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Defining the drug incorporation properties of PLA–PEG nanoparticles

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Abstract

The drug incorporation and physicochemical properties of PLA–PEG micellar like nanoparticles were examined in this study using a model water soluble drug, procaine hydrochloride. Procaine hydrochloride was incorporated into nanoparticles made from a series of PLA–PEG copolymers with a fixed PEG block (5 kDa) and a varying PLA segment (3–110 kDa). The diameter of the PLA-nanoparticles increased from 27.7 to 174.6 nm, with an increase in the PLA molecular weight. However, drug incorporation efficiency remained similar throughout the series. Incorporation of drug into the smaller PLA–PEG nanoparticles made from 3:5, 15:5 and 30:5 copolymers did not influence the particle size, while an increase was observed for the larger systems comprising 75:5 and 110:5 copolymers. An increase in drug content for PLA–PEG 30:5 nanoparticles was achieved by increasing the theoretical loading (quantity of initially present drug). The size of these nanoparticles remained unchanged with the increasing drug content, supporting the proposed micellar type structure of the PLA–PEG 30:5 nanoparticles. The morphology of these systems remained unchanged both at low and high theoretical drug loadings. Formulation variables, such as an increase in the aqueous phase pH, replacement with the base form of the drug and inclusion of lauric acid in the formulation did not improve the incorporation efficiency of drug into PLA–PEG 30:5 nanoparticles. While poly(aspartic acid) as a complexation agent did not improve the drug incorporation efficiency of procaine hydrochloride, it did so for another water soluble drug diminazene aceturate. This may be attributed to a stronger interaction of diminazene aceturate with poly(aspartic acid) relative to procaine hydrochloride, as confirmed by thermodynamic analysis of isothermal titration calorimetric data. The drug incorporation and physicochemical characterisation data obtained in this study may be relevant in optimising the drug incorporation and delivery properties of these potential drug targeting carriers.

Keywords: PLA–PEG; Drug incorporation; Nanoparticles; Micelles; Calorimetry; Targeting

1. Introduction

Site specific delivery is a recognised strategy for improving the therapeutic efficiency and safety of

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drugs (Couvreur et al., 1990; Gregoriadis, 1991; Sinko and Kohn, 1993). Colloidal carriers, such as nanoparticles or polymeric micellar systems, comprise one drug delivery system which is currently being widely investigated for their potential to achieve targeted drug delivery (Porter et al., 1992; Gref et al., 1995; Song et al., 1995; Hawley et al., 1997). However, once injected intravenously these carriers are quickly removed from the circulation by macrophages located in the reticuloendothelial system, thus hindering site specific delivery of drugs to other organs or tissues in the body. In an attempt to reduce or minimise particle interaction with opsonins, which facilitate this phagocytic process, the concept of steric stabilisation of particulates was introduced. To this end, different types of drug carriers with prolonged blood circulation times have been designed, e.g. polymer coated particulates (Illum et al., 1987; Stolnik et al., 1994; Dunn et al., 1994), diblock polymer grafted particulates (Gref et al., 1994; Emile et al., 1996) and diblock polymeric micelles (Kwon and Kataoka, 1995; Piskin et al., 1995). Poly(ethylene glycol) (PEG) has been used to provide a steric barrier and to modify the surface of these systems (Torchilin and Papisov, 1994; Stolnik et al., 1995a).

Nanoparticulates, surface modified with PEG, are being increasingly investigated. The in vivo behaviour of particulate systems prepared from diblock polymers with PEG as the hydrophilic component, such as PLA–PEG, has been widely examined (Gref et al., 1994; Verrecchia et al., 1995). We have studied extensively the physicochemical properties of drug free PLA–PEG systems (Riley et al., 1997, 1999). However, there is currently a lack of studies in the literature which focus on the drug encapsulation properties of a series of such assemblies. The incorporation of water insoluble drugs such as lidocaine and prednisolone (Peracchia et al., 1997) and oligonucleotides (Emile et al., 1996) into PLGA–PEG and PLA–PEG nanoparticles, respectively, have been reported recently. These systems generally suffer from poor drug incorporation efficiencies, especially of water soluble drugs, due to their small size and hence large surface area, which promotes drug loss into the aqueous phase during particle formation. Elucidating the drug incorporation characteristics of these systems will therefore impact on reducing the amount of drug carrier required for patient administration as well as reducing drug loss and hence minimising manufacturing costs.

In this study, we have therefore focused on examining the drug incorporation properties of a series of PLA–PEG nanoparticles using a model water soluble drug, procaine hydrochloride. The physicochemical characteristics of these drug-loaded systems have been assessed in terms of size, surface charge and morphology. Formulation variables, which have been employed to improve the drug incorporation efficiency of other nanoparticulate systems, were also applied to nanoparticles prepared from a PLA–PEG 30:5 copolymer. Finally the enhancement of drug incorporation efficiency using poly(aspartic acid) as a complexing agent was investigated for two model water soluble drugs, procaine hydrochloride and diminazene aceturate.

2. Materials and methods

2.1. Materials

Poly(lactic acid)–poly(ethylene oxide) PLA–PEG diblock copolymers, with a fixed PEG chain length (5 kDa) and a varying PLA segment (3–110 kDa), were synthesised by a ring opening polymerisation method (Riley et al., 1999). The weight ratio of PLA to PEG was determined by ^{1}H NMR for each copolymer. The nomenclature used is therefore based on the NMR determined weight ratios. Poly(DL–lactide-co-glycolide) (PLGA) (50:50; 67:33; 75:25 and 95:5, average $M_w = 10\,000$; 10 184; 10 048 and 9258 Da, respectively) was synthesised by Zeneca Pharmaceuticals (Macclesfield, UK) and was used as obtained. Procaine hydrochloride, diminazene aceturate, poly(aspartic acid) ($M_w = 26\,000$ Da), HEPES (as sodium salt), Trizma® hydrochloride (Tris–HCl) and lauric acid ($C_{12}H_{23}O_2Na$) were purchased from Sigma Chemical Co. (St. Louis, MO). Acetonitrile (HPLC grade) was obtained from Fisher Scientific (Leicestershire, UK). Water used for all

experiments was ultrapure Elgastat® Option 3 water (Elga Ltd., UK). All other chemicals used were of pharmaceutical grade.

2.2. Methods

2.2.1. Preparation of nanoparticles from a series of PLA–PEG copolymers; the effects of theoretical drug loading and formulation variables

Nanoparticles were prepared according to a modified nanoprecipitation method (Fessi et al., 1989). The starting procedure was as follows. PLA–PEG polymer (unless otherwise stated 50 mg) and a specified quantity of drug (procaine hydrochloride) were weighed accurately and dissolved in acetonitrile (5 ml). The organic phase was added into the aqueous phase (15 ml) and stirred magnetically (Stem AS-601, UK) at room temperature until complete evaporation of the organic solvent had taken place. Drug free nanoparticles were prepared according to the same procedure omitting the drug. All samples were prepared in duplicate.

To investigate the influence of various formulation parameters on procaine hydrochloride incorporation efficiency into PLA–PEG nanoparticles, the following alterations were made to the starting procedure:

- amount of a drug in the organic phase (theoretical drug loading) was increased from 1.0 to 20.0 w/w
- to assess the effect of aqueous phase pH, water pH 5.8 was replaced with 1 mM HEPES buffer adjusted to pH 9.3.
- to study the influence of a negatively charged excipient lauric acid (0.84 mg), was added to the formulation at a 1:1 fatty acid: drug molar ratio.
- to determine the influence of replacing the salt form of the drug with the base form, procaine hydrochloride was converted to procaine dihydrate as reported previously (Govender et al., 1999).

2.2.2. Preparation of PLA–PEG 30:5 nanoparticles with poly(aspartic acid) as a complexation agent

The effect of poly(aspartic acid) as a complexation agent for increasing drug incorporation efficiency into PLA–PEG 30:5 nanoparticles was also investigated. Poly(aspartic acid) (0.63 mg) dissolved in 5 ml 25 mM Tris–HCl buffer pH 5.3 and PLA–PEG 30:5 dissolved in 5 ml acetonitrile were separately but simultaneously added dropwise to procaine hydrochloride (1.04 mg), dissolved previously in 10 ml 25 mM Tris–HCl buffer pH 5.3 and stirred overnight. The charge ratio of poly(aspartic acid):drug($-/+$) was 1.2:1. The above was repeated with poly(aspartic acid) (0.29 mg) and another cationic drug, diminazene aceturate (1.04 mg).

2.2.3. Separation of free from incorporated drug

The nanosuspension was filtered (1 μm filters, Whatman, Japan) and then ultracentrifuged (Beckman L-8 60M Ultracentrifuge, UK) at 55 000 rpm ($311\,000 \times g$) for 3 h at 20°C. The supernatant containing the dissolved free drug was discarded and the pellet freeze-dried (Edwards Modulyo, UK) for 48 h. The nanoparticle recovery was calculated using Eq. (1). The individual values for two replicate determinations and their mean values are reported.

$$\text{Nanoparticle recovery (\%)} = \frac{\text{Mass of nanoparticles recovered} \times 100}{\text{Mass of polymeric material, drug and any formulation excipient used in formulation}} \quad (1)$$

2.2.4. Determination of drug incorporation efficiency

Freeze-dried nanoparticles loaded with procaine hydrochloride were dissolved in acetonitrile (50 ml) (a common solvent for PLA–PEG and the drug). Procaine hydrochloride or procaine dihydrate in the solution were measured by ultraviolet spectroscopy at 289 and 286 nm, respectively (Beckman DU® 64 spectrophotometer, UK) (prior studies established no absorbance interference from the PLA–PEG polymer under the same conditions).

For diminazene aceturate quantification, 1 M NaOH solution (15 ml) was added to the freeze-dried contents and mechanically shaken (IKA® Labortechnik, Germany) for 2 h to promote solubilisation of the entrapped diminazene aceturate/

Pasp complexes and degradation of PLA-PEG to its soluble components. The sample was then made up to 50 ml NaOH and analysed for drug content by UV–vis spectroscopy at 426 nm. Drug free PLA–PEG nanoparticles dissolved in 1 M NaOH were used as the reference solution.

Drug incorporation efficiency was expressed both as drug content (% w/w) and drug entrapment (%); represented by Eqs. (2) and (3), respectively. The individual values for two replicate determinations and their mean values are reported.

$$\text{Drug content (\% w/w)} = \frac{\text{mass of drug in nanoparticles} \times 100}{\text{mass of nanoparticles recovered}} \quad (2)$$

$$\text{Drug entrapment (\%)} = \frac{\text{mass of drug in nanoparticles} \times 100}{\text{mass of drug used in formulation}} \quad (3)$$

2.2.5. Isothermal titration microcalorimetry (ITM)

The thermodynamic parameters defining the interaction between poly(aspartic acid) and diminazene aceturate and procaine hydrochloride was determined by ITM. Calorimetric experiments were undertaken using a thermal activity monitor (TAM 2277, Thermometric AB, Sweden). A sample of diminazene aceturate (3 ml, 0.40 mg/ml) or procaine hydrochloride (3 ml, 0.49 mg/ml) was placed in a sample cell and inserted into the instrument. Once the thermal equilibrium was reached, the titration was performed by consecutive injections (20 μl) of a solution of poly(aspartic acid) at 3 mg/ml for diminazene aceturate and 15 mg/ml for procaine hydrochloride, (centrifuged previously at 13 000 rpm for 5 min to remove any air bubbles or dust (Hettich Zentrifugen, EBA 12, Germany)). All solutions were prepared in 25 mM Tris–HCl buffer and adjusted to pH 5.3. The titrant was added by means of a Hamilton microlab syringe mounted in a computer-operated syringe drive (Lund 6100 syringe pump). A control experiment was performed by injecting the same aliquots of poly(aspartic acid) into the buffer (3 ml, 25 mM Tris–HCl buffer, pH 5.3). The experimental method set up via the Digitam® 3 software allowed for data collection over a 7-min period for the injection and a 5-min baseline period before the next injection. This was found to be adequate for the interaction to proceed to completion at each injection point and reach the baseline before the next injection. Data presented are the mean of a minimum of two replicate titrations. The signs of the released heat values were reversed for data analysis because the output from the instrument employed in this study is from the perspective of the equipment and not the system under study. The pH change of all samples after the experiment did not exceed ± 0.2. The heat energy values were then analysed using specific ligand binding models.

2.2.6. Analysis of binding isotherms

The interaction of drug with poly(aspartic acid) was evaluated using McGhee–von Hippel model, for the binding of non-interacting ligands (such as drug molecules) to a lattice of ligand binding residues, such as poly(aspartic acid). In this model the relation between free drug concentration, D_f, and binding density (moles of bound drug per mole of total binding sites on the polymer), v, is given by Eq. (4)

$$\frac{v}{D_f} = K(1-nv)\left(\frac{1-nv}{1-(n-1)v}\right)^{n-1} \quad (4)$$

where K denotes the association constant, and n is the number of binding sites that are occupied by interaction of one drug molecule on the polymer. The binding density can be represented by Eq. (5)

$$v = \frac{[\text{drug}]_{\text{bound}}}{P_t N} \quad (5)$$

where N denotes number of binding sites on one poly(aspartic acid) molecule, and P_t is the total polymer concentration. On the reaction heat content after i injections, Q_i, is related to bound drug concentration by Eq. (6)

$$Q_i = [\text{drug}]_{\text{bound}} V_i \Delta H \quad (6)$$

where ΔH denotes the enthalpy of interaction of the drug to the polymer and V_i is the total solution volume in the cell of the microcalorimeter after i injections. The observed released heat after

each injection includes the heat of dilution of drug and polymer. The heat of dilution was determined from the end part of the titration curves, because usually after 20 injections the interaction was completed and the released heat was rather constant and fluctuating around an average value (see Fig. 4). However, a blank titration (injection of polymer solution into the cell that contained buffer solution without drug) was run (included in Fig. 1) to ensure that the heat of dilution remained rather constant throughout the titrations and close to the measured dilution heat from the titration.

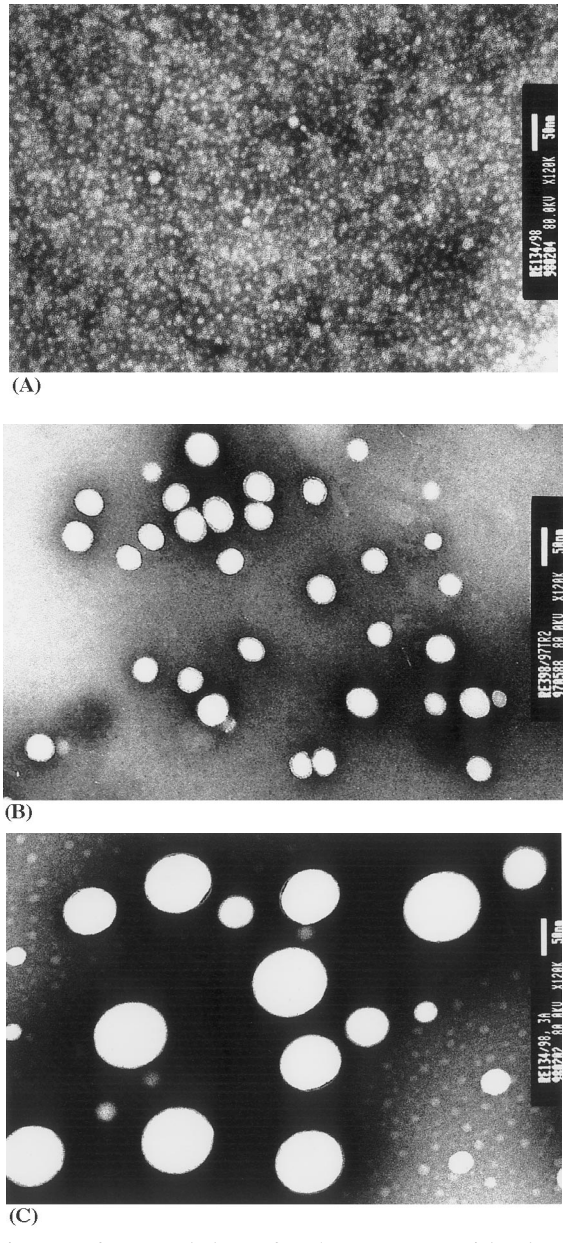

Fig. 1. Surface morphology of PLA-PEG nanoparticles theoretically loaded with 2% w/w procaine hydrochloride. (A) PLA–PEG 3:5, (B) PLA–PEG 30:5, (C) PLA–PEG 110:5

The binding parameters (n, N, K, ΔH) were determined by applying the non-linear least square fits to Eqs. (4)–(6) using the routines available in the Origin 5 program package (Microcal Software, Inc). Because in the calculations of the binding parameters, the concentrations of drug and polymer were used rather than their activities, therefore the abbreviation, obs (for the observation), will be affixed to the determined parameters to specify this condition.

The free energy (ΔG_{obs}) and entropy (ΔS_{obs}) of interaction were determined using $\Delta G_{obs} = -RT \ln(K_{obs})$, and $\Delta S_{obs} = (\Delta H_{obs} - \Delta G_{obs})/T$.

2.2.7. Physicochemical characterisation

2.2.7.1. Particle size. Nanoparticle size was determined using photon correlation spectroscopy (PCS) (Malvern S4700 PCS system, Malvern Instruments Ltd, Malvern, UK). The analysis was performed at a scattering angle of 90° and at a temperature of 25°C using samples diluted with filtered water (0.2 μm filter, Minisart®, Germany). For each sample, the mean diameter ± standard deviation (S.D.) of six determinations were calculated applying multimodal analysis. Values reported are the mean diameter ± S.D. for two replicate samples.

2.2.7.2. Zeta potential. The zeta potential of the particles was determined by laser doppler anemometry (Malvern Zetasizer IV, Malvern Instruments Ltd, Malvern, UK). All analyses were performed on samples diluted with 1 mM HEPES buffer (adjusted to pH 7.4 with 1 M HCl) in order to maintain a constant ionic strength. For each sample the mean value ± S.D. of four determinations were established. Values reported are the mean value ± S.D. for two replicate samples.

Table 1
Physicochemical characterisation of nanoparticles prepared from a series of PLA–PEG copolymers (theoretical procaine HCl loading = 2% w/w)

Copolymer	Particle size ± S.D. (nm) (polydispersity)		Zeta potential ± S.D. (mV)[a]	
	Drug free	Drug loaded	Drug free	Drug loaded
3:5	27.6 ± 1.3 (0.14 ± 0.10)	27.7 ± 0.8 (0.15 ± 0.08)	[b]	[b]
15:5	54.6 ± 0.4 (0.15 ± 0.02)	49.0 ± 0.9 (0.08 ± 0.06)	−6.5 ± 0.7	−6.7 ± 1.2
30:5	72.4 ± 2.8 (0.12 ± 0.03)	68.9 ± 2.2 (0.12 ± 0.04)	−6.4 ± 1.5	−7.8 ± 1.5
75:5	106.6 ± 2.9 (0.10 ± 0.33)	116.5 ± 1.2 (0.09 ± 0.04)	−14.2 ± 0.6	−14.9 ± 1.3
110:5	152.4 ± 1.2 (0.18 ± 0.03)	174.6 ± 1.4 (0.07 ± 0.03)	−28.0 ± 0.4	−26.3 ± 2.1

[a] 1 mM HEPES buffer.
[b] Unable to measure due to insufficient scattered light intensity.

2.2.7.3. Particle morphology. Morphological evaluation of the nanoparticles was performed using transmission electron microscopy (TEM) (Jeol Jem 1010 electron microscope, Japan) following negative staining with phosphotungstic acid solution (3% w/v) (adjusted to pH 4.74 with KOH).

3. Results and discussion

3.1. Drug incorporation into various PLA–PEG series

Procaine hydrochloride was incorporated at a theoretical drug loading of 2% w/w into nanoparticles made from PLA–PEG diblock copolymers with a fixed PEG block (5 kDa) and a varying PLA segment (3–110 kDa). The sizes of drug free nanoparticles increased from 27.6 to 152.4 nm with an increase in the molecular weight of the PLA block of the polymer from 3 to 110 kDa (Table 1). This was expected on the basis of theoretical approaches to the structure of polymeric micelles (Tuzar and Kratochvil, 1993). The PLA–PEG nanoparticle size remained unchanged with incorporation of drug into the 3:5; 15:5 and 30:5 series, while a slight increase was observed for the 75:5 and 110:5 series (Table 1). These results may be rationalised on the basis of our recent comprehensive physicochemical characterisation of this series of PLA–PEG nanoparticles, in the absence of incorporated drug (Riley et al., 1999). The particle size of the PLA–PEG 3:5, 15:5 and 30:5 assemblies was found to depend solely on the length of the PLA block. This suggested that these copolymers form micellar type assemblies, where the PLA blocks associate as linear chains within the particle core (Tuzar and Kratochvil, 1993). In contrast the particle size of the PLA–PEG 75:5 and 110:5 nanoparticles was found to increase as the concentration of copolymer dissolved in the organic phase during particle preparation was increased. This difference was attributed to the PEG block exerting a weaker influence during particle formation as the molecular weight of the PLA block was increased. In effect, the PLA–PEG 75:5 and 110:5 copolymer behaved more like PLA homopolymer itself (Stolnik et al., 1995b), with the PLA blocks agglomerating as entangled chains following precipitation into the aqueous phase. Therefore, there is free space amongst the linear core-forming PLA chains of the micellar type PLA–PEG 3:5, 15:5 and 30:5 nanoparticles. Thus, drug molecules can be incorporated into these regions of the solid core without influencing the particle size of the assembly. However, there is less free space amongst entangled chains of the PLA–PEG 75:5 and 110:5, and the core has to expand to allow incorporation of drug to occur.

Low surface negativity of the 15:5 and 30:5 nanoparticles is due to the end carboxyl acid groups of the PLA chains being capped with PEG. The slightly higher surface negativity of nanoparticles prepared from the 75:5 and 110:5 series is considered to be due to the presence of

PLA homopolymer, because higher ratios of D,L lactide to PEG were used in the synthesis of these polymers (Riley et al., 1999). Incorporation of procaine hydrochloride into these PLA–PEG nanoparticles did not affect their surface negativity (Table 1).

As shown in Table 2, drug content and drug entrapment values were similar for nanoparticles prepared from the various PLA–PEG copolymers from the series, i.e. at a theoretical drug loading of 2% w/w the average drug content and drug entrapment was ~ 0.24 and 8.3% w/w, respectively. These results were initially surprising because higher drug incorporation efficiencies were expected for those systems with larger PLA block segments due to their bigger size which result in a smaller surface area for drug loss as well as a larger core for an increased drug entrapment. For instance, Celikkaya et al. (1996) reported an increasing drug entrapment with PLA–PEG microparticles as the molecular weight of the PLA block was increased. These authors attributed the increase in drug entrapment to the increasing particle size. Although the particle size of the PLA–PEG systems studied here increased from PLA–PEG 3:5 to 110:5, this may be counteracted by changes in the internal structure of the PLA core as discussed above. Furthermore, we recently found (Riley et al., 1997) that the aggregation number of the PLA–PEG nanoparticles increased sharply with an increase in the molecular weight of the PLA block. The low aggregation number of the PLA–PEG 3:5 micellar type nanoparticles is believed to be due to the weaker hydrophobic interactions between the low molecular weight PLA chains. Hence, these low molecular weight copolymers form fairly loosely packed assemblies. However, the number of hydrophobic interactions between the lactic acid units of associating PLA chains increases with the length of the chain, resulting a higher packing density of the PLA–PEG sub-units. This may imply that the mobility of the core also decreases with an increase in the PLA block molecular weight, leading to a decreasing available space within the core for drug incorporation, despite a larger particle diameter. Therefore, although the particle diameter increases, the available internal space for drug incorporation may be decreasing. Another alternative reason for the results in Table 2 may be that an increase in the hydrophobic PLA block segment from 3:5 to 110:5 could result in the core becoming more hydrophobic. Therefore, compatibility of the hydrophilic drug with the PLA PEG core may decrease with an increasing length of the PLA block.

As discussed above, equivalent drug incorporation efficiencies were achieved in this study for PLA–PEG nanoparticles ranging in size from 27.7 to 174.6 nm. These results are important therapeutically because they imply that the drug incorporation efficiency for this drug delivery system is not compromised, even when particle sizes as small as ~ 28 nm may be required specifically.

TEM evaluation showed that the drug loaded nanoparticles from the 3:5; 30:5 and 110:5 copolymer series were spherical and discrete (Fig. 1A–C). This confirmed that drug incorporation into PLA–PEG nanoparticles, prepared from both the lower and higher copolymer series, did not adversely influence particle morphology.

Table 2
Drug incorporation efficiency of nanoparticles prepared from a series of PLA–PEG copolymers (theoretical procaine HCl loading = 2% w/w)

PLA–PEG	Nanoparticle recovery (%)[a]	Drug content (% w/w)[a]	Drug entrapment (%)[a]
3:5	50.16 (50.35; 49.96)[b]	0.27 (0.26; 0.27)	6.44 (6.33; 6.55)
15:5	89.15 (87.97; 90.32)	0.24 (0.25; 0.23)	10.67 (10.92; 10.41)
30:5	69.16 (64.46; 73.86)	0.24 (0.24; 0.23)	7.94 (7.67; 8.20)
75:5	67.60 (70.34; 64.85)	0.20 (0.19; 0.21)	6.62 (6.61; 6.63)
110:5	74.65 (75.63; 73.67)	0.27 (0.25; 0.28)	9.77 (9.50; 10.04)

[a] Mean of the two replicate determinations which are shown in parenthesis.
[b] The relatively low nanoparticle recovery for this system as compared to the others is due to their much smaller size.

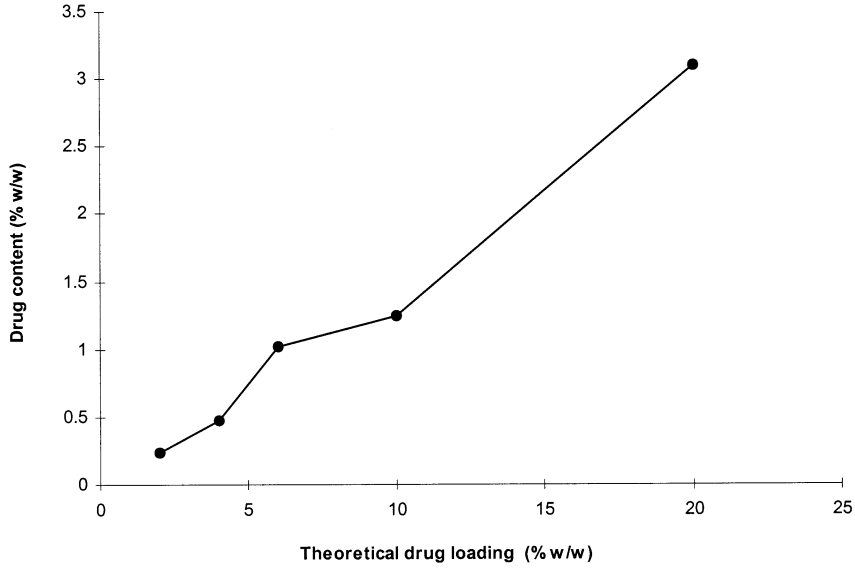

Fig. 2. Influence of procaine hydrochloride theoretical loading on drug content of PLA–PEG 30:5 nanoparticles.

3.2. Influence of theoretical drug loading

The influence of the theoretical loading of a drug into PLA–PEG 30:5 nanoparticles was examined. An increase in the theoretical loading from 1 to 20% w/w led to a corresponding increase in drug content from 0.24 to 3.1% w/w (Fig. 2). Clearly, at higher theoretical loadings there were a greater number of drug molecules available for entrapment into the nanoparticles, thus leading to the higher drug incorporation efficiencies observed. Generally, drug incorporation efficiency into these nanoparticles is limited by their large surface area as well as the water soluble nature of the drug. These two factors accelerate drug loss into the aqueous phase during nanoprecipitation. The results of this study are encouraging, because the 3.1% w/w drug content achieved at an equivalent theoretical loading of 20% w/w is higher than that achieved by Celikkaya et al. (1996) for rifampicin (i.e. 2.3% w/w), although PLA–PEG particles described by Celikkaya et al. (1996) were much bigger (2.3 μm). A similar trend of increasing drug content with the increasing theoretical loading of adriamycin into PLA–PEG micelles has been reported by Piskin et al. (1995).

Table 3
Physicochemical characterisation of PLA–PEG 30:5 nanoparticles prepared with varying theoretical loadings of procaine HCl

Theoretical drug loading (% w/w)	Nanoparticle recovery (%)[a]	Particle size ± S.D. (nm) (polydispersity)	Zeta potential ± S.D. (mV)
0	71.76 (68.78; 74.74)	72.5 ± 2.8 (0.13 ± 0.03)	−6.4 ± 1.5
2	69.16 (64.46; 73.86)	68.9 ± 2.2 (0.12 ± 0.03)	−7.8 ± 1.5
4	62.79 (68.55; 57.03)	66.1 ± 1.2 (0.16 ± 0.02)	−8.5 ± 2.1
6	59.60 (57.72; 61.47)	64.7 ± 1.4 (0.14 ± 0.02)	−7.9 ± 0.3
10	69.83 (66.77; 72.89)	67.1 ± 1.6 (0.14 ± 0.02)	−8.9 ± 1.8
20	66.08 (64.96; 67.20)	68.5 ± 2.8 (0.12 ± 0.03)	−6.1 ± 1.0

[a] Mean of two replicate determinations which are shown in parenthesis.

The nanoparticle recoveries remained unchanged with increasing theoretical drug loadings (Table 3). These findings differ from our previous work with PLGA nanoparticles, also prepared by nanoprecipitation (Govender et al., 1999). In that study, an increasing theoretical loading of procaine hydrochloride led to a decrease in the PLGA nanoparticle recovery. This was attributed to destabilisation of the nanosuspension, which was stabilised solely by the presence of charged groups on the surface i.e. electrostatic stabilisation. With the PLA–PEG systems, steric rather than electrostatic stabilisation is mainly responsible for preventing particle agglomeration (Riley et al., 1999). Hence, in the present study, the presence of excess cationic drug in the aqueous phase in its ionised form did not affect the stabilising properties of the PEG chains on the nanoparticle surface, as a consequence nanoparticle recovery remained unchanged. This would be in agreement with our previous findings on the colloidal stability of PLGA nanoparticles (Stolnik et al., 1995b). It was reported that uncoated PLGA nanoparticles flocculated at sodium sulphate concentrations of 0.05 M, while those surface modified with PLA–PEG flocculated at a much higher concentration of 0.5 M. Therefore, an additional advantage of sterically stabilised drug loaded particles over those that are electrostatically stabilised, is a reduction in particle loss during drug encapsulation.

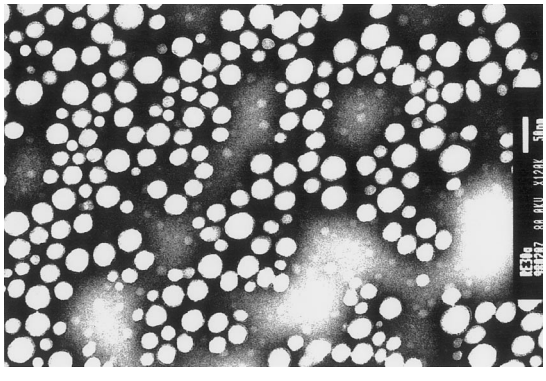

Fig. 3. Surface morphology of PLA–PEG 30:5 nanoparticles theoretically loaded with 20% w/w procaine hydrochloride.

Although the drug content increased from 0.24 to 3.1% w/w, the sizes of the nanoparticles remained unchanged (Table 3). This finding further confirmed the micellar type structure of the 30:5 nanoparticles, i.e. the core although solid, comprises polymeric chains linearly packed which implies that space is available within the core itself even at increasing drug content values.

The surface zeta potential of the PLA–PEG nanoparticles remained unchanged with an increase in the drug content from 0.24 to 3.1% w/w (Table 3). This may further confirm that the colloidal stability of this system remains unaffected with increasing theoretical loadings of the ionised cationic drug.

The nanoparticles were shown to maintain their original morphology, both at low (2% w/w) (Fig. 1B) and high (20% w/w) (Fig. 3) theoretical drug loadings. Despite being only ~ 68 nm in size, these nanoparticles can therefore incorporate drug at high theoretical loadings without any adverse effect on their morphology.

3.3. Influence of formulation variables

Previous studies in our group showed that the drug incorporation efficiency of procaine hydrochloride into PLGA nanoparticles could be improved significantly by employing an aqueous phase pH of 9.3; replacing procaine hydrochloride with its base form, procaine dihydrate and including the fatty acid, lauric acid into the formulation (Govender et al., 1999). Increasing the aqueous phase pH to 9.3 and using the base form of the drug, reduced the aqueous drug solubility thereby increasing the drug incorporation efficiency. Lauric acid, being negatively charged was employed to interact with the positively charged drug and the resultant complex coprecipitated with PLGA, thus reducing drug loss. Therefore, it was decided to apply the same variables to the procaine hydrochloride loaded PLA–PEG 30:5 system to determine whether similar beneficial effects could be obtained. However, the results in Table 4 indicate that the three chosen variables did not increase the drug content and drug entrapment values of procaine hydrochloride into PLA–PEG 30:5 nanoparticles as compared with the PLGA sys-

Table 4
Influence of formulation variables on drug incorporation efficiency of PLA–PEG 30:5 nanoparticles (comparison with PLGA nanoparticles)

Formulation description	PLA–PEG 30:5			PLGA (Govender et al., 1999)		
	Nanoparticle recovery (%)[a]	Drug content (% w/w)[a]	Drug entrapment (%)[a]	Nanoparticle recovery (%)[a]	Drug content (% w/w)[a]	Drug entrapment (%)[a]
Starting preparation[b]	69.16 (64.46; 73.86)	0.24 (0.24; 0.23)	7.94 (7.67; 8.20)	65.1 (65.2; 65.0)	0.35 (0.35; 0.34)	11.02 (11.18; 10.85)
Replacing procaine HCl with procaine dihydrate	69.75 (76.61; 62.89)	0.26 (0.23; 0.28)	8.80 (8.80; 8.80)	92.4 (91.9; 92.9)	0.92 (0.95; 0.89)	41.46 (42.62; 40.30)
Changing aqueous phase pH from 5.8 to 9.3	77.88 (75.82; 79.93)	0.19 (0.18; 0.22)	7.41 (6.81; 8.01)	93.4 (92.8; 94.0)	1.27 (1.26; 1.28)	58.26 (57.61; 58.90)
Including lauric acid at a 1:1 molar ratio	63.03 (62.07; 63.99)	0.25 (0.25; 0.25)	7.73 (7.61; 7.85)	88.8 (89.0; 88.7)	0.79 (0.78; 0.79)	34.81 (34.72; 34.89)

[a] Mean of the two replicate determinations which are shown in parenthesis.
[b] Starting preparation is 2% w/w theoretical loading of procaine HCl (1.04 mg), water pH 5.8 (15 ml), acetonitrile (5 ml).

Table 5
Drug incorporation into PLGA nanoparticles with varying lactide:glycolide ratios (theoretical procaine HCl loading = 2% w/w).

PLGA lactide:glycolide ratio	Nanoparticle recovery (%)[a]	Drug content (% w/w)[a]	Drug entrapment (%)[a]
50:50	65.12 (65.24; 65.00)	0.35 (0.35; 0.34)	11.02 (11.18; 10.85)
67:33	46.74 (45.63; 47.85)	0.38 (0.39; 0.37)	8.70 (8.63; 8.77)
75:25	48.78 (49.25; 48.31)	0.38 (0.37; 0.38)	9.02 (9.04; 8.99)
95:5	47.70 (47.06; 46.34)	0.38 (0.39; 0.37)	8.65 (8.91; 8.40)

[a] Mean of the two replicate determinations that are shown in parenthesis.

tem, i.e. the drug content and drug entrapment for PLA–PEG 30:5 nanoparticles remained virtually unchanged at ~0.24 and 8% w/w, respectively. The exact reason for the differences in the effect of these variables with PLGA and PLA–PEG nanoparticles is not clearly understood. It was initially thought that perhaps the differences in matrix composition between the PLGA and PLA–PEG systems may be a possible reason, i.e. the PLGA matrix would be more amorphous and that of the PLA–PEG nanoparticle more crystalline. However, the drug incorporation data in Table 5, which compare the influence of the lactide/glycolide ratio on drug incorporation, showed minimal differences between preparations with an increasing lactide proportion in the polymer. Therefore, differences in the lactide/glycolide matrix composition could not be a possible reason for the lower drug incorporation observed with the PLA–PEG 30:5 nanoparticles. Although the PLGA nanoparticles of the starting preparation formulation were almost two and a half times larger than those of PLA–PEG 30:5 system, the drug incorporation efficiencies were quite similar (Tables 4 and 5). Furthermore, although PLA–PEG 110:5 nanoparticles were 174.6 nm in size, they had a similar drug incorporation efficiency to those of PLA–PEG 30:5 which were only 68.9 nm in size (Tables 1 and 2). These results therefore imply that the smaller size of the PLA–PEG nanoparticles as compared to those of PLGA, could not be responsible for the observed differences in drug incorporation efficiencies achieved using various formulation approaches.

It was interesting to note that the formulation variables investigated reduced the size of the PLGA nanoparticles while the sizes of the PLA–PEG 30:5 nanoparticles remained unchanged (Table 6). To investigate further this phenomenon, the preparation of these nanoparticles was repeated, but the inclusion of procaine hydrochloride was omitted. The results confirmed

Table 6
Influence of formulation variables on the physicochemical properties of drug loaded PLA–PEG 30:5 nanoparticles (comparison to PLGA nanoparticles)

Formulation description	PLGA (Govender et al., 1999)		PLA–PEG(30:5)	
	Particle size ± S.D. (nm) (polydispersity)	Zeta potential ± S.D. (mV)[a]	Particle size ± S.D. (nm) (polydispersity)	Zeta potential ± S.D. (mV)
Starting preparation[b]	184.1 ± 1.9 (0.08 ± 0.02)	−52.9 ± 0.8	68.9 ± 2.2 (0.12 ± 0.03)	−7.8 ± 1.5
Replacing procaine HCl with procaine dihydrate	135.0 ± 1.3 (0.11 ± 0.03)	−48.4 ± 1.4	60.8 ± 0.9 (0.17 ± 0.02)	−7.4 ± 1.4
Changing aqueous phase pH from 5.8 to 9.3	146.0 ± 3.0 (0.09 ± 0.04)	−50.6 ± 0.6	66.8 ± 1.7 (0.16 ± 0.02)	−8.1 ± 1.8
Including lauric acid at a 1:1 molar ratio	118.8 ± 1.4 (0.12 ± 0.03)	−44.1 ± 1.8	61.3 ± 1.7 (0.15 ± 0.02)	−8.3 ± 1.3

[a] 1 mM HEPES buffer.
[b] Starting preparation is 2% w/w theoretical procaine HCl loading (1.04 mg), water pH 5.8 (15 ml), acetonitrile (5 ml).

that the variables employed influenced the particle size of PLGA nanoparticles, whereas for the PLA–PEG systems they had no effect (Table 7). Therefore, we propose that it is this difference in particle size effect with the various formulation variables which may be responsible for the differences in drug incorporation efficiencies. Because the nanoparticle recovery increased (Table 4) and the particle size decreased for the PLGA systems (Table 7) when the various formulation variables were employed, this may have led to an increase in the number of particles available for drug entrapment as well as to a greater available surface area for drug associated with the particle. Therefore, this may also be responsible for the improved drug incorporation efficiencies observed with this system. Indeed, de Chasteigner et al. (1996) have also attributed an increase in the incorporation efficiency of itraconazole into polycaprolactone nanoparticles to a decrease in particle size with inclusion of the surfactant, sodium deoxycholate into the preparation. With PLA–PEG 30:5, nanoparticle recovery (Table 4) and size (Table 7) remained unchanged when the various formulation variables were employed. Hence, these formulation variables may have influenced the precipitation behaviour of the PLGA polymer and not that of the PLA–PEG 30:5 polymer.

Table 7
Influence of formulation variables on particle size of drug free PLA–PEG 30:5 nanoparticles (comparison to PLGA nanoparticles)

Formulation description	Nanoparticle size ± S.D. (nm) (polydispersity)	
	PLA–PEG 30:5	PLGA
Starting preparation[a]	72.5 ± 2.8 (0.13 ± 0.03)	157.1 ± 1.9 (0.08 ± 0.02)
Replacing procaine HCl with procaine dihydrate	60.8 ± 0.9 (0.17 ± 0.02)	135.0 ± 1.3 (0.11 ± 0.03)
Changing aqueous phase pH from 5.8 to 9.3	66.3 ± 1.2 (0.15 ± 0.03)	123.6 ± 2.3 (0.09 ± 0.03)
Including lauric acid at a 1:1 molar ratio	65.6 ± 0.7 (0.1 2± 0.04)	106.4 ± 0.9 (0.104 ± 0.13)

[a] Starting preparation = polymer (50 mg), 0% w/w drug loading, water pH 5.8 (15 ml), acetonitrile (5 ml).

The following explanation is proposed for the possible differences in behaviour of these polymeric systems. The particle growth rate during precipitation may be influenced by adsorbed material on the particle surface, which act as growth inhibitors (Shaw, 1970). It is postulated that during precipitation of the PLGA polymer, the salts from the buffer, procaine dihydrate and lauric acid in the aqueous phase, may have adsorbed or been close to the charged surface of the particles during the growth phase, in this way possibly behaving as growth inhibitors, thus, explaining the decreased sizes observed with this polymer. It may seem that for the PLA–PEG system, the extending PEG chains on the surface prevent this phenomenon thereby causing particle growth during nanoprecipitation to remain unaffected. Therefore, it may be that the differences in drug incorporation efficiencies between these two polymeric systems may be due to differences in their precipitation behaviour.

3.4. Enhancing drug incorporation by complexation with poly(aspartic acid)

In an attempt to increase drug incorporation into PLA–PEG 30:5 nanoparticles, poly(aspartic acid) was adding as a complexation agent to the formulation. The rationale for its inclusion was that the cationic procaine hydrochloride would interact electrostatically with the poly(carboxylic acid) groups on the poly(aspartic acid) chain and the complex should associate with the PLA–PEG chains thereby increasing drug incorporation. However, the results, shown in Table 8, indicate that poly(aspartic acid) ($pK_a = 4$) failed to improve procaine hydrochloride ($pK_a = 9$) incorporation, even though both species would be almost 100% ionised at the aqueous phase pH of 5.3 and therefore, electrostatic interactions would be possible. Another water soluble cationic drug, diminazene aceturate, was also employed to assess the drug enhancement potential of poly(aspartic acid). Contrary to the results with procaine hydrochloride, at an equivalent theoretical loading of 2% w/w, inclusion of poly(aspartic acid) led to an increase in the drug incorporation efficiency of

Table 8
Characterisation of drug loaded PLA–PEG (30:5) nanoparticles with Pasp as a complexing agent (theoretical drug loading = 2% w/w)

Physicochemical properties and drug incorporation	Procaine HCl without poly(aspartic acid)	Procaine HCl with poly(aspartic acid)	Diminazene aceturate without poly(aspartic acid)	Diminazene aceturate with poly(aspartic acid)
Nanoparticle recovery[a]	90.37 (91.67; 89.06)	80.29 (88.88; 75.70)	48.08 (40.54; 55.62)	77.25 (84.56; 69.94)
Nanoparticle size ± S.D. (nm) (polydispersity)	64.8 ± 1.6 (0.10 ± 0.02)	63.85 ± 1.5 (0.10 ± 0.03)	70.6 ± 2.4 (0.16 ± 0.01)	10.2 ± 3.5 (0.14 ± 0.05)
Zeta potential (mV)[a]	6.9 ± 1.7	6.4 ± 1.2	6.8 ± 0.9	−13.7 ± 0.3
Drug content (%w/w)[b]	0.20 (0.20; 0.19)	0.21 (0.22; 0.19)	0.26 (0.27; 0.24)	0.58 (0.56; 0.59)
Drug entrapment (%)[b]	8.65 (8.99; 8.30)	8.43 (9.71; 7.14)	5.90 (5.29; 6.51)	21.77(23.25; 20.29)

[a] 1 mM HEPES buffer.
[b] Mean of the two replicate determinations which are shown in parenthesis.

diminazene aceturate, i.e. drug content increased from 0.26 to 0.58% w/w and drug entrapment increased from 5.9 to 21.8%. The difference in the effect of poly(aspartic acid) on both these drugs was initially surprising, because both are cationic drugs and have amino groups in their structure which will be protonated at pH 5.3 and therefore an interaction with the negatively charged polymer was to be expected for both molecules.

Therefore, in an attempt to determine the reasons for the above differences, isothermal titration microcalorimetry was used to study the heat changes associated with the interaction of poly(aspartic acid) and diminazene aceturate and procaine hydrochloride. Surprisingly, totally different heat released profiles were obtained for the two drugs (Fig. 4). Low endothermic heat released values were measured for the interaction of

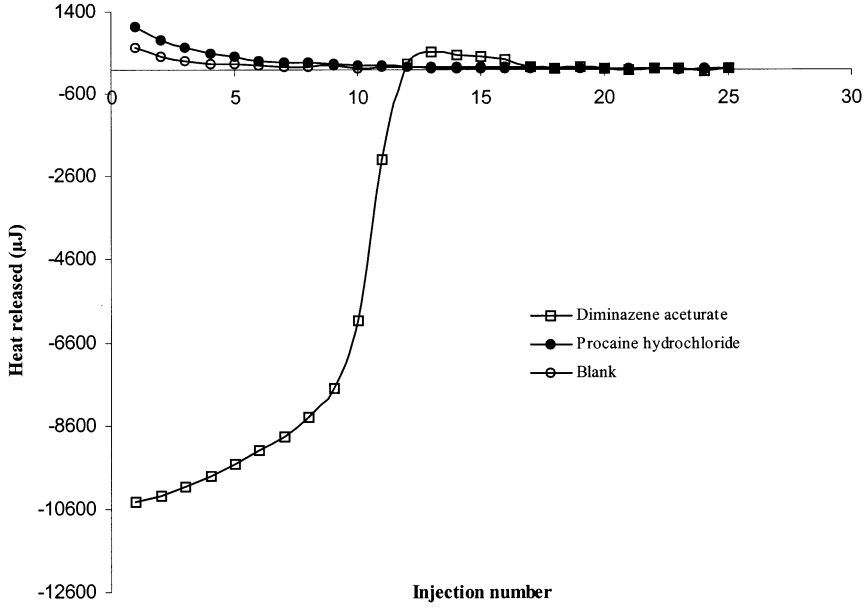

Fig. 4. Integrated heat release values for the interaction of Pasp with diminazene aceturate and procaine hydrochloride. The heat release values for the blank is also shown.

poly(aspartic acid) and procaine hydrochloride while substantial exothermic heat changes were detected for those of poly(aspartic acid) and diminazene aceturate. The equilibrium binding parameters, shown in Table 9, were determined from the curves fitted to the experimental calorimetric data. It is seen that K_{obs} for diminazene is much larger than K_{obs} for procaine. Therefore, the higher incorporation efficiency of diminazene into nanoparticles may be explained by the higher affinity of the drug to poly(aspartic acid) and consequently during the particle formation a larger amount of drug is bound to the polymer in comparison to procaine molecules.

It is seen from Table 9 that the calculated value of N for both drugs is similar and ~ 190. On the other hand, the calculated mean number of monomers of poly(aspartic acid) equals to 189. Comparing this value with the calculated values for N, indicates that one binding site is located on each monomer of poly(aspartic acid). In other investigations (data not shown) we found that the binding sites are the carboxylic groups of the polymer. However, calculated n (occupied binding sites on the polymer by drug) for procaine is close to 1, while for diminazene it is close to 2. Therefore, diminazene molecules occupy two sites on the polymer, whereas procaine molecules occupy just one. Therefore a higher binding constant for diminazene molecules, than for procaine molecules is expected. Also, it is seen from Table 9, that for diminazene, ΔH_{obs} and ΔTS_{obs} are both negative and equal to -10.84 and -2.49 kcal M^{-1}, respectively. Therefore, the interaction of diminazene is enthalpically driven, which shows that high exothermic interactions occurred between the drug and polymer. Usually formation of high extensive hydrogen bonds causes an exothermic reaction (Ohyama and Cowan, 1996), which may be the case for diminazene. In comparison, the calculated ΔH_{obs} and ΔTS_{obs} for procaine equals 0.2 and 4.12 kcal M^{-1}, respectively, which indicates that the interaction between procaine and poly(aspartic acid) is entropically driven. Although the entropy value of the procaine interaction with poly(aspartic acid) is larger than its value for diminazene, the high enthalpy change of reaction for diminazene compensates for the effect of entropy change for the procaine reaction, and therefore, the interaction is stronger for diminazene than for procaine. In summary, a larger number of binding sites and extensive hydrogen bonding for diminazene than for procaine leads to a higher value of association constant for diminazene than for procaine.

Structural differences between these two drug molecules may be responsible for the difference in interaction with poly(aspartic acid). Diminazene aceturate may have a greater charge density than procaine hydrochloride since it has a larger number of amino groups which may enhance its interaction with poly(aspartic acid). Therefore, this study confirmed the potential of poly(aspartic acid) as a complexation agent for drug incorporation efficiency improvement into PLA–PEG nanoparticles. However, the different results obtained with both drugs studied, indicate that a more detailed study is required to determine the structural properties of drugs suitable for this approach of enhancing the efficiency of drug incorporation (These studies are currently being investigated in our laboratories).

4. Conclusions

PLA–PEG nanoparticles have shown potential for drug targeting due to their prolonged circula-

Table 9
The equilibrium thermodynamic parameters of the interaction of diminazene aceturate and procaine hydrochloride with poly(aspartic acid)

Drug	K_{obs} (M^{-1})	N	N	ΔH_{obs} (kcal/mol)	ΔG_{obs} (kcal/mol)	$T\Delta S_{obs}$ (kcal/mol)
Diminazene aceturate	1.4×10^{6}	180	1.89	−10.84	−8.34	−2.49
Procaine hydrochloride	7.4×10^{2}	198	0.99	0.2	−3.92	4.12

tion times and decreased liver uptake. However, a lack of data on the drug incorporation properties of these systems exist. In this study therefore, the drug incorporation characteristics of nanoparticles prepared from a series of PLA–PEG copolymers, using a model water soluble drug, procaine hydrochloride, were investigated. Also, their physicochemical properties, such as size, surface charge and morphology were determined.

Procaine hydrochloride was incorporated into nanoparticles prepared from diblock PLA–PEG polymers with a fixed PEG portion (5 kDa) and an increasing PLA molecular weight segment (3–110 kDa). Although particle size increased with drug incorporation into the nanoparticles, their drug incorporation efficiencies were similar. This was attributed to a change in the internal structure of the core, which becomes less mobile as the molecular weight of the copolymer and hence the aggregation number of the assembly increases. This results in a reduction of the free space available to accommodate the drug. These studies revealed that the drug incorporation efficiency is not compromised, even when the small nanoparticles, within this series, may be required for specific targeting purposes.

An increase in the theoretical drug loading led to an increase in drug content of PLA–PEG 30:5 nanoparticles. Since the size remained unchanged, with an increasing drug content, this supported the proposed micellar-type structure of PLA–PEG 30:5 nanoparticles. Although the core is solid, the polymeric chains are loosely packed with free space available for drug incorporation.

TEM studies showed drug loaded nanoparticles prepared from the various PLA–PEG copolymers, to be spherical and discrete. The morphology of PLA–PEG 30:5 nanoparticles remained unchanged both at low and high theoretical loadings.

Formulation variables, such as an increase in the aqueous phase pH, base form of the drug and inclusion of lauric acid into the formulation which were successful previously in improving the incorporation of drug into PLGA nanoparticles, did not do so for PLA–PEG 30:5 nanoparticles. This was attributed to the possible differences in precipitation behaviour of these polymers.

Inclusion of poly(aspartic acid) as a complexation agent, improved the drug incorporation efficiency of diminazene but not that of procaine hydrochloride. Isothermal titration microcalorimetry proved useful in confirming that a stronger interaction occurred between poly(aspartic acid) and diminazene aceturate than with procaine hydrochloride.

Therefore, these studies have revealed important characterisation data for PLA–PEG drug loaded nanoparticles. The results of this study may impact on the future optimisation of drug incorporation and delivery properties of these potential drug targeting carriers.

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