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PLGA nanoparticles prepared by nanoprecipitation: drug loading and release studies of a water soluble drug

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Abstract

The nanoprecipitation technique for preparation of nanoparticles suffers the drawback of poor incorporation of water soluble drugs. The aim of this study was therefore to assess various formulation parameters to enhance the incorporation of a water soluble drug (procaine hydrochloride) into poly(DL-lactide-co-glycolide) (PLGA) nanoparticles prepared by this technique. Approaches investigated for drug incorporation efficiency enhancement included the influence of aqueous phase pH, replacement of procaine hydrochloride with procaine dihydrate and the inclusion of excipients: poly(DL-lactide) (PLA) oligomers, poly(methyl methacrylate-co-methacrylic acid) (PMMA–MA) or fatty acids into the formulation. The nanoparticles produced were submicron size (<210 nm) and of low polydispersity. It was found that an aqueous phase pH of 9.3, replacement of procaine hydrochloride with procaine dihydrate and the incorporation of PMMA–MA, lauric and caprylic acid into the formulation could enhance drug incorporation efficiency without the size, morphology and nanoparticle recovery being adversely influenced. For instance changing the aqueous phase pH from 5.8 to 9.3 increased nanoparticle recovery from 65.1 to 93.4%, drug content from 0.3 to 1.3% w/w and drug entrapment from 11.0 to 58.2%. However, the presence of high ratios of lauric acid and procaine dihydrate in the formulation adversely affected the morphology and size of the nanoparticles. Also, PLA oligomers were not considered a feasible approach since it decreased drug entrapment from 11.0 to 8.4% and nanoparticle recovery from 65.1 to 19.6%. Drug release from nanoparticles appears to consist of two components with an initial rapid release followed by a slower exponential stage. This study has demonstrated that formulation variables can be exploited in order to enhance the incorporation of a water soluble drug into PLGA nanoparticles by the nanoprecipitation technique.

Keywords: Nanoparticles; Drug incorporation; PLGA; In vitro release; Nanoprecipitation

1. Introduction

The potential of site specific drug delivery in optimising drug therapy [1] has given impetus to significant advancements in the pharmaceutical engineering of novel dosage forms such as nanoparticles, which are solid colloidal polymeric carriers less than 1 μm in size [2]. Several review articles have highlighted the ability of such nanoparticles to reduce associated adverse effects of various drugs [1,3,4]. Some of the commonly reported methods of preparing nanoparticles from biodegradable polymers include solvent evaporation [5], monomer polymeri-

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sation [6], nanoprecipitation [7] and the salting out procedure [8]. The nanoprecipitation method developed by Fessi et al. [9] represents an easy and reproducible technique and has been widely used by several research groups to prepare nanoparticles [7,10,11]. This method is based on the interfacial deposition of a polymer following displacement of a semi-polar solvent miscible with water from a lipophilic solution [9].

A nanoparticle system with maximal drug loading and a high entrapment efficiency will reduce the quantity of carrier required for the administration of sufficient amount of active compound (drug) to the target site as well as drug wastage during manufacturing. Mainly water insoluble drugs have been incorporated into nanoparticles using the nanoprecipitation technique with typical drug content values being: indomethacin, 2.0% w/w [9] or 5.8% w/w [12]; dexamethasone, 0.9% w/w [9] and itraconozole, 4.1% w/w [13]. However, in our hands this technique suffers the drawback of a poor incorporation efficiency of water soluble drugs due to rapid migration and therefore loss of drug into the aqueous phase. Furthermore, while the literature is replete with studies investigating drug incorporation into particles by the solvent evaporation method [14–16], a lack of published data on approaches to promote the incorporation of water soluble drugs by the nanoprecipitation method exists.

Hence, the main aim of the present study was to assess formulation parameters to enhance the incorporation of a water soluble drug into PLGA nanoparticles by the nanoprecipitation technique. PLGA was selected since the poly(esters), including poly(lactic acid), poly(glycolic acid) and their copolymers, have emerged as the most widely used and studied class of biodegradable polymers for pharmaceutical use due to their biocompatibility and biodegradability [17]. The physicochemical characteristics, particle morphology and in vitro release behaviour of the drug loaded nanoparticles have also been elucidated. In all investigations, procaine hydrochloride has been used as a model drug due to its water solubility, ease of analysis, ready availability and cost. Also, due to its cationic nature it is possible to promote electrostatic interactions with anionic excipients.

2. Materials and methods

2.1. Materials

Poly(DL-lactide-co-glycolide) (PLGA, 50:50, M_w = 10 000 Da) was synthesised by Zeneca Pharmaceuticals (Macclesfield, UK) and was used as obtained. Poly(DL-lactide) (PLA) oligomers (M_w = 2000 Da) were synthesised in our laboratories. Procaine hydrochloride (pK_a = 9), HEPES (as sodium salt), Phosphate buffered saline (PBS) tablets, caprylic acid ($C_8H_{15}O_2Na$) and lauric acid ($C_{12}H_{23}O_2Na$) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Poly(methyl methacrylate-co-methacrylic acid) $[-CH_2C(CH_3)(CO_2CH_3)-]_x$ $[-CH_2C(CH_3)(CO_2H)-]_y$ (PMMA–MA) (M_w = 34 000 Da) was purchased from Aldrich Chemical Co. (Milwaukee, USA). 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

Nanoparticles were prepared according to a modified nanoprecipitation method [9]. The starting procedure was as follows. PLGA polymer (50 mg) and a specified quantity of drug were accurately weighed and dissolved in acetonitrile (5 ml). The organic phase was added dropwise into the aqueous phase (15 ml) and stirred magnetically 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 drug incorporation efficiency, the following alterations were made to the starting procedure:

- to assess the effect of aqueous phase pH, water pH 5.8 was replaced with 1 mM HEPES buffer adjusted to pH 6.2, pH 7.9, pH 8.6 and pH 9.3.
- to study the influence of other formulation excipients (PLA oligomers, PMMA–MA and fatty

acids), these were added in specified quantities to the organic phase.

- to determine the influence of replacing the salt form of the drug with the base form, procaine hydrochloride was converted to procaine dihydrate as follows. Procaine dihydrate was obtained by alkalinisation of procaine hydrochloride (2 g) to pH 12.5 with a 2 M NaOH solution. The precipitate obtained was vacuum filtered and washed several times with water. Aqueous alcohol (70% w/w) was then added dropwise to the precipitate with gentle heating until it dissolved and then placed on an ice bath to promote crystallisation. The crystals obtained were separated from the alcoholic solution by vacuum filtration and dried in a desiccater. The dried procaine dihydrate crystals were characterised by infra-red (Philips PU 9716 Infrared Spectrophotometer) and ultraviolet spectroscopy (Pharmacia LKB Biochrom Ultrospec 4000 Spectrophotometer).

2.2.2. Separation of free from incorporated drug

The nanosuspension was filtered (1μm filters, Whatman, Japan) and then subjected to ultracentrifugation (Beckman L-8 60M Ultracentrifuge) at 55 000 rpm (311 000×g) for 3 h at 20°C. The supernatant containing the dissolved free drug was discarded and the pellet freeze-dried (Edwards Modulyo Freeze-drier) for 48 h. The nanoparticle recovery, which is also referred to as nanoparticle yield in the literature, was calculated using Eq. (1). The individual values for two replicate determinations and their mean values are reported.

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

2.2.3. Determination of drug incorporation efficiency

Freeze-dried nanoparticles were dissolved in acetonitrile (50 ml) (a common solvent for PLGA and the drug). Procaine hydrochloride and procaine dihydrate in the solution were measured by ultraviolet spectroscopy at 292 nm and 286 nm respectively (Pharmacia LKB Biochrom Ultrospec 4000 Spectrophotometer) (Prior studies established no absorbance interference from PLGA polymer under the same conditions). Drug incorporation efficiency was expressed both as Drug Content (% w/w), also referred to as drug loading in the literature, and Drug Entrapment (%); represented by Eqs. (2) and (3) respectively. The individual values for two replicate determinations and their mean values are reported.

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

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

2.2.4. Physicochemical characterisation

2.2.4.1. 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 appropriately diluted with filtered water (0.2 μm filter, Minisart®, Germany). For each sample, the mean diameter±standard deviation of six determinations were calculated applying multimodal analysis. Values reported are the mean diameter±standard deviation for two replicate samples.

2.2.4.2. 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 appropriately 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±standard deviation of four determinations were established. Values reported are the mean value±standard deviation for two replicate samples.

2.2.4.3. Particle morphology. Morphological evaluation of the nanoparticles was performed using Trans-

mission 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).

2.2.4.4. In vitro release study. The in vitro drug release behaviour of the nanoparticles was determined using a modified ultrafiltration technique [12]. The study was performed on nanoparticles containing 10% w/w theoretical drug loading and prepared in HEPES buffer pH 9.3 as the aqueous phase. This formulation was chosen since it provided for a relatively high drug content of 3.6% w/w (this will facilitate ease and accuracy of sample analysis), and the nanoparticle morphology and size was not adversely affected. Free drug was removed by washing twice with HEPES Buffer pH 9.3 (25 ml) and ultrafiltration of the nanosuspension. This method of separation of free from incorporated drug was found to be comparable to that by the ultracentrifugation method (3.2% w/w). The nanosuspension (5 ml) was added directly into a stirred ultrafiltration cell (Model 8050, Amicon, USA) containing PBS (45 ml, 10 mM, pH 7.4) and moderately stirred. At specified time intervals aliquots of the release medium (3 ml) were filtered through the ultrafiltration membrane (Diaflo® ultrafiltration membranes with a molecular weight cut off point of 300 000 Da, XM300, Amicon, USA) using less than 2 bar nitrogen gas. The withdrawn sample was replaced with equal volumes of fresh dissolution medium. Procaine hydrochloride was quantitated by UV at 289 nm (λ_{max} of procaine hydrochloride as determined in a solution of HEPES buffer pH 9.3 and PBS in a ratio of 1:9). The percentage drug released at each time point was corrected for dilution by sample replacement. The dissolution study was performed in duplicate and the mean values are reported. A control experiment to determine the release behaviour of the free drug, procaine hydrochloride dissolved in 1 mM HEPES buffer pH 9.3 was also performed. This was done by adding HEPES buffer pH 9.3 (5 ml) containing procaine hydrochloride (0.8 mg) to PBS (45 ml) and performing the test as for the samples.

2.2.5. Statistical analyses

All statistical analyses were undertaken using the ANOVA test with a Minitab® statistical software programme.

3. Results and discussion

3.1. Influence of the theoretical loading of procaine hydrochloride

The starting procedure involved the production of PLGA nanoparticles with procaine in its salt form and using water pH 5.8 as the aqueous phase. In order to establish the maximum amount of drug that could be incorporated into nanoparticles at such conditions, the initial approach involved increasing the theoretical loading of procaine hydrochloride in the formulation from 1 to 10% w/w. The results showed that this led to a corresponding increase in drug content from 0.2 to 4.6% w/w; however the corresponding drug entrapment decreased from 14.5 to 6.3% (Table 1).

The particle size data show that nanoparticles produced were of submicron size and of low polydispersity (Table 1) which indicated a relatively narrow

Table 1
Characterisation of procaine hydrochloride loaded PLGA nanoparticles prepared in water pH 5.8 as the aqueous phase

Theoretical drug loading (% w/w)	[a]Nanoparticle recovery (%)	Nanoparticle size±S.D. (nm) (polydispersity)	Zeta potential ±S.D. (mV)	Drug content[a] (% w/w)	Drug entrapment[a] (%)
0	92.3 (92.4; 92.2)	157.1±1.9 (0.08±0.02)	−49.2±0.7	–	–
1	80.8 (83.0; 78.6)	164.0±1.1 (0.06±0.03)	−50.3±0.6	0.2 (0.2; 0.2)	14.5 (14.2; 14.8)
2	65.1 (65.2; 65.0)	184.1±1.7 (0.09±0.02)	−52.9±0.8	0.3 (0.3; 0.3)	11.0 (11.2; 10.8)
4	56.6 (57.8; 55.5)	198.0±3.4 0.10±0.03)	−54.1±0.6	0.6 (0.6; 0.6)	8.9 (9.0; 8.9)
6	29.8 (31.0; 28.7)	203.6±2.1 (0.10±0.04)	−54.2±0.5	1.5 (1.4; 1.6)	7.6 (8.0; 7.2)
10	13.6 (13.5; 13.7)	209.5±2.7 (0.09±0.07)	−55.1±0.9	4.6 (4.6; 4.6)	6.3 (6.3; 6.3)

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

particle size distribution. An increase in particle size from 157.1 nm to 209.5 nm with an increase in the theoretical drug loading was also observed (Table 1). The increase in drug content of the nanoparticles with increased theoretical drug loading may have resulted in the increased particle sizes displayed. Also, TEM studies on nanoparticles theoretically loaded with 2% w/w procaine hydrochloride (Fig. 1) showed them to be spherical and discrete. Drug-free PLGA nanoparticles had a negative surface charge of −49.2 mV (Table 1) which can be attributed to the presence of end carboxyl groups of the polymer on the nanoparticle surface, as reported previously for drug-free PLGA nanoparticles [18,19]. Zeta potential measurements showed slight increases in negativity (from −49.2 mV to −55.1 mV) with an increase in theoretical drug loadings. These findings are contrary to what was expected, namely a decrease in the surface negativity due to interaction of carboxyl groups and the cationic drug on the particle surface. The increase in nanoparticle size with increases in the theoretical drug loading of procaine hydrochloride (Table 1) may possibly have influenced the surface charge of the PLGA nanoparticles. An increase in surface negativity has also been reported by Redhead [19] for Rose Bengal incorporation into PLGA nanoparticles. The results of this study, however, differ from those of de Chasteigner et al. [13] who reported a decrease in the negative surface charge when itraconazole was loaded into polycaprolactone nanoparticles. The differences in results could be due to the different drug and polymer employed in these studies.

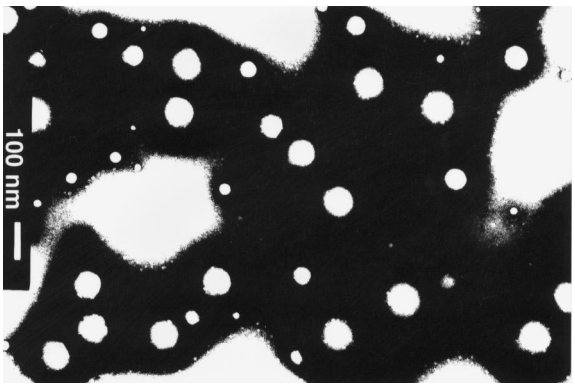

Fig. 1. Morphology of PLGA nanoparticles theoretically loaded with 2% w/w of procaine hydrochloride and prepared in water pH 5.8 as the aqueous phase.

The importance of an enhanced nanoparticle drug incorporation efficiency has been emphasised earlier. Since a high nanoparticle recovery is required for reducing manufacturing costs and its size and morphology important for quality control and biodistribution [1], it was also necessary to study the influence of formulation variables on these parameters. The selection of an optimal formulation in our study was therefore based on that which provided a combination of good morphology (in terms of sphericity and discreteness), extreme unaffected particle sizes and a high nanoparticle recovery, drug content and drug entrapment. For instance, although a 10% w/w theoretical loading led to a relatively high 4.6% w/w drug content, this formulation was not selected for further studies due to a drug entrapment of only 6.3% which implied a very high drug wastage of 93.7% during the preparation procedure, and a nanoparticle recovery of only 13.6% (Table 1).

The preparation with a 2% w/w theoretical drug loading which provided a drug content of 0.3% w/w and a drug entrapment of 11.0%, good morphological features and a relatively high nanoparticle recovery of 65.1% and particle size of 184.1 nm (Table 1 and Fig. 1) was selected as the optimal starting formulation for comparison to other studies. These drug entrapment and drug content values (as well as those at other theoretical drug loadings) are still too low and therefore disadvantageous since it indicates a high drug wastage of 89.0% during the particle preparation procedure and that a large quantity of carrier would be required to achieve sufficient amount of drug at a target site. The low drug incorporation efficiency may be attributed to the water soluble nature of procaine hydrochloride. This led to its rapid partitioning into the aqueous phase and hence decreased entrapment into the nanoparticles during polymer deposition. The large surface area of the nanoparticle geometry may have also contributed to loss of drug into the aqueous phase during preparation. Low drug incorporation efficiency of another water soluble drug, 5-fluorouracil, into PLGA nanoparticles has also been established by Niwa et al. [20]. Also, similar drug content and drug entrapment trends with increasing theoretical

drug loadings, as shown in Table 1, were observed by Niwa et al. [14] who encapsulated a water soluble drug (nafarelin acetate) into PLGA nanoparticles by a spontaneous emulsification solvent diffusion method. These researchers attributed the decreased drug entrapment with increasing theoretical drug loadings to an enhanced drug leakage into the aqueous phase at high loadings, which may also apply for our study.

Another reason for the decreasing drug entrapment with increasing theoretical drug loadings in the present study could be the corresponding decrease in nanoparticle recovery (Table 1) which would also lead to an enhanced drug loss.

The low procaine hydrochloride incorporation efficiency into PLGA nanoparticles achieved under the conditions of this investigation indicated clearly that other formulation approaches were necessary in order to improve drug entrapment and drug content.

3.2. Influence of aqueous phase pH

The aqueous phase pH will influence the ionisation of a drug and hence its solubility. It was therefore likely that increasing the aqueous phase pH thereby decreasing the solubility of procaine hydrochloride could enhance drug entrapment into nanoparticles. Nanoparticles with a theoretical loading of 2% w/w procaine hydrochloride were prepared as for the previous study except that water pH 5.8 was replaced with buffer adjusted to pH values of 6.2; 7.9; 8.6 and 9.3. The profiles show an increasing drug entrapment and drug content trend with an increase in the aqueous phase pH from 5.8 to 9.3. (Fig. 2). For example, employing water pH 5.8 as the aqueous phase resulted in only 0.3% w/w drug content and 11.0% drug entrapment while HEPES buffer pH 9.3 dramatically increased drug

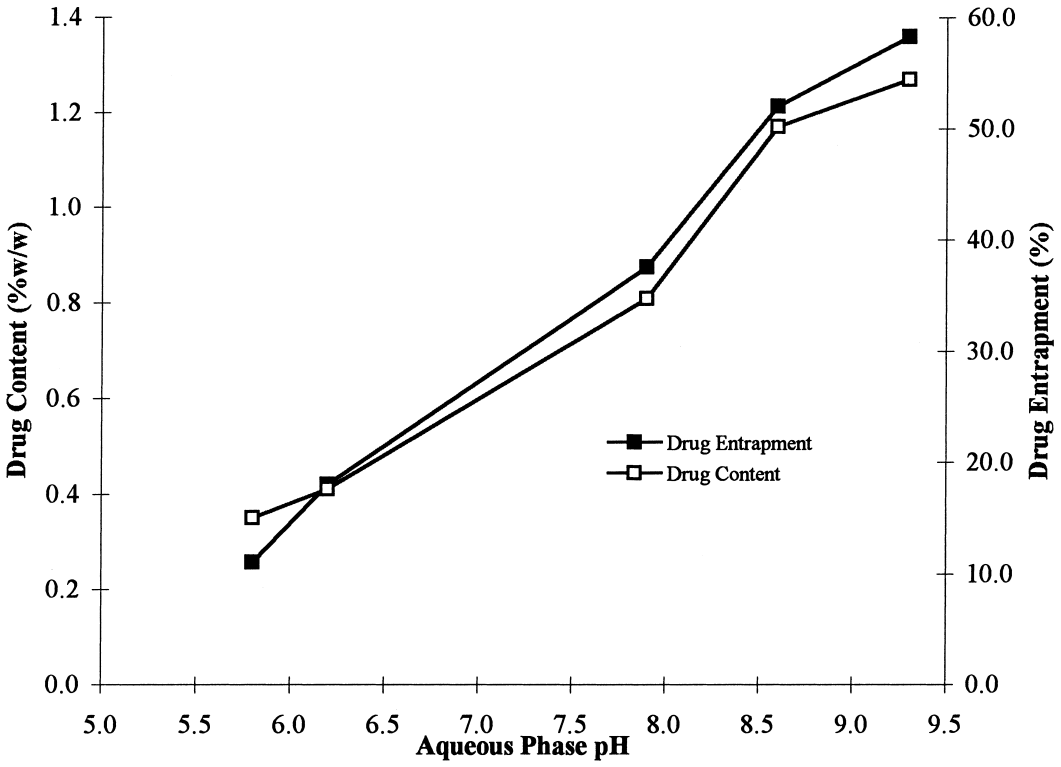

Fig. 2. Effect of aqueous phase pH on drug incorporation efficiency (drug entrapment and drug content) of PLGA nanoparticles theoretically loaded with 2% w/w procaine hydrochloride.

content to 1.3% w/w and drug entrapment to 58.2%. These results represent an almost four times increase in drug content and a five times increase in drug entrapment. Therefore, as compared to water pH 5.8, drug wastage has been reduced from 89.0% to 41.8%.

This increased drug content and entrapment is most likely due to a change in the degree of drug ionisation. Namely, in an aqueous phase pH at 9.3, procaine hydrochloride was only 33.4% ionised and therefore less soluble than in water at pH 5.8 where it would be 99.9% ionised. This may have therefore reduced migration of drug into the aqueous phase at pH 9.3, enhancing drug entrapment and drug content in that way into PLGA nanoparticles. These findings are consistent with those recently reported in the literature, where the drug entrapment of savoxepine base, using the salting out technique of particle preparation, was increased from 7.9 to >83% [8] while that of an anti-proliferative agent, using the solvent evaporation technique, was increased from 28.2 to 84.3% with an increase in aqueous phase pH from 6.5 to 8.6 [15].

The studies also illustrate that, at equivalent drug loadings, drug entrapment and drug content was profoundly higher for particles prepared in HEPES buffer pH 9.3 than those prepared in water pH 5.8 (Tables 1 and 2). Interestingly, the nanoparticle recovery was also significantly higher for preparations made in HEPES buffer pH 9.3 than in water pH 5.8 ($P<0.05$) (Tables 1 and 2). When the theoretical drug loading was increased progressively to 10% w/w, the nanoparticle recoveries when employing HEPES buffer pH 9.3 were high and remained between 86.1 and 93.4% while those for nanoparticles prepared in water pH 5.8 decreased from 92.3 to only 13.6%. Since a surfactant was not employed in the present particle preparation procedure, they would be stabilised solely by the presence of charged groups at the surface of the PLGA nanoparticles. Hence, the presence of electrolytes and salts in the aqueous medium can lead to instability of the nanosuspension [18]. Therefore, the larger quantity of procaine hydrochloride salt in the aqueous phase as well as its greater ionisation degree in water pH 5.8 as compared to in HEPES buffer pH 9.3 at equivalent drug loadings most probably contributed to destabilisation of particles hence decreasing the nanoparticle recovery. The greater ionisation degree of surface carboxylic acid groups at the higher pH of 9.3 may have also contributed to an improved nanosuspension stability [18].

Drug-free nanoparticles prepared in HEPES buffer pH 9.3 were significantly smaller than those prepared in water pH 5.8 ($P<0.05$) (Tables 1 and 2). It is postulated that the greater ionisation of carboxyl groups at a higher pH would promote greater particle repulsion leading to the smaller particle sizes observed. It would also oppose its precipitation hence leading to an improved colloidal stability. This may imply that the choice of an aqueous phase with the nanoprecipitation technique influences the polymer precipitation/particle formation mechanism and thus the size of nanoparticles. The size of nanoparticles prepared in HEPES buffer pH 9.3 also increased with an increase in the theoretical drug loading which is consistent with the trend showed for particles prepared in water pH 5.8 (Tables 1 and 2).

Table 2
Characterisation of procaine hydrochloride loaded PLGA nanoparticles prepared in HEPES Buffer pH 9.3 as the aqueous phase

Theoretical drug loading (% w/w)	Nanoparticle[a] recovery (%)	Nanoparticle size±S.D. (nm) (polydispersity)	Zeta potential ±S.D. (mV)	Drug content[a] (% w/w)	Drug entrapment[a] (%)
0	87.4 (88.4; 86.4)	123.6±2.3 (0.09±0.03)	−46.2±2.0	–	–
1	90.0 (93.4; 86.7)	129.1±1.9 (0.09±0.02)	−48.3±1.1	0.7 (0.7; 0.7)	62.0 (63.0; 61.0)
2	93.4 (92.8; 94.0)	146.0±3.0 (0.09±0.04)	−50.6±0.6	1.3 (1.3; 1.3)	58.2 (57.6; 58.9)
4	88.0 (89.2; 86.8)	158.9±3.5 (0.11±0.07)	−53.0±0.9	2.0 (2.0; 2.0)	44.7 (45.6; 43.9)
6	86.1 (84.2; 88.0)	161.8±2.8 (0.06±0.04)	−54.8±1.1	2.6 (2.6; 2.6)	37.7 (36.8; 38.7)
10	87.1 (85.2; 89.1)	186.5±2.3 (0.07±0.05)	−55.3±1.2	3.2 (3.2; 3.2)	28.3 (27.9; 28.7)

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

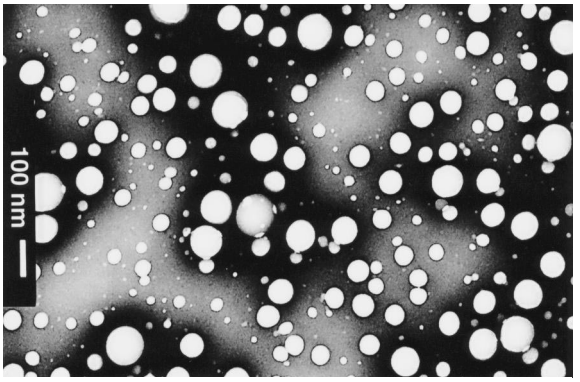

Fig. 3. Morphology of PLGA nanoparticles theoretically loaded with 2% w/w of procaine hydrochloride and prepared in HEPES buffer pH 9.3 as the aqueous phase.

TEM examination further confirmed the suitability of HEPES buffer pH 9.3 as an appropriate aqueous phase by showing the nanoparticles to be smaller and as spherical and discrete (Fig. 3) as those prepared in water pH 5.8 (Fig. 1). Hence, the substitution of an aqueous phase pH of 5.8 with that of 9.3 proved successful in enhancing drug incorporation efficiency and improving the nanoparticle recovery. Furthermore, it also generated nanoparticles with favourable morphological characteristics without the size being adversely influenced.

3.3. Influence of replacing procaine hydrochloride with procaine dihydrate

Possible drawbacks of employing an aqueous medium with a pH of 9.3 for the preparation of drug loaded PLGA nanoparticles could be the promotion of polymer degradation at alkaline conditions [21] and the unsuitability of a high pH for intravenous injection into patients. Although both of these drawbacks may be overcome by replacing the suspension medium with the appropriate buffer after particle preparation, another alternative approach to decrease the solubility of drug in the aqueous phase is the replacement of procaine hydrochloride with its base, procaine dihydrate in the formulation. For this study, nanoparticles containing 2% w/w theoretical loading of either procaine hydrochloride or procaine dihydrate were prepared and their drug incorporation efficiencies compared.

At 2% w/w theoretical drug loading, use of the dihydrate form instead of the salt form led to an increase in drug entrapment from 11.0 to 41.4% and drug content from 0.3 to 0.9% w/w (Table 1 and Table 3). Therefore, drug wastage has been reduced from 89.0 to 58.6% and drug content enhanced three times. The dihydrate form being less water soluble (1 g in 200 ml) than the salt form (1 g in 1 ml) most probably decreased leakage of the drug into the aqueous phase and/or alternatively improved its association with the hydrophobic PLGA matrix thus leading to higher drug content and drug entrapment values.

Drug content increased from 0.4 to 4.1% w/w with an increase in the theoretical drug loading from 1 to 10% w/w (Table 3) while a comparison of the drug entrapment trends for procaine hydrochloride and procaine dihydrate shows an interesting phenomenon. Unlike for procaine hydrochloride, where a decrease in drug entrapment was observed with increasing theoretical drug loadings (Table 1), studies with procaine dihydrate revealed an initial drug entrapment increase from 36.2 to 44.1% with

Table 3
Characterisation of procaine dihydrate loaded PLGA nanoparticles prepared in water pH 5.8 as the aqueous phase

Theoretical drug loading (% w/w)	Nanoparticle[a] recovery (%)	Nanoparticle size±S.D. (nm) (polydispersity)	Zeta potential ±S.D. (mV)	Drug content[a] (% w/w)	Drug entrapment[a] (%)
0	92.3 (92.4; 92.2)	157.1±1.9 (0.08±0.02)	−49.2±0.7	–	–
1	91.7 (92.9; 90.5)	148.3±1.2 (0.09±0.02)	−49.0±0.6	0.4 (0.4; 0.4)	36.2 (35.6; 36.9)
2	92.4 (91.9; 92.9)	135.0±1.3 (0.11±0.03)	−48.4±1.4	0.9 (0.9; 0.9)	41.4 (42.6; 40.3)
4	85.2 (86.4; 84.0)	81.8±1.0 (0.20±0.02)	−41.0±3.3	2.1 (2.1; 2.0)	44.1 (46.1; 42.2)
6	83.1 (83.1; 83.1)	56.2±1.9 (0.28±0.03)	−35.3±2.8	2.8 (2.9; 2.7)	39.0 (39.9; 38.1)
10	83.6 (85.2; 82.0)	20.2±0.2 (0.42±0.02)	Could not measure	4.1 (4.0; 4.3)	34.8 (34.4; 35.3)

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

theoretical drug loadings from 1 to 4% w/w. Thereafter, drug entrapment decreased to 34.8% with further increases in the theoretical drug loading. A possible reason for this difference could be that while nanoparticle recovery decreased with increased theoretical drug loadings for procaine hydrochloride (Table 1), the nanoparticle recovery for procaine dihydrate remained consistently high (Table 3). The results obtained in this study also suggest that there may have existed a maximum amount of procaine dihydrate that could be entrapped into the PLGA nanoparticles. The drug entrapment trend displayed in this study differs from those of Niwa et al. [14] who reported a decrease with increasing theoretical drug (nafarelin acetate) loadings, but it is concordant with those of Yamakawa et al. [16] who showed that drug (neurotensin analogue) entrapment increased with an increase in theoretical drug loadings up to 10% w/w but further increases led to a decreasing drug entrapment.

Interestingly, an increase in the theoretical drug loading to 10% w/w led to a decrease in particle size from 157.1 nm to 20.2 nm (Table 3), opposite to the trend established for nanoparticles loaded with procaine hydrochloride (Table 1). Corresponding TEM studies showed an adverse effect on particle morphology as the theoretical drug loading was increased. Nanoparticles with a 2% w/w theoretical drug loading were spherical and discrete (Fig. 4A) while those with theoretical drug loadings of 4% w/w were less discrete and showed a slight tendency of 'fragmenting' (Fig. 4B). Finally, the preparation with 10% w/w theoretical drug loading appeared simply as an agglomerate of 'fragments' (Fig. 4C). Clearly, high procaine dihydrate theoretical loadings adversely affected the precipitation of PLGA polymer to form spherical particles. The reason for this occurrence is not yet understood. Interestingly, the effect is different than when procaine hydrochloride is incorporated at increasing theoretical loadings. In that instance, the nanoparticle recovery decreased as theoretical drug loadings increased because nanoparticles agglomerated and were removed during filtration. On the contrary, high theoretical drug loadings of procaine dihydrate does not appear to cause particle agglomeration but rather affects their formation. A difference in these two situations is that in the first case, there is a high concentration of ionised, water soluble drug in the aqueous medium while in the second case there is a high concentration of less ionised and less water soluble drug in the system.

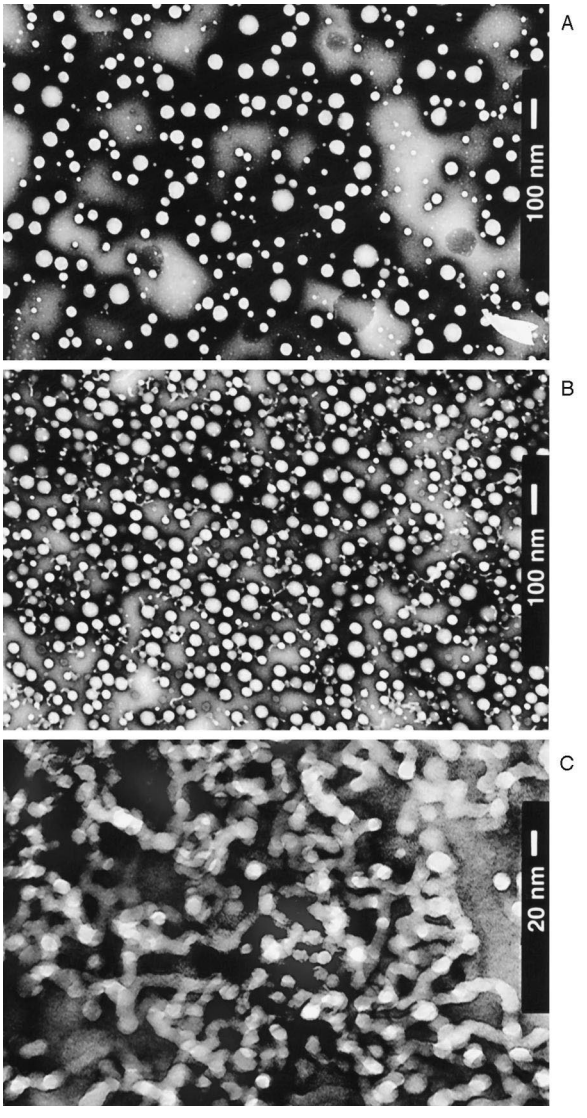

Fig. 4. (a) Morphology of PLGA nanoparticles theoretically loaded with 2% w/w procaine dihydrate and prepared in an aqueous phase pH of 5.8. (b) Morphology of PLGA nanoparticles theoretically loaded with 4% w/w procaine dihydrate and prepared in an aqueous phase pH of 5.8. (c) Morphology of PLGA nanoparticles theoretically loaded with 10% w/w procaine dihydrate and prepared in an aqueous phase pH of 5.8.

The decreased particle size observed with an

increase in procaine dihydrate loading implies an increase in the number of particles and therefore an increase in surface area for drug adsorption. This may explain the reduced negative surface charge also displayed (Table 3).

Therefore, although an improved drug incorporation efficiency was obtained by replacement of procaine hydrochloride with procaine dihydrate, this approach may be limited to nanoparticles requiring only a low theoretical drug loading due to its adverse effects on nanoparticle formation and size at higher theoretical drug loadings.

3.4. Influence of PLA oligomers

An additional strategy employed to increase drug entrapment and drug content included the blending of short chain PLA oligomers ($M_w = 2000$ Da) with PLGA polymer ($M_w = 10\,000$ Da) so that the increased level of carboxyl groups with introduction of oligomers may promote ionic interactions with the drug and increase its incorporation efficiency into the nanoparticles. The results for a 1:1 polymeric mixture is reported in Table 4. It can be seen that drug content increased slightly with the introduction of PLA oligomers to 0.9% w/w as compared to 0.3% w/w for the formulation without oligomers. However, the PLA oligomers led to a decreased drug entrapment from 11.0% to 8.4% and a dramatic decrease in nanoparticle recovery from 65.1% to only 19.6%. It is possible that the oligomers did not coprecipitate with the PLGA polymer and that they contributed to destabilisation of the nanosuspension leading to the decreased nanoparticle recovery. These results are in contrast to those of Niwa et al. [14] who reported an increased drug entrapment into nanoparticles by an emulsification solvent diffusion method when low molecular weight PLGA (4500 Da) was blended with a high molecular weight PLGA (127 598 Da) polymer. The difference in results may be due to the fact that our system did not include a surfactant while theirs used polyvinyl alcohol which may have prevented destabilisation of the nanosuspension.

3.5. Influence of PMMA–MA

PMMA–MA was chosen to determine whether an increased content of carboxyl groups in the matrix could increase the entrapment of a cationic drug. It was expected that PMMA–MA would have complexed with the cationic procaine hydrochloride by ionic interactions, and since it is insoluble in the aqueous phase, coprecipitated with PLGA and decreased migration of drug into the aqueous phase. The results (Table 4) indicate that inclusion of PMMA–MA into the formulation led to only a slightly improved drug incorporation efficiency. Drug content increased from 0.3% w/w to 0.5% w/w while drug entrapment increased from 11.0% to 18.4%. At this charge ratio of 5:1 (carboxyl: amino groups), the molar ratio of PMMA–MA to drug was 0.03:1. Perhaps, a more pronounced effect would have been observed if the charge/molar ratios were

Table 4
Influence of excipients (PLA oligomers, PMMA-MA and fatty acids) on drug incorporation efficiency[a]

Additional excipient	Nanoparticle[b] recovery (%)	Nanoparticle size ±S.D. (nm) (polydispersity)	Zeta potential ±S.D. (mV)	Drug content[b] (% w/w)	Drug entrapment[b] (%)
–	65.1 (65.2; 65.0)	157.1±1.9 (0.08±0.02)	−49.2±0.7	0.3 (0.3; 0.3)	11.0 (11.2; 10.9)
PLA oligomers (1:1)[c]	19.6 (20.0; 19.2)	171.7±2.2 (0.07±0.02)	−27.1±0.7	0.9 (0.9; 0.9)	8.4 (8.8; 8.0)
PMMA−MA (5:1)[d]	72.8 (72.5; 73.1)	152.4±1.8 (0.09±0.02)	−49.7±0.9	0.5 (0.5; 0.5)	18.4 (18.3; 18.5)
Caprylic acid (1:1)[e]	88.9 (89.8; 88.1)	123.5±1.6 (0.11±0.04)	−44.7±0.6	0.5 (0.5; 0.5)	22.0 (21.1; 23.0)
Caprylic acid (3:1)[e]	87.9 (85.2; 90.7)	55.2±1.0 (0.18±0.02)	−22.1±3.1	0.7 (0.6; 0.7)	29.3 (28.0; 30.7)
Lauric acid (1:1)[e]	88.8 (89.0; 88.7)	118.8±1.4 (0.12±0.03)	−44.1±1.8	0.8 (0.8; 0.8)	34.8 (34.7; 34.9)
Lauric acid (3:1)[e]	81.5 (78.0; 85.1)	55.8±1.5 (0.19±0.02)	−28.6±4.4	1.2 (1.2; 1.2)	50.0 (47.2; 52.8)

[a] (Theoretical procaine hydrochloride loading = 2% w/w).
[b] Mean of the two replicate determinations which are shown in parenthesis.
[c] Ratio of PLGA polymer to PLA oligomers (25 mg each).
[d] Charge ratio of carboxyl to amino groups.
[e] Molar ratio (fatty acid:drug).

further optimised for maximal complex formation. For instance, it has been shown that the interaction of poly(aspartic acid) and tobramycin depended on the molar ratio of the poly(acid) and drug being optimised [22].

3.6. Influence of fatty acids

Fatty acid salts, lauric acid and caprylic acid, were also investigated for their influence on drug incorporation efficiency. The rationale for their incorporation into the formulation was similar to that for PMMA–MA. It can be seen that the fatty acids were effective in enhancing drug content and drug entrapment (Table 4). At a 1:1 (fatty acid:drug) molar ratio, lauric acid and caprylic acid were successful in enhancing drug content from 0.3 to 0.8 and 0.5% w/w and drug entrapment from 11.0 to 34.8 and 22.0%, respectively. Lauric acid therefore had a greater effect in increasing drug content and drug entrapment than caprylic acid ($P<0.05$). This could be due to the longer carbon chain and hence greater lipophilicity of lauric than caprylic acid which reduced the loss of drug into the aqueous phase. This would be in agreement with the findings of Yamakawa et al. [16].

Although fatty acids further enhanced drug incorporation at a higher molar ratio of 3:1, particle size and surface negativity was reduced and particle morphology adversely influenced (Table 4). A similar effect may have acted for a decrease in the particle size and surface negativity, as for the procaine dihydrate studies, when fatty acids were included in the formulation. Nanoparticles prepared with lauric and caprylic acid at a 1:1 ratio were spherical and discrete (not shown). However, the morphology of those prepared with each fatty acid at a molar ratio of 3:1 lacked uniform sphericity and appeared 'distorted' (Fig. 5).

An attempt was made to further promote drug incorporation by combining the effect of fatty acid and an increased aqueous phase pH of 9.3 (Fig. 6). Nanoparticles prepared in HEPES buffer pH 9.3 with increasing theoretical drug loadings and without lauric acid were compared to those containing lauric acid at a 1:1 molar ratio. However, as can be seen in Fig. 6, drug content values for nanoparticles prepared in HEPES buffer pH 9.3 with lauric acid as a formulation component were similar to that for nanoparticles prepared in HEPES buffer pH 9.3 without lauric acid, i.e. no significant differences were observed for these two formulations ($P>0.1$). Similar drug entrapment values for both preparations were also obtained (not shown). The reduced ionisation of procaine HCl in HEPES buffer pH 9.3 as compared to water pH 5.8 may have reduced electrostatic interactions with lauric acid, thus preventing the effect of the fatty acid on drug incorporation efficiency enhancement as displayed in water pH 5.8. The results in this study may confirm that electrostatic interactions are indeed responsible for the increase in drug incorporation efficiency with the fatty acids and PMMA–MA when water pH 5.8 was the aqueous phase. Thus, it is proposed that lauric acid would be beneficial when water pH 5.8 is used as the aqueous phase but may be unnecessary when HEPES buffer pH 9.3 is used.

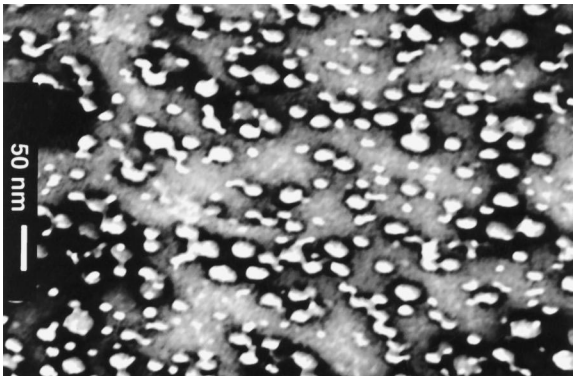

Fig. 5. Morphology of PLGA nanoparticles containing lauric acid at a 3:1 fatty acid:drug molar ratio. The nanoparticles were theoretically loaded with 2% w/w procaine hydrochloride and prepared in an aqueous phase pH of 5.8.

3.7. In vitro release study

In vitro release studies were performed on a nanosuspension containing a 10% w/w theoretical loading of procaine hydrochloride which was prepared in HEPES Buffer pH 9.3. The mean values are shown in Fig. 7 (The coefficient of variation for each data point was less than 5%). The control profile shows that 98% of the drug was released at the first sampling time of 15 min and 100% by 30 min. The

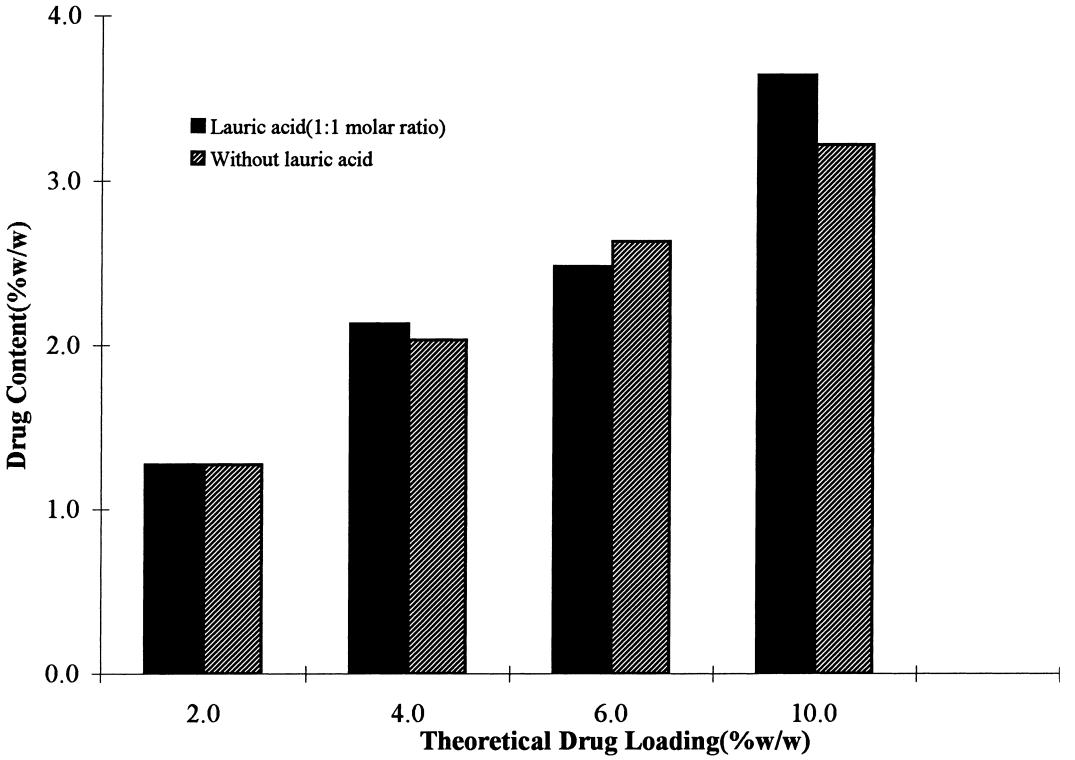

Fig. 6. Effect of procaine hydrochloride theoretical loading on drug content of nanoparticles prepared in an aqueous phase pH of 9.3 with and without lauric acid.

drug release from the nanoparticles appeared to have two components with an immediate release of about 65% at the first sampling time of 15 min. This was followed by a slower exponential release of the remaining drug over the next 4–6 h. The rapid initial release of procaine hydrochloride was probably due to drug which was adsorbed or close to the surface of the nanoparticles and the large surface to volume ratio of the nanoparticle geometry because of their size [12]. It may also be due to the water soluble nature of procaine hydrochloride. Upon addition of the nanosuspension to the dissolution medium procaine hydrochloride partitioned rapidly into the release medium accounting for the 'burst effect' observed. This effect has been reported by other research groups [15,23,24]. The exponential delayed release may be attributed to diffusion of the dissolved drug within the PLGA core of the nanoparticle into the dissolution medium.

4. Conclusions

Initially PLGA nanoparticles loaded with procaine hydrochloride were prepared by the nanoprecipitation method in water pH 5.8 as the aqueous phase. Small, spherical and submicron sized ($<$210 nm) nanoparticles were obtained. However, drug content and drug entrapment were very low. This study therefore investigated the influence of various formulation variables on enhancing the incorporation efficiency of procaine hydrochloride, a model for a water soluble drug. An increase in the aqueous phase pH from 5.8 to 9.3 enhanced the drug content and drug entrapment which may be due to a decreased degree of ionisation and hence lower solubility in the aqueous phase. Nanoparticle recovery was also higher for particles prepared in HEPES buffer pH 9.3 than in water pH 5.8. The drug-loaded nanoparticles prepared in HEPES buffer pH 9.3 also displayed

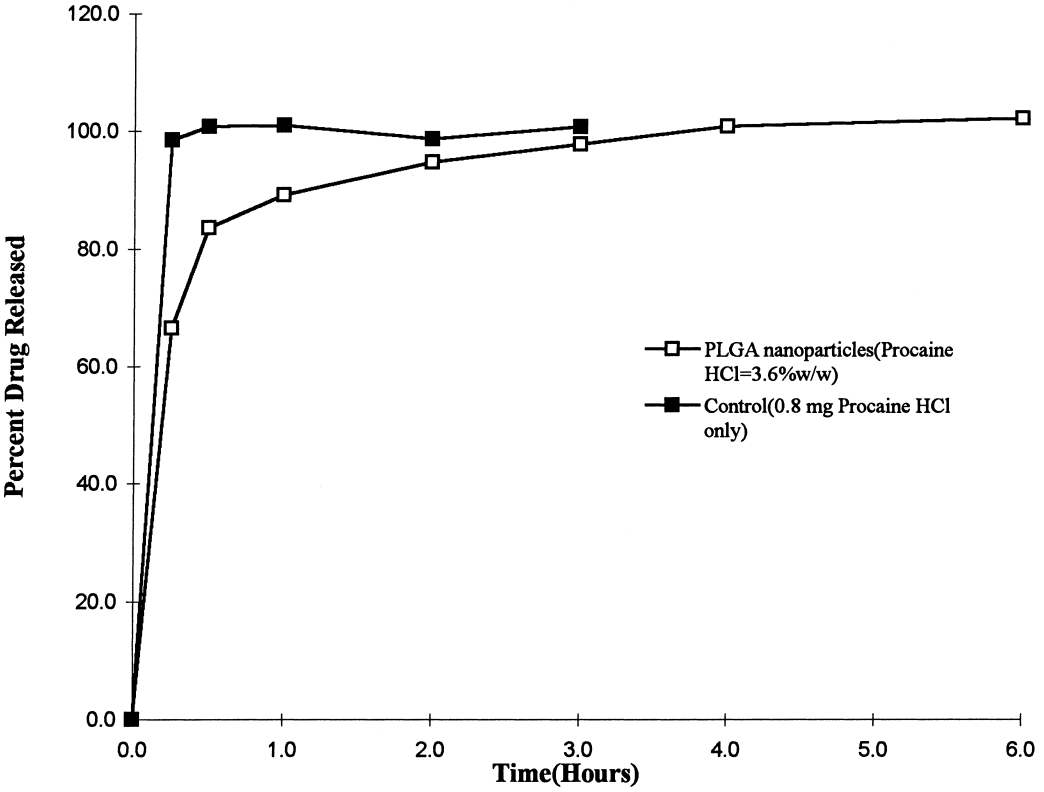

Fig. 7. In vitro release profile of procaine hydrochloride from PLGA nanoparticles (Drug content = 3.60% w/w).

favourable morphological characteristics, all of these indicating that high ionisation of carboxyl groups from PLGA chains and relatively low ionisation and lower solubility of the drug are favourable conditions for the production of nanoparticles. An alternative approach of replacing procaine hydrochloride with procaine dihydrate in the formulation also increased drug content and drug entrapment. However higher procaine dihydrate theoretical loadings of 4 and 10% w/w adversely influenced the particle formation. It appears that higher drug loadings of the base affected the process of PLGA precipitation and formation of spherical nanoparticles. The above approaches, of increasing the aqueous phase pH and replacement of procaine hydrochloride with procaine dihydrate, emphasised the importance of a decreased ionisation and reduced solubility of drug in the aqueous phase for enhancement of drug incorporation efficiency.

The effects of the addition of charged excipients containing carboxylic acid groups on drug incorporation efficiency was also examined. Although PLA oligomers enhanced drug content slightly, it was not considered as a feasible approach since it decreased drug entrapment and also reduced nanoparticle recovery dramatically. PMMA–MA also proved to be a useful excipient for improving drug incorporation efficiency since it increased drug content and drug entrapment. At a 1:1 and 3:1 (fatty acid:drug) molar ratio, lauric acid and caprylic acid were successful in enhancing drug content and drug entrapment. However, at a 3:1 molar ratio the morphology of nanoparticles were adversely influenced by lauric acid. Drug incorporation efficiency was not enhanced by combining the approaches of preparing nanoparticles in HEPES buffer pH 9.3 with lauric acid as a component. Hence, the results achieved for the inclusion of PLA oligomers, PMMA–MA and fatty acids highlighted the potential of the drugs inter-

action with charged groups to enhance drug incorporation efficiency without the need to adjust the aqueous phase pH.

Procaine hydrochloride release from nanoparticles appeared to have two components. An initial rapid release due to surface associated drug was followed by a slower exponential release of the drug which was dissolved in the core.

This study has therefore shown that formulation variables can be exploited in order to enhance the incorporation of a water soluble drug into PLGA nanoparticles by the nanoprecipitation technique.

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