were rapidly cleared from the systemic circulation by the liver with approximately 70–80% of the radioactivity associated with the liver 3 h post injection (Fig. 5 and Table VI). However, particles prepared from the higher molecular weight PLA–PEG copolymers (13:5 and 25:5) provided extended circulation times for the complex with approximately 25% of the injected dose in blood 3 h after administration and a reduced liver uptake of approximately 50% of the injected dose. It should be noted, that this reduction in the liver accumulation was less pronounced for the PLA–PEG 13:5 and 25:5 micellar particles than the reduction in liver uptake previously observed for polystyrene nanoparticles coated with a layer of PEG (Stolnik *et al.*, 1994). It is likely that this could be due to a lower PEG surface coverage (PEG density) provided by the PLA–PEG 25:5 system (as suggested by the colloidal stability test and modelling) as compared to a polystyrene system coated with poloxamine 908. It has been shown elsewhere, that the density of the PEG layer on the surface of nanoparticles greatly influences the blood circulation time both for physically adsorbed PEG or PEG grafted onto the matrix of the particles (Dunn *et al.*, 1994; Stolnik *et al.*, 2001). However, it is also likely that the difference in particle size between the two systems would also be an important factor in determining the biodistribution.

It is interesting that the micellar like particles obtained from the lower molecular weight PLA–PEG copolymers (PLA–PEG 3:5, 6:5, 10:5), that showed effective steric stabilization (Fig. 4), were cleared rapidly from the systemic circulation and accumulated mainly in the liver. Effective steric stabilization of particles has been identified to be a critical factor for the extended circulation of both nanoparticles and liposomes, by reducing interactions with the opsonic factors that cause injected particulates to be recognised and removed by phagocytosis (Stolnik *et al.*, 1995). Hence, this indicates that other factor(s) affect their fate. Whether the differences observed in the biodistribution of PLA–PEG particles can be attributed mainly to the size differences between those that exhibit prolonged circulation times (diameter above 70 nm), and those that are rapidly cleared (diameter below 70 nm), or other factors such as a possible difference in the arrangement or mobility of

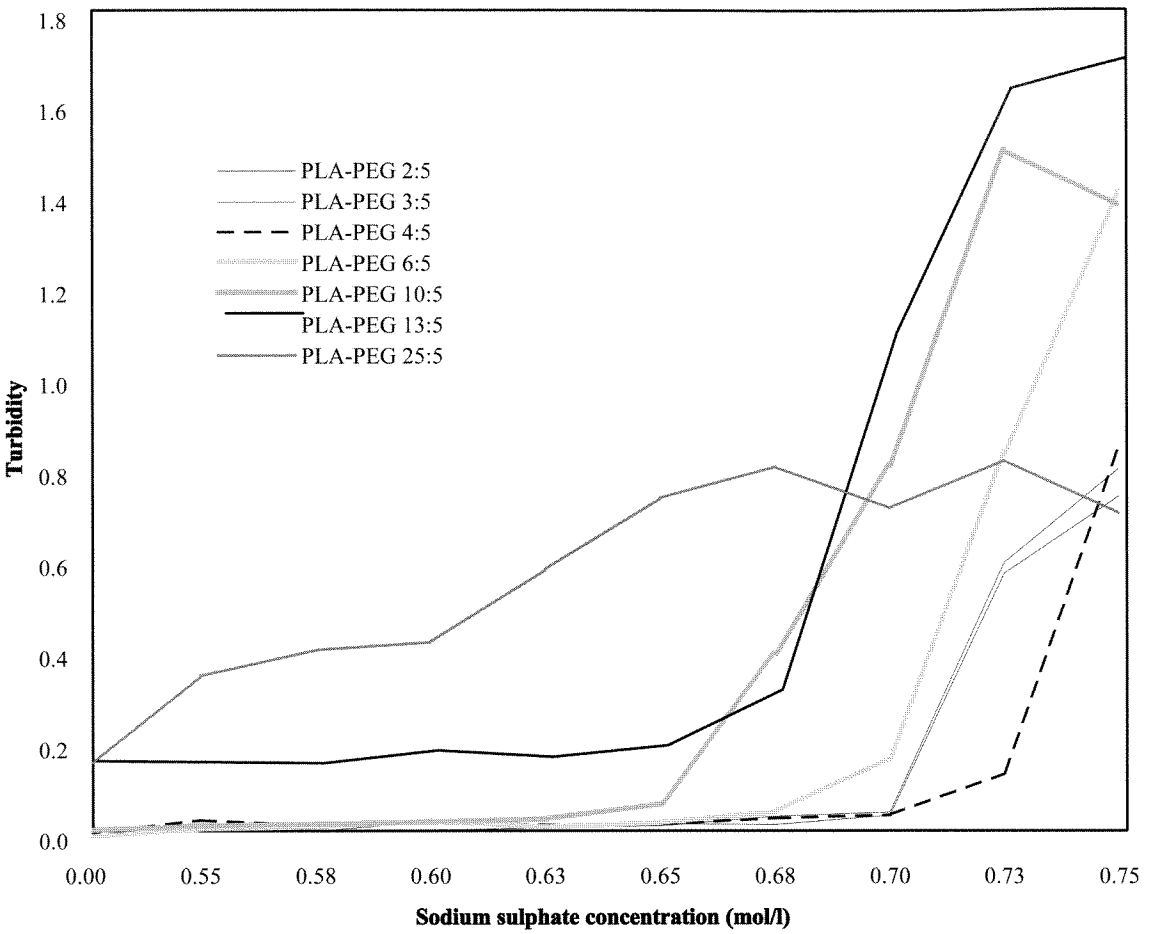

FIGURE 4 Critical flocculation concentration for dispersions of PLA–PEG micellar type particles.

the PEG chains in the corona, can only be a matter of speculation at the present time. It can be noted that recent literature has suggested that the effect of attached PEG moieties on the blood circulation of colloids is limited to particles in a size range between approximately 60 and 200 nm in diameter (Moghimi *et al.*, 1993; Litzinger *et al.*, 1994). For instance, irrespective of the PEG surface coverage, particles with a diameter of above approximately 200–300 nm exhibit enhanced accumulation in the spleen. This is believed to be the result of mechanical filtration (a function of splenic construction and intrasplenic microcirculation), followed by eventual phagocytosis in the red pulp macrophages (Moghimi *et al.*, 1993). Those processes that could provide a lower size limit for extended blood circulation are less well understood. Increased accumulation of nanoparticles in the liver has been observed and attributed to the possible penetration of such small particles through the fenestrae in the endothelial lining of the liver and their association with parenchymal cells (Litzinger *et al.*, 1994). These fenestrations have been reported to have a mean diameter of approximately 100 nm (Wisse, 1970). Hence, the small sizes of PLA–PEG 3:5, 6:5 and 10:5 micellar-like particles (26.6, 30.3 and 42.7 nm, respectively) could well be a crucial factor that influenced their accumulation in the liver. Experiments on the intrahepatic distribution of PLA–PEG micellar-like systems have been performed in our group to assess differential cellular uptake within

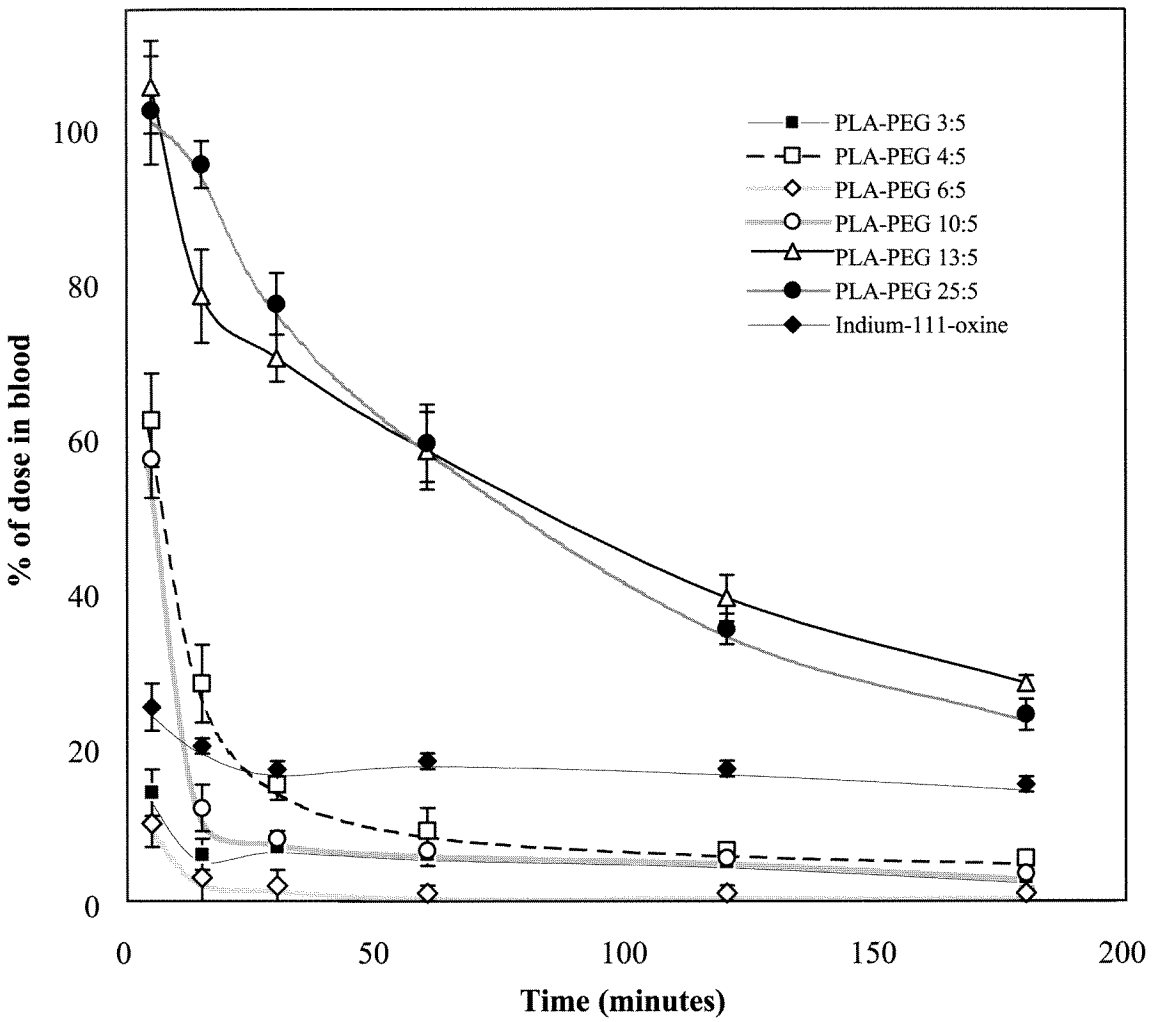

FIGURE 5 Blood clearance profiles for a series of PLA–PEG micellar systems in rats post intravenous administration.

the liver (hepatocytes, endothelial cells, Kupffer cells). Our preliminary results suggest that small sterically stabilised particles can distribute mainly to the parenchymal cells of the liver after intravenous injection.

CONCLUSION

In the present investigation, a range of PLA–PEG copolymers, with a constant size of the PEG moiety of 5 or 2 kDa, and an increasing size of the PLA moiety were assessed for their *in vitro* and *in vivo* properties. The results from the physicochemical characterisation suggests that in an aqueous medium (selective solvent) these copolymers assemble into micellar-like particles, where the PLA segments form the core and the PEG chains form a corona protruding into the aqueous environment. The micellar-like particles so formed are not as dynamic as micelles obtained from conventional non-ionic surfactants (such as Triton X-100). Indeed, the PLA–PEG molecules do not appear to migrate between unimer and micellar states. The micellar particle core is maintained in a solid-like

state, especially for the PLA–PEG copolymers with higher molecular weight PLA moieties. The kinetic stability of PLA–PEG micellar-like particles has implications for their possible use as drug carriers. The solid-like nature of the particle core will preserve a micellar structure even after rapid dilution in body fluids for example after intravenous injection and so prevent rapid drug release. However, an equally important factor for effective drug incorporation within the PLA matrix will be the manner in which the PLA chains pack together and the available space for drug molecules.

The *in vivo* data showed that the PLA–PEG micellar-like particles with a size smaller than approximately 70 nm in diameter accumulated in the liver, despite the presence of a stabilizing PEG corona. In contrast, particles with a diameter above 70 nm showed prolonged blood circulation times and a reduced liver uptake. This, together with the results from other research groups, indicates that particulates with a size smaller than the fenestrae in the endothelial lining of the blood vessels of the liver can extravasate. Whether such small particles can interact with parenchymal cells and be internalised, needs to be studied further. Initial results have shown an increased uptake of nanoparticles into these cells. The present work demonstrates that by controlling the size of the PLA moiety, PLA–PEG copolymers can be used for the production of small micellar-like particles capable of accumulating in the liver (hepatocytes?) or freely circulating in the blood stream.

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