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Keywords

controlled release, haloperidol, nanoparticles, PLGA end groups, drug-delivery

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**Production of haloperidol loaded PLGA nanoparticles
for extended controlled drug release of haloperidol**

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Abstract

We have developed an emulsion-solvent evaporation method for producing haloperidol loaded PLGA nanoparticles with up to 2 % (wt/wt. of polymer) drug content, in-vitro release duration of over 13 days and less than 20% burst release. The free haloperidol is removed from the nanoparticle suspension using a novel solid phase extraction technique. This leads to a more accurate determination of drug incorporation efficiency than the typical washing methods. We have discovered that PLGA end groups have a strong influence on haloperidol incorporation efficiency and its release from PLGA nanoparticles. The hydroxyl terminated PLGA (uncapped) nanoparticles have a drug incorporation efficiency of more than 30% as compared to only 10% with methyl terminated PLGA (capped) nanoparticles. The in-vitro release profile of nanoparticles with uncapped PLGA has a longer release period and a lower initial burst as compared to capped PLGA. By varying other processing and materials parameters, we also controlled the size, haloperidol incorporation and haloperidol release of our haloperidol loaded PLGA nanoparticles.

Introduction

Biodegradable microparticles and nanoparticles are promising candidates for controlled drug delivery and can deliver small molecular weight drugs, peptides or genes to the tissue of interest. The therapeutic agent of interest is encapsulated within the polymer matrix of biodegradable particles to achieve extended release (Allemann et al., 1996; Soppimath and Aminabhavi, 2002). The drug is released slowly over an extended time interval; the polymer degrades and is metabolized by the body. The polymers used most extensively for long-term drug delivery are poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and their copolymer poly(lactide-co-glycolide acid) (PLGA) (Jain et al., 1998).

Nanoparticles have been successfully used for systemic, oral, pulmonary and transdermal routes for various purposes (M J Cappel, 1991). A nanoparticulate drug delivery system, once designed, can be evaluated on the basis of four important performance metrics. These are: particle size, drug incorporation efficiency, drug content and the drug release characteristics. Hence a basic understanding of all the factors controlling the above mentioned performance metrics is of paramount importance in designing a nanoparticulate drug delivery system for a particular drug.

Haloperidol is an extensively used, highly potent antipsychotic drug. An uninterrupted supply of antipsychotic medication therapy is vital for patient health. A long-term drug delivery system would be an ideal candidate to improve drug adherence and to ensure a continuous supply of optimum dosage levels of the drug. The aim of our research is to understand the various factors that affect the crucial performance metrics of haloperidol-loaded nanoparticles including- size, drug incorporation, loading and release.

We have produced haloperidol-loaded PLGA nanoparticles using an emulsification-solvent evaporation method and have developed a novel solid phase extraction technique to remove the non-encapsulated haloperidol (free haloperidol) from the formulation. Subsequent to production, the particles were extensively studied for factors influencing haloperidol incorporation, particle size and haloperidol release from the particles. We have determined that the PLGA end groups, the haloperidol-PLGA interaction and the PLGA hydrophobicity most strongly influence the haloperidol incorporation and its release from nanoparticles.

Materials and Methods

Materials

Poly (D,L-lactic-co-glycolic acid) (PLGA) 50:50 DL 3A (inherent viscosity, 0.37 dL/g), 50:50 DL 3M (inherent viscosity, 0.36 dL/g), 95:5 (inherent viscosity, 0.68 dL/g) were purchased from Alkermes, USA. Polyvinyl alcohol (PVA) (MW, 25,000, 88% hydrolyzed) was purchased from Polysciences Inc., USA. Haloperidol, PBS, ammonium acetate, HEPES were purchased from Sigma, USA. Acetonitrile, DCM and acetone were purchased from Fisher scientific. All the solvents were HPLC grade.

Nanoparticle preparation

An emulsification-solvent evaporation method was used to prepare haloperidol loaded PLGA nanoparticles. Haloperidol and 100 mg of PLGA was dissolved in 3 ml of DCM. 50 ml of surfactant solution (250 mg of PVA dissolved in 50 ml of pH 10 HEPES buffer) was added to the organic phase and an O/W emulsion was prepared by

homogenizing at 12000 rpm for 7 minutes (Kinematica Polytron Benchtop Homogenizer, Brinkmann Instruments). The nanodroplets were then stirred at 400 rpm under atmospheric conditions for 2-3 hours to evaporate the DCM and form polymer nanoparticles. Unless otherwise noted, the following set of parameters was chosen to prepare the nanoparticles: PLGA 50:50 uncapped, molecular weight 51 kD at a concentration of 33.3 mg/ml in DCM; initial haloperidol concentration of 0.83 mg/ml in DCM; aqueous phase of pH 10; PVA as surfactant at a concentration of 1% wt/vol.; homogenization at 12000 rpm for 7 minutes.

Free drug extraction

This method consists of passing the nanoparticulate suspension through a cartridge packed with porous particles of a polymeric sorbent that selectively captures basic analytes, while allowing the nanoparticles (with encapsulated drug) to pass through as effluent. The sorbent captures haloperidol, a basic drug, through a combined reversed-phase and mixed-cation-exchange chromatographic mechanisms. Specifically, our nanoparticulate suspensions were passed through Oasis Mixed-Cation-Exchange (MCX) cartridges (Waters, USA) pre-conditioned with methanol and water to solvate the sorbent. The effluent nanoparticle suspension was taken for drug incorporation studies and in-vitro release studies.

Nanoparticle characterization

The size and size distribution of the nanoparticles were measured by laser dynamic light scattering. (DLS, 90 plus Particle Size Analyzer, Brookhaven Instruments,

USA). Scanning electron microscopy (SEM, JEOL 6300F FEG HRSEM, USA; 5 kV) was used to determine the shape and surface texture of the nanoparticles. One ml of the nanoparticulate suspension was dried under vacuum, coated with platinum in a sputter coater (Cressington 108 Sputter Coater) and examined by SEM.

The haloperidol content and incorporation efficiency were measured using HPLC (Waters, USA) with a reversed phase Symmetry C18 5.0 micrometer column (4.6mm x 150mm). The mobile phase used for the column was 38 % acetonitrile and 62 % 10mM, pH 4.8 ammonium acetate solution. After being passed through the MCX cartridge, a 1-ml of nanoparticulate suspension was dissolved in 40 ml of mobile phase and a 50 μ l aliquot of this sample was injected in HPLC machine with an auto injector (Waters 717plus Autosampler). The column effluent was detected at 254 nm by UV spectrophotometry (Waters 2487 Dual wavelength absorbance detector). A calibration curve for haloperidol was obtained using a series of haloperidol standards prepared in the mobile phase. The calibration curve was linear in the range of concentrations measured. The encapsulation efficiency was obtained as the ratio of the amount of haloperidol incorporated in the nanoparticles to the total amount of haloperidol used. Drug content was calculated as the ratio of the mass of drug inside the nanoparticles to the total initial mass amount of the polymer. Since the polymer recovery was close to 90%, this method of drug content calculation gave similar results as the usual method of taking it as the ratio of the drug amount inside the particles to the total mass recovered. Thus, this method of calculating drug content provides a lower limit of drug content and the true drug content could be ~10%-12% higher.

In-vitro release study

The *in-vitro* release study of the haloperidol-loaded PLGA nanoparticles was carried out in stirred dissolution cells at 37.4°C by suspending the nanoparticulate suspension in large quantity of pH 7.4 PBS solution such that the total amount of haloperidol inside the suspended nanoparticles is less than 10% of its solubility limit in PBS buffer. This ensures the correct *in-vitro* conditions to study the release behavior of a hydrophobic drug (Chorny et al., 2002b). One ml aliquots were taken out of the dissolution cells at pre-determined time intervals, replaced by fresh PBS buffer and analyzed for released haloperidol.

Results and Discussion

A novel method to eliminate free drug and determine drug incorporation

We compared the haloperidol incorporation in PLGA nanoparticles determined after using the standard method of multiple washings and our method of solid phase extraction. Two batches of haloperidol loaded nanoparticle suspensions were each divided into three groups: (i) Raw nanoparticle suspension, (ii) Washed nanoparticle suspension, and (iii) Stripped nanoparticle suspension. The Raw nanoparticle suspension refers to the nanoparticulate suspension obtained just after solvent evaporation. The Washed nanoparticle suspensions refer to raw nanoparticles that were centrifuged and washed thrice with distilled water. The Stripped nanoparticle suspensions refer to the raw suspension passed through MCX cartridges.

Table 1 shows the % haloperidol in the nanoparticle suspension (Raw, Washed and Stripped) for two initial concentrations of haloperidol. The raw suspension has a

relatively large amount of haloperidol, ~90%, because it contains both free and encapsulated haloperidol. The Washed suspension has a lower value of % haloperidol, ~70%, indicating that some of the free drug is indeed removed by the washing method. The Stripped suspension has the lowest value, < 40%, owing to the complete removal of free drug.

These data suggest that the nanoparticle suspension prepared using the emulsification solvent-evaporation method contains some drug that is encapsulated within the polymer nanoparticles while the remaining drug is suspended outside the particles or sticks loosely on the particle surfaces. Thus the incomplete removal of free drug by washing techniques leads to an overestimation of the drug incorporation efficiency and drug content inside the particles.

Some researchers have addressed the problem of incomplete free drug removal by proposing alternative techniques of free drug removal from the nanoparticulate suspension (Baichello et al., 1999), (Aberturas et al., 2002). These methods are either cumbersome or they require an exact knowledge of the solubility behavior of the drug in the aqueous phase, in the presence of the surfactant. Alternatively, our method of solid phase extraction is a simple and efficient method to remove the free drug completely from the nanoparticulate suspension.

Size and size distribution

The mean nanoparticle size and size distribution is affected by the organic solvent, polymer concentration and processing parameters. SEM images indicate that the nanoparticles are spherical in shape, and thus will be described by an effective diameter

and a size polydispersity obtained by DLS (Figure 1). For the same polymer concentration of 33.3 mg/ml PLGA, the effective diameter for particles prepared with acetone is 275 ± 25 nm while it is 524 ± 220 nm for particles prepared with DCM (Figure 1 a, c). The particles obtained from acetone have a unimodal population distribution with a polydispersity of 0.04 (Figure 1a). With DCM, the particles have a bimodal population distribution and, consequently, a high polydispersity of ~ 0.29 (Figure 1c). Decreasing the PLGA concentration in DCM from 33.3 mg/ml to 20 mg/ml changes the particle size distribution from bimodal to unimodal (Figure 1 c, b). Figure 2 shows that the effective particle diameters for a range of PLGA concentration in both acetone and DCM. Bimodal distributions of particles were obtained with DCM at higher polymer concentrations while acetone gives unimodal distributions of particles at all polymer concentrations. The diameters of the two populations obtained from DCM are shown by dotted lines while the mean diameter is shown by full line.

The polymer concentration and organic solvent selection are critical in producing unimodal nanoparticles. DCM, a water immiscible solvent, forms nanoparticles by a true emulsification mechanism in which the larger emulsion droplets are broken into smaller droplets by the application of external energy (Bodmeier and McGinity, 1987). At higher polymer concentrations, the energy applied through homogenization is insufficient to overcome the resistive viscous forces provided by the dissolved PLGA in the organic phase and the dissolved surfactant (PVA) in the aqueous phase, leading to heterogeneous droplets and a bimodal size distribution.

In contrast, acetone, a water-soluble solvent, gives a smaller sized, more uniform, unimodal population even in the absence of homogenization through a nanoprecipitation

mechanism (Chorny et al., 2002a; Fessi et al., 1989; Quintanar-Guerrero et al., 1998). Acetone rapidly diffuses into the aqueous phase resulting in the precipitation of the polymer (PLGA) that forms nanoparticles. Thus, the preparation of nanoparticles using acetone is less sensitive to the polymer concentration and the homogenization parameters than when using DCM. Birnbaum et. al. (Birnbaum et al., 2000a) have reported similar trends with DCM and acetone for their PLGA nanoparticles.

Drug incorporation efficiency and drug content

Effect of initial haloperidol concentration. Figure 3a shows the haloperidol incorporation efficiency values for various initial concentrations of PLGA 50:50 (33.3 mg/ml, 25 mg/ml and 16.6 mg/ml) in DCM. There are two trends evident in Figure 3a. First, the haloperidol incorporation efficiency decreases upon increasing the initial haloperidol concentration for a fixed initial polymer concentration. Second, for a fixed initial drug concentration, the haloperidol incorporation efficiency increases upon increasing the initial PLGA concentration. However, the haloperidol content in the nanoparticles has a constant value of ~1 % for PLGA 50:50 particles, irrespective of the initial polymer or drug concentrations for the range tested (Figure 3b). Similar trend was observed for capped PLGA 95:5 particles (data not shown).

The initial drug to polymer ratio in the organic phase is critical in determining the drug incorporation, although the larger values of this ratio lead to smaller values of drug incorporation. This unexpected finding is furthered by the observation that the haloperidol content in the nanoparticles is independent of the initial haloperidol concentration. These results combine to suggest that the final haloperidol content in

these PLGA nanoparticles has an upper limit, which cannot be increased by simply increasing the initial haloperidol to polymer ratio in the emulsion. Rather increasing the initial haloperidol to polymer ratio in the emulsion leads to an increase in the amount of free drug, while the amount of encapsulated drug remains constant. Chorny et. al. (Chorny et al., 2002b) also reported an upper limit on their drug (tyrphostin AG-1295) amount that can be incorporated in a fixed amount of PLGA. Barichello et. al. (Baichello et al., 1999) suggested that a low affinity between PLGA and their drug (valproic acid) may be responsible for extremely low incorporation efficiency of 5.6 %. Hence, the physical encapsulation of haloperidol in PLGA is limited by the haloperidol-PLGA interaction.

Effect of PLGA end groups. We found that the end groups have a significant effect on the haloperidol incorporation and its release behavior from the nanoparticles. Two types of PLGA polymers were used to produce haloperidol-loaded nanoparticles: uncapped (carboxyl acid end group) and capped (methyl ester end group) PLGA (Figure 4). The haloperidol incorporation efficiency with uncapped PLGA 50:50 is 32 ± 15 %, which is three times higher than with capped PLGA 50:50, 8 ± 5 %. Also, the haloperidol incorporation values for capped PLGA 50:50 (~8%) and capped PLGA 95:5 (~12%) are comparable, thus indicating that the L:G ratio is less important than the endgroups. The importance of endgroups has previously been suggested for PLGA microspheres loaded with the drugs gentamicin (Nagata S, 1994) and leuprorelin (Takada S, 1998). There is also a report for PLGA nanoparticles in which the end group doubles the protein loading (Gaspar M M, 1998).

Based on these results, we propose that the presence of carboxylic acid groups (-COOH) increases the hydrogen bonding between the PLGA chains and the haloperidol molecules, which hinders the drug diffusion out of the polymer nanoparticle during solvent evaporation. Thus the amount of haloperidol incorporated in the uncapped PLGA matrix is higher due to the tendency for hydrogen bonding between -COOH end groups and haloperidol (Figure 4b). The -COCH₃ end group of capped PLGA and the -CH₂OH end group of both capped and uncapped PLGA are much less capable of forming a hydrogen bond with haloperidol. This is further verified if we consider two nanoparticles samples: uncapped and capped PLGA nanoparticles, prepared with 2.5 mg of haloperidol and 100 mg of polymer of molecular weight ~50 kD corresponding to $\sim 1.2 \times 10^{18}$ -COOH end groups in the uncapped PLGA. If each -COOH group corresponds to one additional haloperidol molecule (by virtue of hydrogen bonding) then the uncapped PLGA nanoparticles would contain ~0.75 mg more haloperidol than the capped PLGA nanoparticles. The initial haloperidol amount was 2.5 mg, so this corresponds to an increase of ~30% in haloperidol incorporation, which is comparable to the observed increase of ~24%. Hence the polymer end groups exercise significant effect on haloperidol incorporation and content in the nanoparticles.

In-vitro release study

The release rate is strongly influenced by the PLGA end groups and the PLGA copolymer composition (L:G ratio). Figure 5 shows the cumulative % of haloperidol released as a function of time for nanoparticles made from PLGA 50:50, uncapped and capped. It can be seen that the particles from uncapped PLGA have a lower initial burst

of ~40% as compared to that of ~70% from capped PLGA. Also, ~95% of the haloperidol is released in 4 days from uncapped PLGA nanoparticles while it takes only 2 days with capped PLGA.

There are two opposing factors that contribute to the drug release from PLGA nanoparticles. The rate of hydrolysis is higher for uncapped PLGA, corresponding to a faster release. Conversely, the strength of haloperidol-PLGA interactions is greater for the uncapped PLGA due to the –COOH groups, yielding slower drug release. In our haloperidol-PLGA nanoparticles, the slower release observed in uncapped PLGA 50:50 (relative to capped 50:50) indicates that the haloperidol-PLGA interactions dominate the release profile. The prolonged drug release from our nanoparticles prepared using uncapped PLGA is consistent with the extended release response reported for nanospheres of L-asparaginase and PLGA.(Gaspar M M, 1998) In contrast other researchers have reported faster drug release when using uncapped PLGA.(Soppimath and Aminabhavi, 2002) (Lam X M, 2000). These opposing observations can be reconciled by considering the balance between the rate of polymer hydrolysis and the strength of drug-polymer interactions for specific drug-polymer combinations. In our haloperidol-PLGA system and the L-asparaginase-PLGA system (Gaspar M M, 1998) there are strong interactions between the drug molecules and the carboxylic acid end groups of the uncapped PLGA chains. This is evident from the fact that the drug incorporation in uncapped vs. capped PLGA nanoparticles increases by thrice and twice, respectively, in the above two systems. These strong interactions clearly overwhelm the faster hydrolysis typical of uncapped PLGA causing an overall slower release for uncapped PLGA. On the other hand, the rhIGF-I-PLGA system (Lam X M, 2000) and the

nifedipine-PLGA system (Soppimath and Aminabhavi, 2002) have the same drug incorporation for uncapped and capped PLGA indicating the absence of any favorable interaction between the drug and polymer end groups. So in these cases the faster hydrolysis of the uncapped PLGA dominates and the overall release is faster for the uncapped PLGA particles. Clearly, the balance between faster hydrolysis and stronger interactions is specific to the number and type of drug-PLGA interactions and thus will be drug dependent.

In addition to the influence of end groups, the PLGA composition strongly influences release. Figure 6a shows the cumulative % haloperidol release for nanoparticles made from capped PLGA 95:5 and uncapped PLGA 50:50 as a function of time. Both sets of particles have a drug content of ~0.3% and a bimodal size distribution with populations of ~250 nm and ~970 nm in size. It can be seen that the capped PLGA 95:5 particles exhibit low burst release of ~15% as compared to that of ~35% with uncapped PLGA 50:50. The drug release period is ~13 days with capped PLGA 95:5 as opposed to ~2 days with uncapped PLGA 50:50.

Another release study was performed for unimodal particles made from uncapped PLGA 50:50 and capped PLGA 95:5 and the particle diameter was monitored. These particles from both polymers have an initial effective diameter of ~630 nm and the drug loading is ~1.9 % for uncapped PLGA 50:50 particles and ~0.4 % for capped PLGA 95:5 particles. As expected, the haloperidol release is slower from capped PLGA 95:5 nanoparticles, Figure 6b. The particle size, as measured by dynamic light scattering, remains approximately constant for both sets of particles during the in vitro release study, Figure 6c. Comparison of drug release profiles for the same polymer in Figures 6a and

6b suggests that the particle size, size distribution and drug loading also influence drug release rate.

Drug can be released from polymer nanoparticles by the mechanism of diffusion and/or polymer erosion. We have observed that haloperidol release from *bulk* PLGA requires many weeks as it proceeds by both diffusion and polymer erosion (Steven J Siegel, 2002). In contrast, the nanoparticles studied here release the haloperidol without a detectable change in effective diameter. Thus, the mechanism governing release from these nanoparticles is predominantly drug diffusion. Furthermore, the slower release from capped PLGA 95:5 can be understood in terms of drug diffusion, because the increased hydrophobicity of capped PLGA 95:5 as compared to uncapped PLGA 50:50 corresponds to less hydration and swelling and consequently slower drug diffusion out of the polymer matrix. The dramatic differences in drug release characteristics between bulk and nanoparticle PLGA systems highlights the importance of size when designing materials smaller than 1 micron, as is typical in the field of nanotechnology.

Conclusions

Haloperidol-loaded PLGA particles were produced using an emulsification-solvent evaporation method. The size of the particles can be varied from 200 nm to 2000 nm and is most strongly affected by solvent miscibility and polymer concentration in the organic phase. Solid phase extraction was used to remove free drug from the nanoparticle suspension. PLGA with a –COOH end group (uncapped) substantially increases the drug incorporation in these nanoparticles, perhaps through the mechanism of hydrogen bonding. Haloperidol incorporation in uncapped PLGA nanoparticles is three times that

in capped PLGA nanoparticles. Including –COOH end groups on PLGA (uncapped) and increasing the PLA content in the PLGA reduces the initial burst and extends the duration of drug release from the nanoparticles. Uncapped PLGA shows a lower initial burst release (~40%) and a longer period of haloperidol release (~4 days) as compared to capped PLGA (~70% burst release and ~2 day release period). Nanoparticles prepared from uncapped PLGA 95:5 show a lower burst release of ~15 % and have a drug release period of ~13 days. While release in bulk mixtures of PLGA-drug is dominated by the degradation rate of the PLGA, the drug release from PLGA-haloperidol nanoparticles is dominated by the diffusion rate of the drug as controlled by the swelling of the PLGA, which is related to the PLA content, and the interactions between the drug and the PLGA. The distinctions between bulk and nanoparticle drug loaded PLGA systems highlight the importance of size on the production and properties of materials, a cornerstone of nanotechnology, and suggest general approach to engineering nanoparticles for extended drug delivery.

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Tables

Table 1: Comparison of haloperidol incorporation for various preparation methods as indicated.

Initial haloperidol concentration in DCM	% haloperidol in the nanoparticle suspension relative to the initial amount of haloperidol		
	Raw	Washed	Stripped
0.83 mg/ml	92 ± 3	70 ± 8	37 ± 6
1.67 mg/ml	90 ± 5	75 ± 3	23 ± 5

Figure Captions

Figure 1: Nanoparticle size distribution provided by dynamic light scattering histograms and the SEM images of nanoparticles prepared using PLGA 50:50 at (a) 33.3 mg/ml initial concentration in acetone, (b) 20 mg/ml initial concentration in DCM, and (c) 33.3 mg/ml initial concentration in DCM. The nanoparticles in (a) and (b) are unimodal in size while those in (c) are bimodal.

Figure 2: Effect of PLGA 50:50 concentration in the organic phase on the nanoparticle effective diameter for particles prepared with DCM and acetone as the organic solvents. The dotted lines indicate the diameters of the two populations in case of DCM at higher concentrations. The full lines indicate the mean effective diameters for particles prepared with DCM (■) and with acetone (▲).

Figure 3: (a) Haloperidol incorporation efficiency as a function of initial haloperidol loading for various initial concentrations in DCM of uncapped PLGA 50:50 (of MW 51 kD). (b) Final haloperidol content in the nanoparticles as a function of initial haloperidol concentration for various initial concentrations of PLGA 50:50. Legends: ◆: 33.3 mg/ml of PLGA 50:50; ◇: 25mg/ml of PLGA 50:50; ▲: 16.6 mg/ml of PLGA 50:50.

Figure 4: (a) Chemical structure of capped (-COCH₃ terminated) PLGA. (b) Schematic showing the hydrogen bonding between uncapped (-COOH terminated) PLGA and haloperidol.

Figure 5: In-vitro haloperidol release from PLGA 50:50 nanoparticles for □ uncapped (-COOH terminated) and ■ capped (-COCH₃ terminated) PLGA. Nanoparticles prepared with uncapped PLGA exhibit a lower initial burst release and a longer release period as compared to nanoparticles prepared with capped PLGA.

Figure 6: (a) In-vitro haloperidol release from PLGA nanoparticles with bimodal size distribution. Nanoparticles prepared from capped PLGA 95:5 (■) show an extended drug release period and a lower initial burst release as compared to nanoparticles prepared from uncapped PLGA 50:50 (□). (b) In-vitro haloperidol release profile from PLGA nanoparticles with unimodal size distribution and prepared from uncapped PLGA 50:50 and capped PLGA 95:5. (c) Variation of the effective diameter of the particles in part (b) as measured by dynamic light scattering. The error bars represent the standard deviation of the effective diameter from triplicate light scattering data.

Figures

Figure 1:

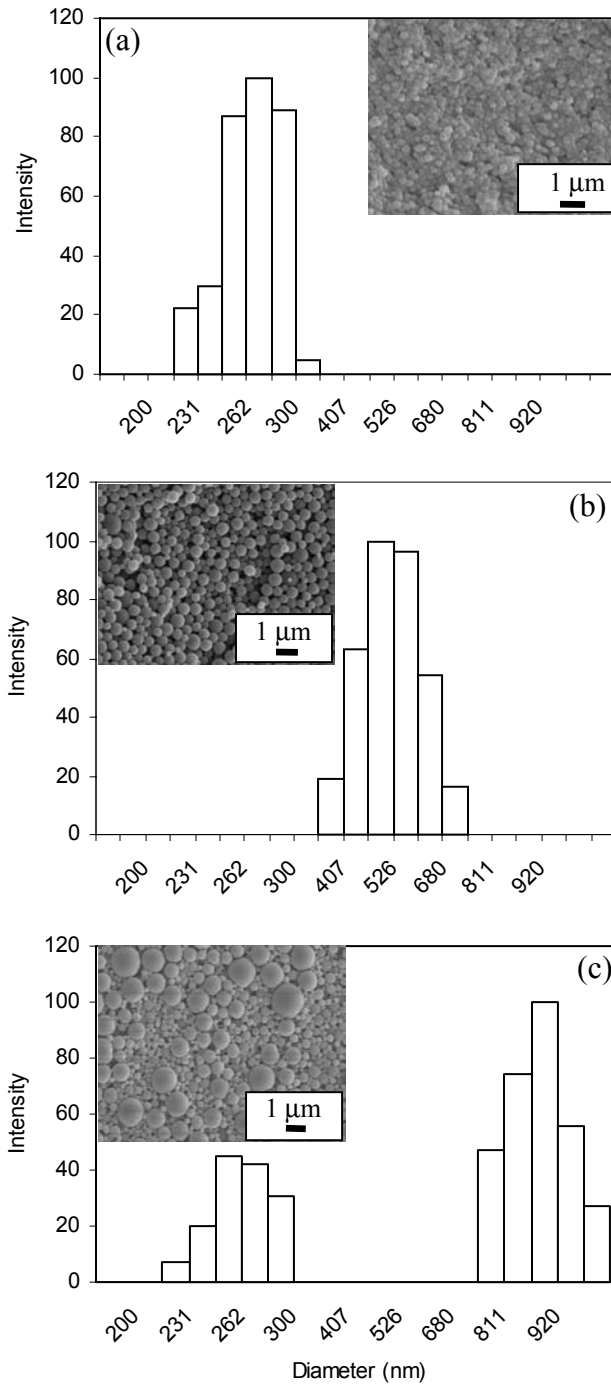


Figure 2:

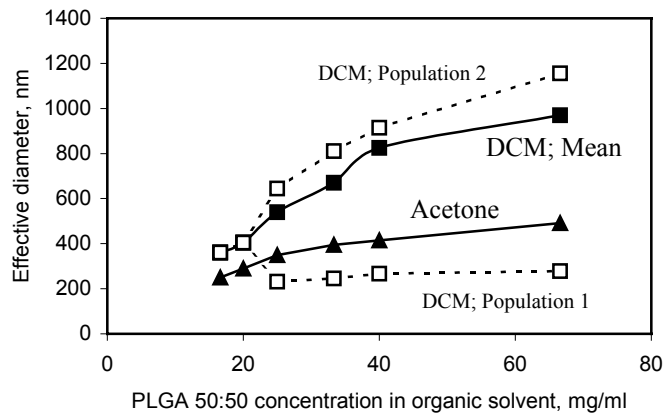


Figure 3

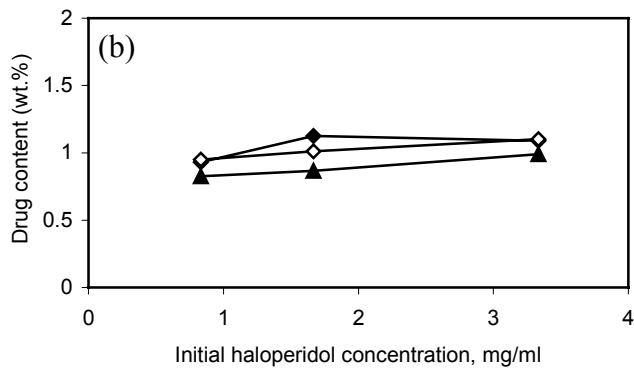
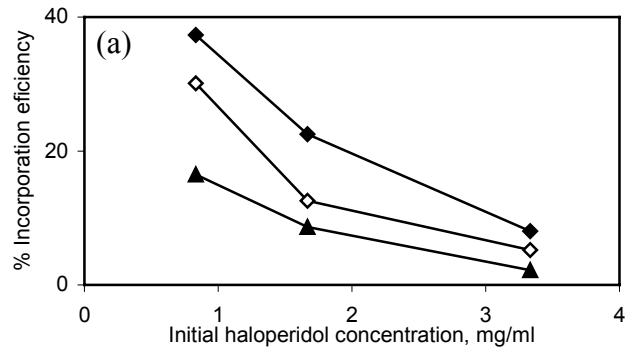
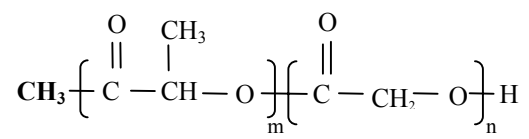


Figure 4

(a)



Capped PLGA

(b)

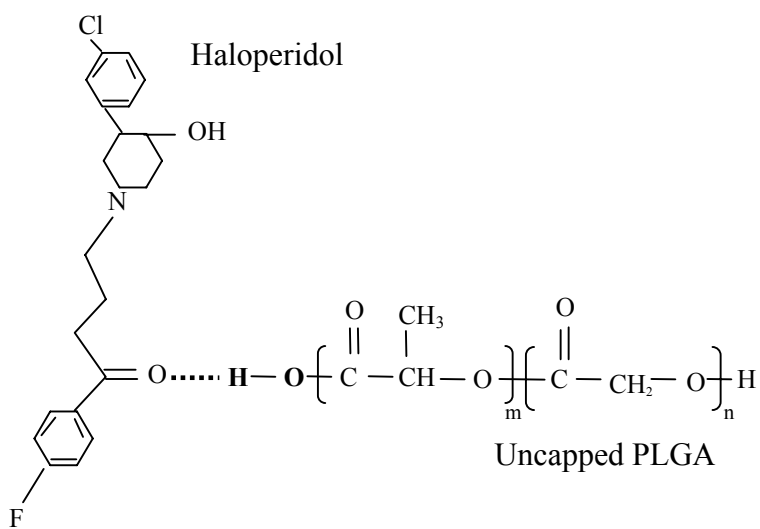


Figure 5

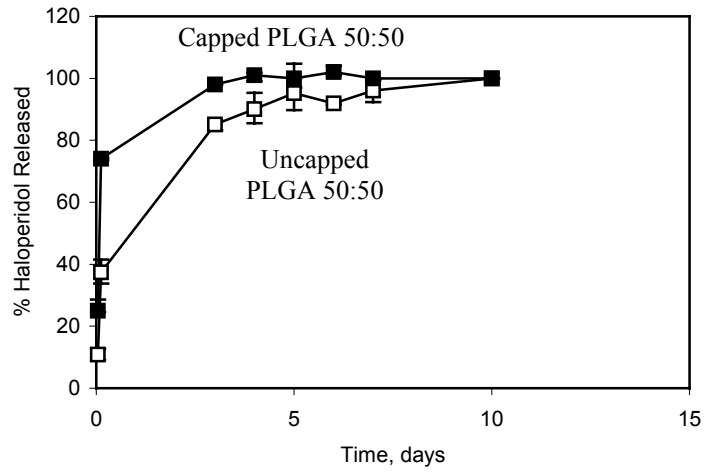


Figure 6

