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Research paper

Polymer and microsphere blending to alter the release of a peptide from PLGA microspheres

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Abstract

The objective of this study was to evaluate the effect of polymer and microsphere blending in achieving both a sufficient initial release and a desired continuous release of a peptide from poly(D,L-lactide-co-glycolide) microspheres. Leuprolide acetate loaded hydrophilic 50:50 PLGA microspheres were prepared by a solvent-extraction/evaporation process and were characterized for their drug load, bulk density, size distribution, surface area, surface morphology, in vitro drug release, and in vivo efficacy. Combining PLGA polymers that varied in their molecular weights in various ratios yielded microspheres with varied drug release profiles commensurate with the hydration tendencies of the polymers. Increasing the component of lower molecular weight 50:50 hydrophilic PLGA polymer, 8.6 kDa increased the initial drug release. A similar microsphere formulation prepared instead with blending microspheres from individual polymers showed a similar increase. In an animal model, microspheres obtained from polymer or microsphere blends attained a faster onset of testosterone suppression as compared to microspheres from higher molecular weight 50:50 hydrophilic PLGA polymer, 28.3 kDa, alone. These studies illustrated the feasibility of blending polymers or microspheres of varied characteristics in achieving modified drug release. In particular the increased initial release of the peptide could help avoid the therapeutic lag phase usually observed with microencapsulated macromolecules.

Keywords: PLGA microspheres; Leuprolide, Peptide release; Polymer and microsphere blend

1. Introduction

Compared with conventional low molecular weight molecules, proteins and peptides have unique requirements and limitations for delivery. These agents in general have short plasma half-lives, are unstable in the gastrointestinal (GI) tract, and also have low bioavailabilities due to their large molecular weights and high aqueous solubility [1,2]. Frequent injections might be acceptable in cases of acute situations but not in chronic conditions. Thus, development of sustained release dosage forms for peptide/protein delivery will help these agents to realize their full potential as drugs while enhancing patient compliance and convenience.

Microspheres prepared from poly(D,L-lactic-co-glycolic) acid (PLGA) polymers have been studied extensively in the last two decades as sustained release dosage forms and have shown improved patient compliance and/or therapeutic efficacy of contraceptive steroids, narcotic antagonists, antimalarials, and anticancer agents [3,4]. Recent studies, especially with luteinizing hormone-releasing hormone (LHRH) analogues, have shown these systems to be effective in the sustained delivery of macromolecules [5–7]. In addition to being biocompatable, degradation rates of PLGA and the accompanying release of encapsulated drug can be controlled by the polymer's physical properties such as molecular weight, hydrophilicity, and the ratio of lactide to glycolide [8–11]. Thus, it is possible to obtain the desired drug release from PLGA microspheres by altering the polymer's characteristics. An extension to this approach is to optimally combine microspheres prepared from different polymers with known drug release or to blend polymers prior to preparing the microspheres. The effect of mixed populations of controlled release particles on the resulting release pattern has been cited in some early literature reports [12–14] but there has been little experimental follow-up to show the feasibility and practical application of blending.

The purpose of this study was to prepare and evaluate

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peptide-loaded microspheres from various blends of two commercial PLGA polymers with different molecular weights as an alternative to modifying the polymer characteristics in achieving enhanced initial drug release. Further, formulations obtained by physically blending microspheres that were prepared from individual polymers were evaluated as an alternative approach to making microspheres from blended polymers. LHRH superagonist analogue leuprolide acetate was selected and peptide-loaded microspheres from hydrophilic PLGA (50:50) polymers were prepared by a solvent extraction/evaporation method. Physico-chemical characteristics of the microspheres were correlated with the in vitro peptide release, and the formulation efficiency in suppressing serum testosterone levels for sustained periods was evaluated in an animal model.

2. Materials and methods

2.1. Materials

PLGA (50:50) polymers, Resomer® RG503H (MW 28, 032) and RG502H (MW 8631) were obtained from Boehringer Ingelheim (Ingelheim, Germany). LHRH analogue, leuprolide as an acetate salt was purchased from Bachem Inc. (Torrance, CA). All other chemicals used were of analytical reagent grade.

2.2. Preparation of microspheres

Microsphere formulations in a typical batch size of 1.5 g were prepared by a solvent-extraction/evaporation method [15]. PLGA polymer or a combination of PLGA polymers in methylene chloride was mixed with a methanolic solution of the peptide. The resulting mixture (dispersed phase, DP) was added to a 0.35% w/w polyvinylalcohol (PVA, MW 30–70 kDa, Sigma) aqueous solution (continuous phase, CP) while stirring at 7000 rev./min using a homogenizer (Silverson L4R, Silverson Instruments Corp., MA). After 5 min at 25°C the stirring rate was decreased to 500 rev./min and the temperature raised to 40°C to slowly extract and evaporate the organic phase over 1 h. After cooling to 25°C, particles were recovered by filtration and dried overnight under vacuum at room temperature.

To evaluate the effect of polymer blends, the 8.6 kDa polymer was combined in various proportions with 28.3 kDa polymer as detailed in Table 1 (formulations C–E). Total polymer concentration was adjusted so that the viscosities of polymer/methylene chloride solutions, as measured by a Brooksfield viscometer, were comparable. The target drug loading for all the formulations was 12.5% w/w. Microspheres prepared from individual polymers were mixed physically in 3:1 (28.3 kDa/8.6 kDa) drug content ratio to obtain formulation F. Characteristics of these microspheres were compared with those of microspheres prepared from a 3:1 polymer mixture (formulation C).

2.3. Characterization of microspheres

The microspheres were characterized for drug content, bulk density, specific surface area, mean particle size, surface morphology, and in vitro drug release and in vivo efficacy.

2.3.1. Drug content

Drug loaded microspheres were quantitatively dissolved in methylene chloride and the peptide was extracted into acetate buffer (pH 4, 0.1 M) by shaking the mixture for 1 h on a wrist action shaker (Burrell, Pittsburgh, PA). The aqueous buffer phase was separated by centrifugation and extracted peptide was quantitated by a reverse phase-HPLC method [16] after some modifications. The extraction was repeated with fresh buffer and the combined peptide amount values were reported as the drug content, and expressed as % w/w of microspheres. Triplicate samples were used for determining the drug content and mean values were reported. HPLC analytical conditions were as follows: chromatograph separation was achieved on a C_{18} μBondapak column (3.9 × 300 mm, Waters) using a variable wavelength detector at 220 nm, a gradient pump (both from Dionex Corp., Sunnyvale, CA) and an autosampler (Thermo Separation Products, Fremont, CA). The mobile phase was a 68:32 isocratic mixture of HPLC grade water and acetonitrile, which was adjusted to pH 4.0 with 0.1% trifluoracetic acid. The flow rate was 1.1 ml/min.

2.3.2. Bulk density

The dry microspheres were quantitatively transferred to a graduated test tube. The test tube was subsequently tapped

Table 1
Manufacturing parameters of peptide-loaded microspheres[a]

Formulation ID	A	B	C	D	E
Polymer(s)	28.3 kDa	8.6 kDa	28.3 kDa/8.6 kDa	28.3 kDa/8.6 kDa	28.3 kDa/8.6 kDa
Ratio			3:1	4:1	5:1
w/w % of polymer in DP[b]	16.3	38.0	24.0	21.1	19.6
Ratio of CH_3OH/CH_2Cl_2 in DP	0.24	0.22	0.20	0.20	0.20

[a] Formulation F was a physical 3:1 combination of A and B.
[b] DP, dispersed phase containing peptide and polymer in methanol and methylene chloride.

20 times from a vertical distance of approximately 0.5 inches and the occupied volume recorded. The tapping process was repeated until the volume occupied by particles remained unchanged. The final volume was recorded as bulk volume, v_b, and the tapped bulk density (g/cc) was calculated as m/v_b, where m was the weight of microspheres employed.

2.3.3. Specific surface area

The specific surface area was determined using an ASAP 2000 surface area analyzer (Micromeritics, Norcross, GA) by BET transformation of the adsorption-desorption isotherms of Kr on the surface of microspheres. The area values were normalized to the sample weight, which was typically in the range of 250–300 mg.

2.3.4. Size distribution

Particle size distribution was determined using a Malvern 2600c Laser Diffraction Particle Sizer (Malvern Instruments, Southborough, MA). The microspheres were suspended in pre-filtered 0.1% aqueous Tween 80 solution and either a 63 mm (for a size range of 0.5–118 μm) or 100 mm (for a size range of 1.9–188 μm) focal length lens was employed to determine particle size. Mean diameter based on volume was determined.

2.3.5. Surface morphology

The surface morphology was examined by scanning electron microscopy (Hitachi Model S800, Japan) after coating the microsphere sample with gold-palladium on an aluminum stub.

2.3.6. In vitro drug release

Approximately 10 mg of peptide loaded microspheres were quantitatively transferred to test tubes and incubated with 10 ml of 0.033 M phosphate buffer (pH 7) at 37°C in a temperature controlled oven. Separate samples were maintained for each time point. The tubes were shaken twice weekly and 8 ml of supernatant were replaced with fresh buffer every 7 days to maintain sink conditions. After sampling, microspheres were separated by centrifugation. To minimize the loss of microspheres, only 80% of the supernatant was removed. Correction for peptide in the remaining 2 ml of supernatant was made in the final calculations of peptide remaining in microspheres. The drug content in the microspheres was quantitated as described earlier by HPLC.

Peptide release was based on the peptide remaining in the microspheres rather than on the released amount of peptide, as the released peptide has limited stability (unpublished laboratory studies) in the in vitro releasing medium under the experimental conditions. Released drug was calculated as the difference between initially loaded drug and that remaining in the microspheres, and expressed as a % of initially loaded amount.

2.3.7. In vivo evaluation

Male Sprague–Dawley rats (Harlan Sprague Dawley, Inc., Chicago, IL) at least 12 weeks old, weighing 200–250 g were employed ($n = 6$ per formulation) to assess serum testosterone levels. Animals were maintained as per the guidelines set forth in *Guide for the Care and Use of Laboratory Animals*, DHEW Pub. No. (NIH) 78-23 (revised). The microspheres were suspended in a mixture of 1% carboxymethylcellulose (7LFPH, USP, Aqualon, Delaware, NJ) and 2% mannitol (USP/EP) and injected into rats subcutaneously just below the neck region, at a drug dose of 3 mg leuprolide/kg body weight based on literature reports [17]. A single injection was given to each animal immediately after collecting an initial sample from the tail vein. Further samples were collected at 0.25, 1, 4, 8, 15, 25, 32, 33, 42, and 43 days after dose administration. On days 32 and 42, animals were challenged with 100 μg/kg of leuprolide acetate, to investigate whether the LH receptors were still down-regulated. Following the challenge doses, additional samples were taken at 6 and 24 h. The lack of an elevation in testosterone levels above 0.5 ng/ml would indicate that the receptors were still occupied. Samples were assayed in duplicate for testosterone levels by radioimmunoassay using a standard commercial kit (Active Testosterone®, Diagnostic Systems Laboratories, Webster, TX).

3. Results and discussion

3.1. In vitro characterization

The physico-chemical characteristics of peptide loaded

Table 2
Characteristics of peptide loaded microspheres[a]

Formulation ID (polymer-ratio)	Drug content (%w/w)	Surface area (m^2/g)	Size[b] (μm)	Bulk density (g/cc)
A (28.3 kDa)	11.88	0.387	18.0	0.54
B (8.6 kDa)	11.34	1.540	21.0	0.30
C (28.3/8.6 – 3:1)	9.87	0.584	28.0	0.52
D (28.3/8.6 – 4:1)	9.48	0.420	28.5	0.55
E (28.3/8.6 – 5:1)	9.75	0.602	20.0	0.56

[a] Formulation F was a physical 3:1 combination of A and B.
[b] Mean diameter based on volume.

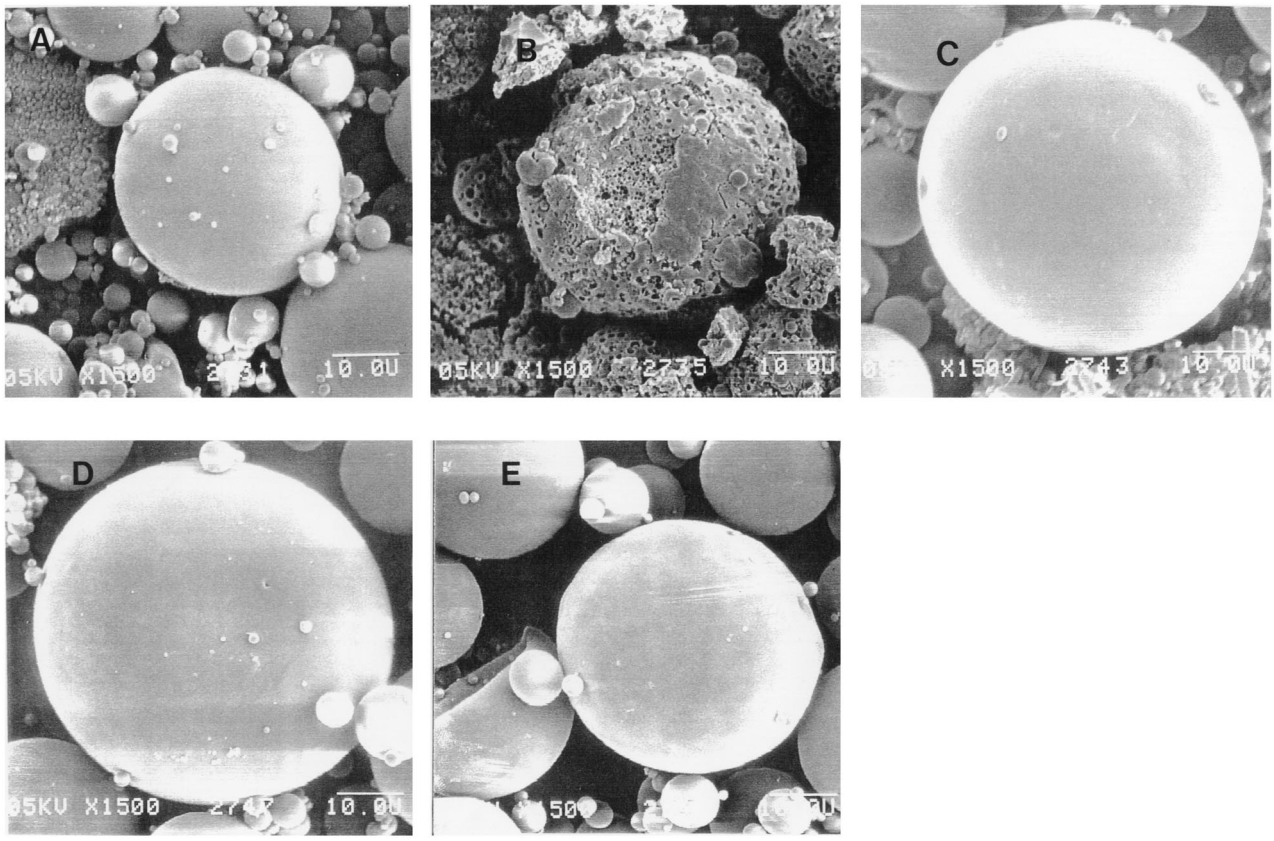

Fig. 1. Scanning electron micrographs of microspheres from polymer combinations at 1500× (28.3 kDa/8.6 kDa: A, 1:0; B, 0:1; C, 3:1; D, 4:1; E, 5:1).

PLGA microspheres are tabulated in Table 2 and scanning electron photomicrographs of representative microspheres from each formulation at 1500× magnifications are shown in Fig. 1. All the formulations except for the 8.6 kDa microspheres had a similar surface morphology. In general, microspheres were spherical, smooth, and non-porous. In contrast, the 8.6 kDa microspheres were very porous with rough surfaces and were expected to have high specific surface area and release drug faster.

The formulations had a similar size distribution with mean diameters in the range of 18–29 μm. As the viscosity of polymer solution can influence the microsphere characteristics including size distribution, similar polymer viscosities in the organic phases were maintained by modifying polymer concentrations (Table 1). In the case of the lower molecular weight polymer, the viscosity was maintained by increasing the polymer concentration. The microspheres used in this study were in an injectable range for convenient subcutaneous and intramuscular injections via a 21- or 23-gauge needle.

Drug content (% w/w) values for 28.6 and 8.6 kDa microspheres were similar; however, microspheres from the polymer combinations have a decreased loading efficiency (Table 2). The encapsulation efficiency values for all the formulations calculated against the target drug loading of 12.5% w/w ranged from 78 to 95%. Slightly higher amounts of methylene chloride were used in preparing the 'polymer combination' batches and possibly due to this, higher residual levels of methylene chloride (in the range of 132 vs. <20 ppm with single polymer formulations; data not shown) were observed. It was possible that as methylene chloride was extracted at an apparent slower rate, a higher amount of peptide was lost into the aqueous phase through the polymer wall that remained soft and permeable for a

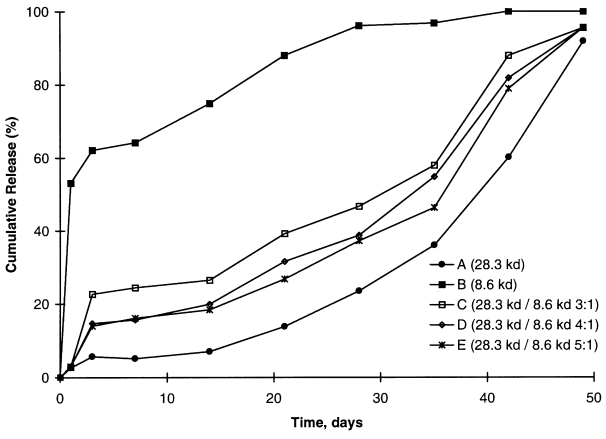

Fig. 2. In vitro peptide release from microspheres prepared from polymer combinations (28.3 kDa/8.6 kDa: A, 1:0; B, 0:1; C, 3:1; D, 4:1; E, 5:1).

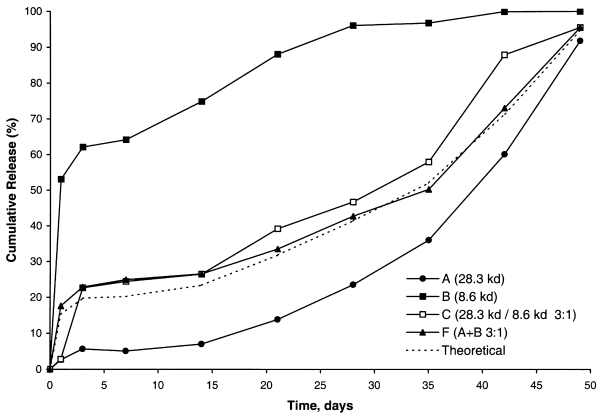

Fig. 3. In vitro peptide release from microspheres prepared from polymer C and microsphere F blends.

relatively longer time in case of combination formulations leading to lower drug content values.

Microspheres prepared from 8.6 kDa polymer in this study had the lowest bulk density, which correlated well with the observed porous surface morphology (Fig. 1B). Low bulk density value can be a qualitative indicator of the formation of hollow microspheres and/or lack of optimum packing of irregular (non-spherical) microparticles. Bulk density values can also be correlated with specific surface area values; formulations with lower bulk density having higher specific surface areas-internal plus external. In this study, 8.6 kDa microspheres with the lowest bulk density had the highest total specific surface area. Formation of porous or hollow microspheres with high specific surface area in general translates into a faster drug release [18]. No major differences in bulk density were observed among 28.3 kDa, and polymer combination microspheres which were predominantly from the 28.3 kDa polymer. The specific surface areas with formulations C–E were higher as compared to that of formulation A, however, not as high as that was seen with 8.3 kDa polymer formulation B

Figs. 2 and 3 show the in vitro cumulative release of peptide. As expected, drug release from 8.6 kDa microspheres was very rapid, with approximately 55% of encapsulated drug being released within 24 h. This high initial release can be attributed to the more rapid hydration of lower molecular weight polymer as well as higher specific surface area. The high initial release was followed by a slower uniform release until exhaustion after 30 days. In contrast, drug release from 28.3 kDa microspheres was very slow and gradual up to 14 days, at which time the polymer apparently started dissolving and subsequently, polymer erosion controlled the drug release. A clear modi-

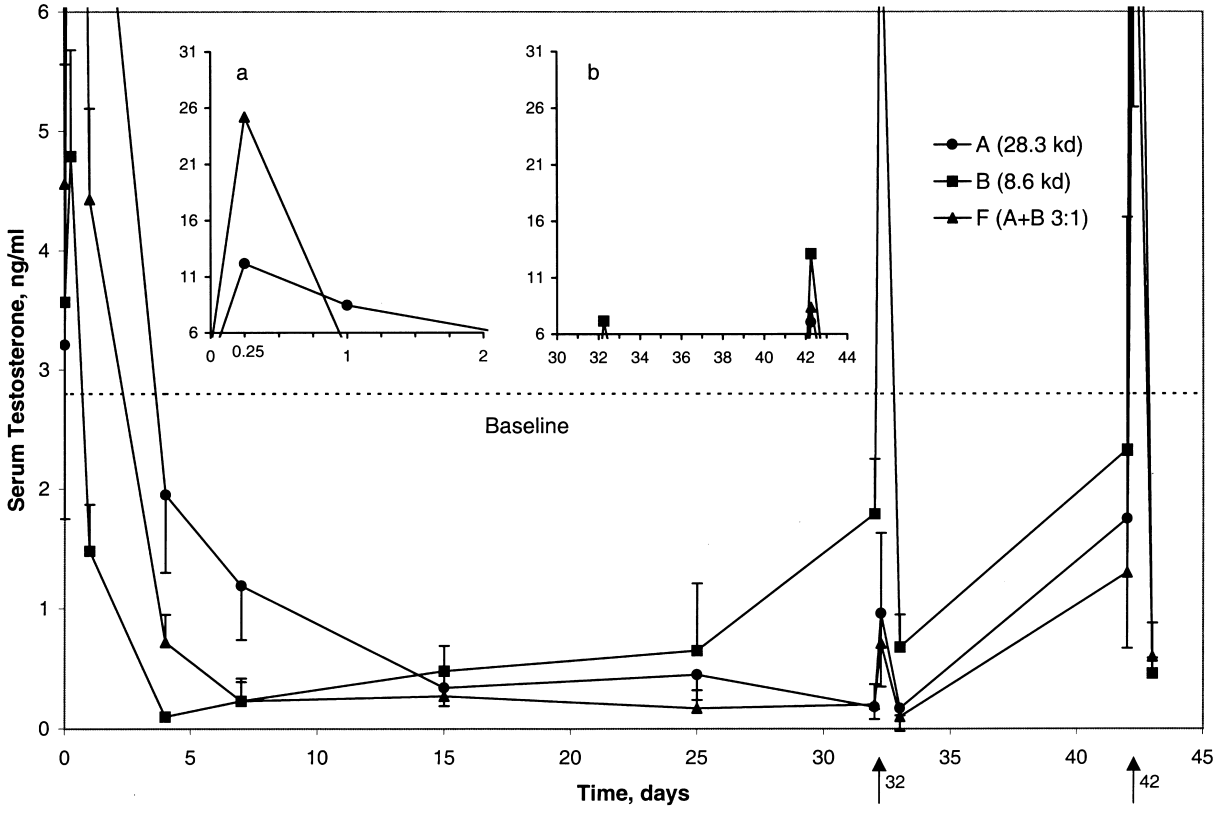

Fig. 4. In vivo testosterone suppression (SEM) with individual polymers, A and B, and microsphere blend, F, formulations. The inserts a and b show the data in the extended range above 6 ng/ml. Mean zero time values for the groups are shown on the *y*-axis while a mean baseline value from 180 rats is shown by the dotted line. ↑ indicates the challenge.

fication in the peptide release, particularly during the initial period was observed with the polymer combination formulations versus the single polymer formulations A and B as well as within polymer combinations C vs. D, and E. As 8.6 kDa polymer content was increased, the initial release of peptide was increased (formulation C). Although no major difference in the initial release was observed between formulations D and E (20 and 16%, respectively), differences were noted after approximately 10 days of study with the higher 8.6 kDa component formulation D favoring a slightly higher release. In general, the release profiles seemed to be similar with the exception of the extent of initial release. Preparations from polymer combinations appeared to have a lag phase (3–14 days) of drug release after the initial release. This lag is believed to be due to the predominance of the 28.3 kDa polymer. Previous studies by Li et. al. [19,20] suggested that gelling and solidification of the polymers occurred separately even in a homogeneous mixture of the two polymers. In such a case, it could be hypothesized that the more hydrated domains of 8.6 kDa polymer within the microsphere matrix accounted for the initial release during the first 3 days of the study, and the more prominent 28.3 kDa domain dictated the subsequent release in the later period.

Fig. 3 shows the comparative cumulative release of peptide from two formulations (C and F) obtained from polymer (C) and microsphere mixing (F). As the % drug content of 28.3 and 8.6 kDa microspheres was very similar (Table 2), a 3:1 ratio of drug content rather than polymer content was used in obtaining formulation F. The profiles were very close, except for some differences on the first day and during the 14–49 day period. PLGA polymers degrade hydrolytically giving rise to an acidic microenvironment in the particle structure [21] which enhances polymer degradation and mass loss. An acidic microenvironment is attained faster in the case of the 8.6 kDa PLGA as this polymer hydrates faster owing to its higher number of carboxylic acid endgroups. Additionally, microspheres from the lower MW polymer had a more porous internal structure which would also facilitate hydration. Thus, microspheres that contain 8.6 kDa PLGA as a combination in their structure are expected to degrade and release drug faster as compared to microspheres that are physically blended, as the hydration of the 8.6 kDa polymer will also hydrate the closely associated 28.3 kDa polymer. This may explain the higher drug release seen with polymer combination formulations at later time points. However, as the noted difference in the drug release from polymer or microsphere blends is not substantial, obtaining microspheres by physical mixing appears to be a suitable formulation alternative.

These studies also showed that the experimental release closely approximated the theoretical (calculated proportionately from profiles of individual polymer formulations) release values (Fig. 3). Thus, it is possible to determine an

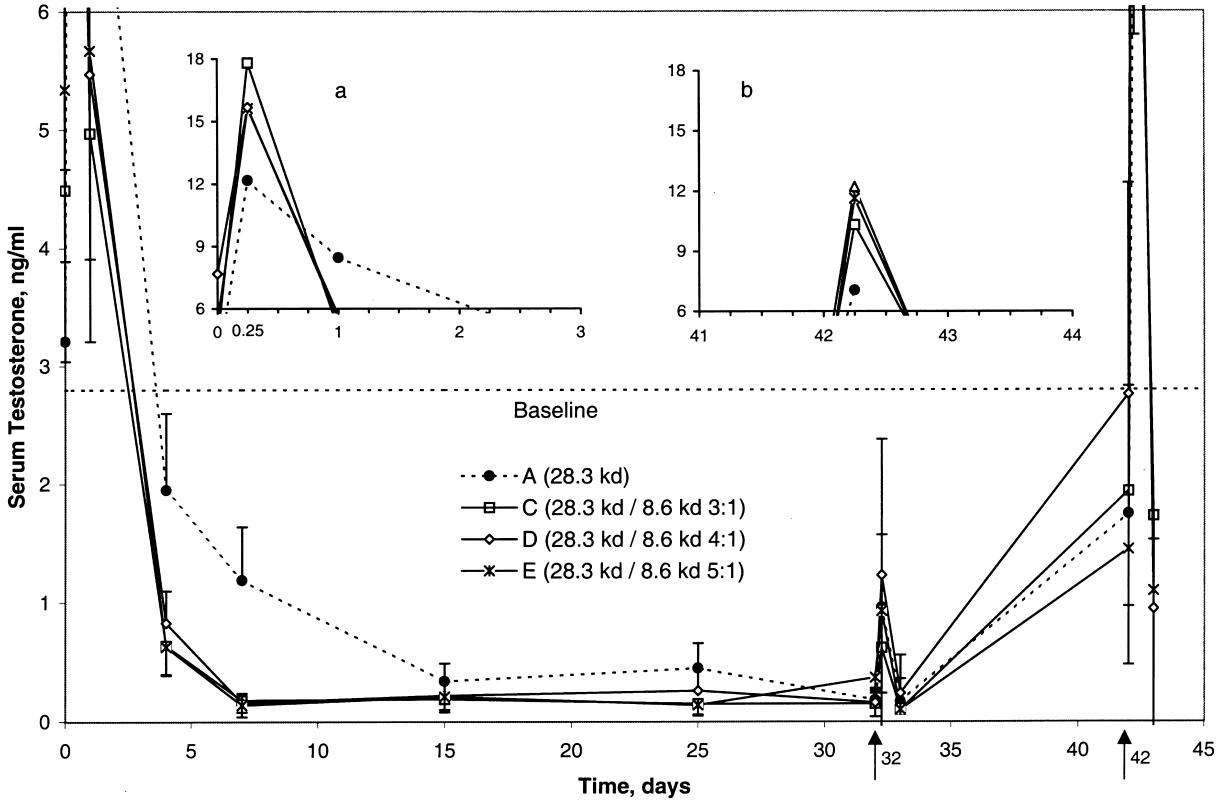

Fig. 5. In vivo testosterone suppression ($n = 6$) with polymer blend formulations C, D, and E. The inserts a and b show the data points above 6 ng/ml and ↑ indicates the challenge.

optimum combination of microspheres without performing a high number of experiments.

3.2. In vivo evaluation

Figs. 4 and 5 show the testosterone suppression obtained with formulations A–F. Fig. 4 depicts the testosterone suppression obtained with 28.3 kDa (A), 8.6 kDa (B), and a 3:1 28.3 kDa/8.6 kDa (F) mixture of microspheres. Leuprolide acetate, being a LHRH superagonist, causes an initial elevation in the serum testosterone levels. Insert a shows the elevated levels of 25.3 and 12.2 ng/ml at 6 h for F and A, respectively. For Formulation B, the testosterone peak occurred prior to 6 h and by that time had already descended from the initial rise to 4.79 ng/ml. Within 4 days, all levels were below baseline. As expected from the in vitro release profiles (Fig. 3), the suppression with 8.6 kDa microspheres was rapid as compared to 28.3 kDa formulation. The effect of substituting 28.3 kDa component microspheres with 8.6 kDa at 25% w/w (3:1 28.3 kDa/8.6 kDa) was clearly evident, as the suppression with the combination microspheres, F, was faster than with 28.3 kDa microspheres but slower than with 8.6 kDa microspheres alone. This effect correlates with the higher amount of drug released in vitro. In addition to achieving faster onset of testosterone suppression, the combination microspheres maintained the suppression for at least 30 days of the study meeting the study objective of rapidly achieving and sustaining suppressed testosterone levels. In contrast, testosterone levels were seen to be elevated with the 8.6 kDa microspheres by 25 days due to faster depletion of the drug.

Fig. 5 shows the testosterone profiles obtained with the microspheres from polymer combinations (C–E). As compared with the 28.3 kDa polymer microspheres alone, all the three polymer combinations yielded faster testosterone suppression (Figs. 4 and 5) suggesting an initial higher release of peptide in vivo as was the case in vitro (Fig. 2). Challenging animals with leuprolide solution at days 32 and 42, showed the formulations to be effective at least until 30 days. Compared to the elevation observed on day 32, the elevation on second challenge was very high indicating the exhaustion of drug levels to completely occupy the receptors. The suppression profiles obtained with formulations C, D and E did not differ much even though the 8.6 kDa component in these formulations varied from 16 to 25% w/w and in vitro, a difference in drug release (C vs. D and E) was noticed. This lack of pharmacological difference cannot be explained, as the testosterone suppression determined in this study is a pharmacological indicator of the drug release in vivo. Once the gonadotropin LHRH receptors are down regulated, the serum levels of leuprolide needed to maintain desensitization are known to be very

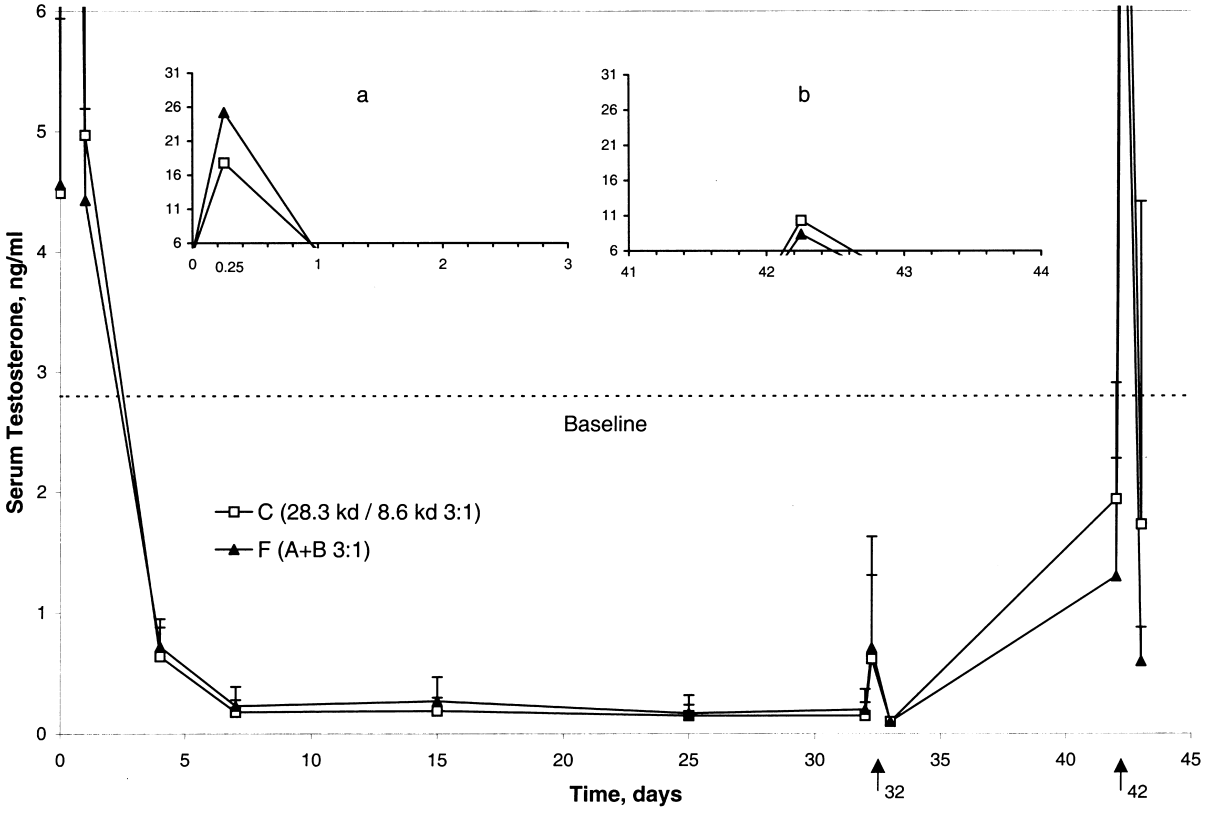

Fig. 6. In vivo testosterone suppression ($n = 6$) with polymer blend formulation C, and microsphere blend formulation F. The inserts a and b show the data points above 6 ng/ml and ↑ indicates the challenge.

low [22], and it is possible that variations in drug release from these formulations in vivo were not significant enough to affect varied suppression profiles. Since serum leuprolide levels nearly nondetectable after the testosterone levels are reduced to below 0.5 ng/ml, a direct assessment of in vivo drug release, were not determined in this study.

Fig. 6 compares the efficacy of formulations F, a 3:1 physical blend of 28.3 and 8.6 kDa microspheres, and C that was prepared from 3:1 28.3 kDa/8.6 kDa polymer blend. The testosterone suppression profiles obtained with these formulations were very similar as expected from the similar in vitro drug release (Fig. 3). Thus physically mixing microspheres from single polymers whose drug release profiles are known, appears to be an attractive approach and can be an alternative to making microspheres from custom made or blended polymers.

In conclusion, this study had shown the feasibility of utilizing blends of polymers or microspheres to prepare formulations that will provide the desired release of peptides and effect earlier suppression of testosterone as well as continued suppression for at least 30 days. These approaches offer two practical alternatives to the expensive and laborious process of customizing the polymer properties for the desired drug release.

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