Available online at www.sciencedirect.com
ScienceDirect
Journal of Controlled Release 119 (2007) 77–85
journal of controlled release
www.elsevier.com/locate/jconrel

Estradiol loaded PLGA nanoparticles for oral administration: Effect of polymer molecular weight and copolymer composition on release behavior *in vitro* and *in vivo* ☆

G. Mittal [a], D.K. Sahana [a], V. Bhardwaj [a], M.N.V. Ravi Kumar [a,b,*]

[a] Department of Pharmaceutics, National Institute of Pharmaceutical Education and Research (NIPER), S.A.S. Nagar, 160 062, Punjab, India
[b] Center for Pharmaceutical Nanotechnology, National Institute of Pharmaceutical Education and Research (NIPER), S.A.S. Nagar, 160 062, Punjab, India

Received 2 December 2006; accepted 23 January 2007
Available online 3 February 2007

Abstract

The present investigation was aimed at optimization of estradiol loaded PLGA nanoparticulate formulations resulting in improved oral bioavailability and sustained release of estradiol by varying the molecular weight and copolymer composition of PLGA. Nanoparticles were prepared following emulsion–diffusion–evaporation method employing didodecyldimethyl ammonium bromide (DMAB) as stabilizer. The effect of polymer molecular weight and copolymer composition on particle properties and release behavior (*in vitro* and *in vivo*) has been reported. Drug release *in vitro* decreased with increase in molecular weight and lactide content of PLGA. Zero order release was obtained with low molecular weight (14,500 and 45,000 Da) PLGA, while high molecular weight (85,000 and 213,000 Da) and different copolymer compositions followed square root of time (Higuchi's pattern) dependent release. The bioavailability of estradiol from nanoparticles was assessed in male Sprague Dawley (SD) rats at a dose of 1 mg estradiol/rat. The *in vivo* performance of the nanoparticles was found to be dependent on the particle size, polymer molecular weight and copolymer composition. The C_{max} of drug in the plasma was dependent on the polymer molecular weight and composition while particle size was found to influence the duration of release, suggesting smaller is better. The histopathological examination revealed absence of any inflammatory response with the formulations prepared of low/high molecular weight or high lactide content polymers for the studied period. Together, these results indicate that nanoparticulate formulations are ideal carriers for oral administration of estradiol having great potential to address the dose related issues of estradiol.
© 2007 Elsevier B.V. All rights reserved.

Keywords: Biodegradable; Biocompatible; Copolymer; Molecular weight; Nanoparticles; Oral delivery

1. Introduction

Estradiol (E2) is most potent natural estrogen and mainly prescribed in the case of postmenopausal symptoms as a part of hormone replacement therapy (HRT), either alone (called unopposed estrogen) or in combination with another female hormone, progestin [1,2]. HRT is given for 2–3 years, if the aim of treatment is symptom control; however, if the main aim is to prevent the long term consequences (eg. osteoporosis) of decreased estrogen levels, then treatment needs to last for at least 5–10 years [3]. Apart from postmenopausal symptoms, estradiol also has therapeutic use as a contraceptive and hypocholesteremic drug. Also, it has been found that estradiol intake may decrease the risk of Alzheimer's disease by promoting the growth and survival of cholinergic neurons and reducing cerebral amyloid deposition [4,5]. Estradiol has good oral absorption but poor bioavailability (~10%), because of high gut wall and first pass metabolism. As a result, oral dose is large with conventional delivery systems, leading to undesired side effects due to increased levels of active metabolites like

☆ In memory of Dr. Anup Kumar Mukhopadhyay (July 07, 1961–November 30, 2006), Associate Professor, Department of Biotechnology, National Institute of Pharmaceutical Education and Research (NIPER).

* Corresponding author. Department of Pharmaceutics, National Institute of Pharmaceutical Education and Research (NIPER), S.A.S. Nagar, 160 062, Punjab, India. Tel.: +91 172 2214683 89 (2055); fax: +91 172 2214692.
E-mail addresses: mnvrkumar@niper.ac.in, mnvrkumar@yahoo.com (M.N.V. Ravi Kumar).

0168-3659/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jconrel.2007.01.016

estrone and estriol in the blood circulation [6]. Some of the serious health risks allied with the use of estradiol are breast cancer and endometrial cancer. All the risks coupled with the estradiol are dose and duration dependent. Therefore, whenever long-term estrogen therapy is required, the lowest effective dose should be recommended [7].

Nanoparticles by virtue of their unique uptake mechanisms, hold the advantage of bypassing the first pass metabolism of the encapsulated drugs prone to such metabolism. Most evidences suggest that nanoparticle uptake occurs preferentially via "M-cells" in the Peyer's patches by the process of endocytosis (lymphoid uptake), thereby delivering the drug loaded particles directly into systemic circulation through the lymphatics and circumventing the first pass metabolism, however, uptake by transcellular and paracellular (non-lymphoid uptake) pathways involving the intestinal absorptive enterocytes cannot be ruled out [8–10]. Nanoparticles also protect the entrapped drug from gastrointestinal interferences (chemical and enzymatic degradation), prolong the systemic circulation time and control the release of drug in blood [11,12]. All of these benefits can contribute to reduction of dose and dosing frequency, thereby reducing the side effects and improving the patient compliance. Recently, we reported estradiol loaded PLGA nanoparticles for oral administration which could provide *in vitro* release for over 30 days and 7 days release *in vivo* on single administration of 1 mg equivalent of estradiol nanoparticles [13]. However, a molecule like estradiol with numerous potential benefits as well as undesirable health risks demands formulations of minimum possible dose to circumvent any dose related complications.

For the past two decades, polymers are being extensively used in drug delivery systems [14]. Polymers by virtue of their ability to sustain the drug release over long periods of time and provide steady plasma concentration may reduce the total dose and some adverse reactions. For example, effective dose of Lupron Depot® (PLA/PLGA microspheres containing leuprorelin acetate) formulation was reduced to 1/4–1/8 of that needed in the repeated parenteral administration of a plain solution [15]. All of these attributes have made the polymers a requisite part of any drug delivery system, be it a conventional or a novel delivery system. A number of polymers have been exploited for formulating the nanoparticulate carrier systems, but poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and their copolymers poly(lactide-co-glycolide) (PLGA) have been extensively employed because of their biocompatibility, biodegradability and versatile degradation kinetics. The physical properties and the FDA approval of the products containing these biodegradable polymers have made them most extensively studied commercially available polymers. The degradation rate of PLGA depends on the molar ratio of lactic and glycolic acid in the polymer chain, molecular weight of polymer, the degree of crystallinity, and glass transition temperature of the polymer (Tg). By manipulating the molecular weight and lactide/glycolide ratio, the degradation time of PLGA and subsequently, release profile can be varied accordingly [11,16]. Therefore, the present study reports the influence of polymer molecular weight and copolymer composition on the release behavior of estradiol *in vitro* as well as *in vivo*.

2. Materials and methods

2.1. Materials

PLGA 50:50 of different molecular weights (14,500, 45,000, 85,000, 137,000 and 213,000 Da) and PLGA of different copolymer compositions; PLGA (50:50; MW-85,000 Da), PLGA (65:35; MW-97,000 Da), and PLGA (85:15; MW-87,000 Da) were purchased from Birmingham Polymers, Inc (Birmingham, AL). Estradiol was a gift sample from Orion Pharma (Espoo, Finland). Didodecyldimethyl ammonium bromide (DMAB) was purchased from Aldrich (St. Louis, MO, USA) while Ethyl acetate (AR grade) and acetonitrile (ACN; HPLC grade) were purchased from Rankem Fine Chemicals (New Delhi, India). ELISA kit was procured from DRG diagnostics (Frauenbergstr., Germany). Ultrapure water (SG Water Purification System, Barsbuttel, Germany) was used for all the experiments.

2.2. Preparation of PLGA nanoparticles

Nanoparticles were prepared by emulsion–diffusion–evaporation method, previously reported by our group [13]. The objective of the present study was to understand the effect of molecular weight and copolymer composition on particle formation and release behavior leading to lowest effective dose preparation of estradiol. Therefore, particles were made of PLGA 50:50 (MW 14,500, 45,000, 85,000, 137,000 and 213,000 Da) as well as PLGA 65:35 (MW 97,000 Da) and PLGA 85:15 (MW 87,000 Da). In brief, PLGA and estradiol (10% w/w of polymer) solution in ethyl acetate was added to the aqueous phase (1% w/v DMAB) under stirring resulting in o/w emulsion. The organic to aqueous phase ratio for the preparation of o/w emulsion was 1:2. The o/w emulsion was subsequently homogenized using a high-speed homogenizer (Polytron PT4000, Switzerland) and finally water was added with constant stirring that facilitated diffusion and evaporation of organic solvent. This resulted in nanoprecipitation and formation of nanoparticles containing estradiol.

2.3. Particle size and surface charge

The size of nanoparticles was determined by means of dynamic light scattering technique (Nano ZS, Malvern Instruments, Malvern, UK), taking the average of 5 measurements. Zeta potential is an indicator of surface charge, which determines particle stability in dispersion. Zeta potential was estimated on the basis of electrophoretic mobility under an electric field, as an average of 30 measurements. The polydispersity index (PDI) which is a dimensionless number indicating the width of the size distribution, having a value between 0 and 1 (0 being for mono-dispersed particles) was also obtained.

2.4. Entrapment efficiency

The percentage of drug incorporated during nanoparticle preparation was determined by centrifuging the drug loaded

nanoparticles and separating the supernatant. The pellet was washed twice with water and then drug content in the pellet was analyzed using validated HPLC method. Waters high-performance liquid chromatography (HPLC) system consisting of 996 Photodiode Array Detector and Merck LiChoCART® 100 RP-18 end-capped 5 μm column (Germany) was used. Acetonitrile (ACN):Water (65:35) was used as the mobile phase with a flow rate of 0.5 ml/min. The injection volume was 15 μl and retention time of estradiol was 7.1 min. The detection wavelength (λ_{max}) for estradiol was 281 nm. Standard solutions were made in acetonitrile (HPLC grade). Linearity range of the calibration curve was found to be 1–10 μg/ml while correlation coefficient varied between 0.9996±0.0002.

2.5. In vitro drug release studies

Dialysis membrane method was used to determine the release of estradiol from the nanoparticulate formulations. Freshly made PLGA nanoparticle dispersions (equivalent to 1 mg drug entrapment) of different molecular weights (14,500, 45,000, 85,000 and 213,000 Da) and copolymer compositions (50:50, 65:35, 85:15) were centrifuged, redispersed in 1 ml of pH 7.4 phosphate buffer and then put in the dialysis bags (Sigma) with a molecular mass cut-off of 12,000 Da. The bags were suspended in vials containing 10 mM ionic strength phosphate buffer of pH 7.4. Volume of the release media was kept 15 ml for low molecular weight (14,500 and 45,000 Da) PLGA nanoparticles, while for the rest of the formulations (high molecular weight and high lactide content) it was optimized to 5 ml considering the sensitivity of the analytical method. All the vials were kept in shaker water bath maintained at 37 °C and 100 rpm. The release medium was completely replaced with fresh buffer at 6 and 12 h on first day, followed by sampling at every 24 h interval till the release was observed. The estradiol content in the release medium was quantified using a validated HPLC method.

2.6. In vivo drug release studies

In vivo studies of selected formulations were carried out in male Sprague Dawley rats weighing between 200–220 g. PLGA nanoparticles (equivalent to a dose of 1 mg estradiol/rat) of different molecular weights (14,500, 45,000 and 213,000 Da) and copolymer composition (65:35) were redispersed in 1 ml of pH 7.4 phosphate buffer and then administered orally using oral gavage needle. After administration of different formulations blood samples were collected (200–300 μl) from the retro-orbital plexus under mild ether anesthesia at 0.5, 1, 2, 6, 12 and every 24 h for 11 days in the heparinized microcentrifuge tubes (50 units heparin/ml of blood). Plasma was separated by centrifuging the blood samples at 10,000 ×*g* for 15 min. Methanol was added to precipitate the plasma proteins. Thereafter, samples were vortexed and centrifuged at 15,000 ×*g* for 15 min. Then supernatant was separated and vacuum dried in Maxi Dry Plus (Heto Lab equipment, Germany). Finally, the residue was reconstituted and estradiol concentration was determined using ELISA kit (DRG diagnostics, Germany) having the linearity range of 0–2000 pg/ml. The competence of nanoparticulate formulations was assessed by administering pure drug orally and measuring the blood levels at 0.5, 1, 2, 6, 12 and 24 h. The drug suspension was made by dispersing the drug in 0.05% DMSO in water to get a concentration of 1 mg/ml. For intravenous administration, estradiol was dissolved in 40% ethanol to obtain a concentration of 1 mg/ml and then solution (1 ml) was administered through femoral vein and the blood levels were monitored over a time period of 0.16, 0.25, 0.5, 1, 2, 4, 6, 12 and 24 h.

2.7. Histopathological studies

After 11 days of administration of nanoparticles, animals were sacrificed and tissue sections collected. Segments from duodenum, jejunum and ileum were isolated and fixed in 10% formal saline. Liver and spleen were also excised and stored in 10% formal saline. All the tissue sections were first dehydrated by treating them with increasing concentrations of alcohol and xylene. The anhydrous tissue samples thus obtained were embedded in the paraffin blocks. Sections of tissues were cut using a microtome and then processed for hydration. Final staining was performed with hematoxylin and eosin. Finally, sections were mounted on the microscopic slide and fixed with coverslips using DPX solution. These sections were observed

Table 1
Effect of polymer (PLGA 50:50) molecular weight on particle characteristics of blank as well as drug loaded nanoparticles

Sr. No.	MW (Da)	Blank nanoparticles			Drug loaded nanoparticles			
		PS (nm)	PDI	ZP [a] (mV)	PS (nm)	PDI	ZP [a] (mV)	EE [b] (%)
1.	14,500	90.9±2.8 [c]	0.117±0.023	72.5±3.4	98.3±2.6 [c]	0.160±0.008	78.9±2.1	51.34±5.59
2.	45,000	92.9±5.6 [c]	0.102±0.010	81.2±2.9	104.5±4.8 [c]	0.091±0.024	85.6±1.9	43.57±2.68
3.	85,000	106.8±5.1 [d]	0.106±0.009	90.7±4.6	118.2±5.1 [d]	0.088±0.045	94.7±3.7	34.57±4.89 [e]
4.	137,000	133.0±2.2	0.076±0.015	96.6±4.8	141.3±4.1 [d]	0.095±0.013	102.8±5.6	50.19±3.69
5.	213,000	143.0±1.9	0.105±0.024	94.7±3.2	155.4±3.9 [d]	0.185±0.025	95.4±4.1	67.82±4.54 [d]

MW: molecular weight, PS: particle size, PDI: polydispersity index, ZP: zeta potential, EE: entrapment efficiency.
Values given are mean±standard deviation ($n=3$).
[a] The zeta potentials reported are in the pH range of 3.96–4.50.
[b] Initial drug loading was 10% w/w of the polymer weight.
[c] $p<0.05$ vs. 3,4,5 nanoparticles.
[d] $p<0.05$ vs. all other nanoparticles.
[e] $p<0.05$ vs. 1,4,5 nanoparticles.

Table 2
Effect of copolymer composition on particle properties of blank as well as drug loaded nanoparticles

Sr. No.	Copolymer composition	Blank nanoparticles			Drug loaded nanoparticles			
		PS (nm)	PDI	ZP [a] (mV)	PS (nm)	PDI	ZP [a] (mV)	EE [b] (%)
1.	50/50	106.8±5.1	0.106±0.009	90.7±4.6	118.2±5.1	0.088±0.045	94.7±3.7	34.57±4.89***
2.	65/35	115.7±2.7	0.112±0.024	80.2±2.5	126.0±2.6	0.124±0.056	89.4±6.5	60.10±2.51
3.	85/15	112.5±4.1	0.087±0.052	91.2±3.4	129.0±4.1	0.091±0.043	98.2±5.8	60.17±2.48

PS: particle size, PDI: polydispersity index, ZP: zeta potential, EE: entrapment efficiency.
Values given are mean±standard deviation ($n=3$).
***$p<0.001$ vs. 2,3 nanoparticles.
[a] The zeta potentials reported are in the pH range of 4.05–4.56.
[b] Initial drug loading was 10% w/w of the polymer weight.

under high magnification (200×/400×/630×) light microscope to check any histopathological changes.

2.8. Statistics

Statistical analysis of the data was performed via one way analysis of variance (ANOVA) using SigmaStat 2.0 software (Jindal Scientific); a value of $p<0.05$ was considered significant.

3. Results and discussion

3.1. Preparations of PLGA nanoparticles

Particle size is an important parameter as it can directly affects the physical stability, cellular uptake, biodistribution and the drug release. In general, the smaller the particle size, the better the performance of the nanoparticles. There are many factors which influence the particle size; polymer molecular weight is one of them.

The particle size increased proportionately with increase in molecular weight from 14,500 to 213,000 Da, for blank as well as drug loaded nanoparticles (Table 1). One possible reason could be the increase in viscosity of the polymer solutions with increasing molecular weight, thereby posing difficulty in stirring them into smaller emulsion droplets in contrast to the low molecular weight polymers because of lower stirring efficiency at the same agitation speed.

Entrapment efficiency did not follow a regular pattern. First, it decreased from 51.3% to 34.5%, as molecular weight was increased from about 14,500 to 85,000 Da; thereafter, a significant increase ($p<0.05$) was observed to 67.8% as molecular weight was increased to 213,000 Da (Table 1). Entrapment efficiency of particles using an o/w method mainly depends on drug partition coefficient in internal and external phases. The rate of polymer solidification/precipitation during nanoparticle preparation may change the drug partitioning into the external aqueous phase and subsequently influence the entrapment percentage. It is possible that increase in viscosity, on increasing the molecular weight might have decreased the diffusion rate of solvent into the external aqueous phase. Polymer precipitated slowly because of slow rate of solvent removal, giving drug molecules more time to come into the aqueous phase, resulting in low entrapment efficiency. However, an increase in the entrapment efficiency was observed for particles with molecular weights of 137,000 and 213,000 Da, which could be due to strong hydrophobic interactions of polymer molecular chains and the drug.

Nanoparticles prepared of different copolymer compositions; PLGA (50:50, MW-85,000 Da; 65:35, MW-97,000 Da and 85:15, MW-87,000 Da) did not show any significant difference in particle size of both, blank as well as drug loaded nanoparticles (Table 2), which could be due to similar molecular weight range of these polymers.

The entrapment efficiency of PLGA 50:50 was found to be lowest while there was a significant increase ($p<0.001$) in the entrapment efficiency of PLGA 65:35, although no difference was observed between PLGA 65:35 and PLGA 85:15 (Table 2). This can be due to the fact that as lactide content of PLGA copolymer was increased (PLGA 65:35), hydrophobicity of copolymer also increased because of hydrophobic nature of PLA. This increased hydrophobicity of the copolymer could have led to increased solid-state solubility of hydrophobic drug in the polymer, resulting in increased entrapment efficiency as compared to that obtained with PLGA 50:50. On contrary, entrapment remained same on further increasing the lactide content (PLGA 85:15), where initial drug loading could be limiting the entrapment efficiency. This warrants further study with PLA alone for better understanding the mechanism of entrapment.

3.2. In vitro drug release studies

Polymer molecular weight is one of the key factors affecting the drug release. Molecular weight is indicative of chain length of the polymer and more is the molecular weight, bigger is the chain length. Furthermore, chain length reflects the hydrophilicity/lipophilicity of the polymer. Increase in chain length increases the lipophilicity and decreases the degradation rate of the polymer. So, by varying the molecular weight, degradation rate of the polymer and release kinetics of the drug can be controlled accordingly [16]. The understanding of the degradation phenomenon of PLGA is important as it determines the rate and mechanism of release of the therapeutic agent. PLGA undergoes bulk degradation through random hydrolytic scission of its backbone ester linkage throughout the matrix [17,18]. The acidic (lactic acid and glycolic acid) monomers and oligomers thus formed further catalyze the degradation of the parent polymer, a process known as autocatalysis. Release of the

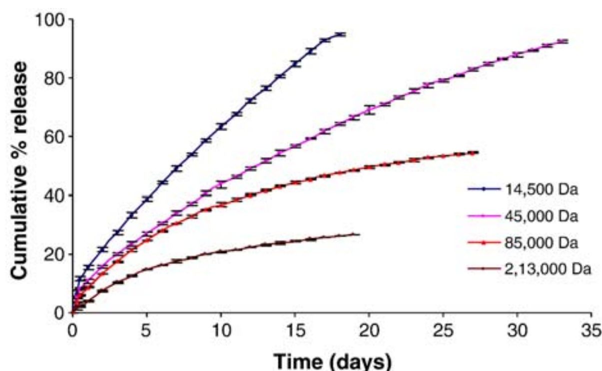

Fig. 1. *In vitro* release profiles of estradiol loaded PLGA (50:50) nanoparticles of different molecular weights with DMAB as stabilizer in pH 7.4 phosphate buffer. Data points shown are mean ± standard deviation ($n=3$).

entrapped therapeutic agent from PLGA matrix has been found to occur through diffusion-cum-degradation mediated process. It has been shown that during the early phases, release occurs mainly through diffusion in the polymer matrix while during the later phases; release is mediated through both diffusion of the therapeutic agent and degradation of the polymer matrix itself [19,20]. However, literature lacks any inclusive report describing the influence of molecular weight and copolymer composition on release behavior from nanosized delivery systems.

Increase in molecular weight from 14,500 to 213,000 Da significantly decreased the release rate of estradiol. PLGA nanoparticles of low molecular weight (14,500 and 45,000 Da) released 94.7 and 92.4% of total drug in 18 and 33 days, respectively at a nearly constant rate. On the other hand, high molecular weight (85,000 and 213,000 Da) PLGA nanoparticles showed only 54.5 and 26.6% release of the total drug in 27 and 19 days, respectively (Fig. 1), after which drug levels went below detectable limits. The cumulative drug release was fitted into different release models namely zero order, first order, Higuchi's square root plot and Hixson–Crowell cube root plot [21]. The model giving a correlation coefficient close to unity was taken as order of release. Zero order patterns were observed with low molecular weight viz; 14,500 and 45,000 Da PLGA nanoparticles with R^2 value of 0.9908 and 0.9783 respectively. It is possible that because of the low molecular weight, degradation is playing a dominating role and controlling the release rate. Rate of degradation is not constant and increases continuously with the time. This keeps the release rate constant (zero order release) which otherwise tends to decrease due to diffusion (a concentration gradient process). Increase in degradation rate with the time can be justified on the basis of two factors namely autocatalysis and glass transition temperature of the polymer (Tg). Acidic monomers and oligomers (with carboxylic acid end group) formed during degradation further catalyze the degradation and increases the degradation rate with the time. Glass transition temperature depends on the polymer molecular weight and normally decrease in molecular weight lowers the Tg that determines a glassy and a rubbery state of the polymer below and above it. In case of low molecular weight PLGA nanoparticles, rapid water hydration (due to less lipophilicity of the polymer) allows the Tg to shift to the lower temperature (called plasticizing effect of the water) which makes polymer chain segments more mobile and thus more labile for degradation [22]. Among the low molecular weights also, PLGA with relatively high molecular weight (45,000 Da) showed much sustained release as compared to the lowest molecular weight (14,500 Da) PLGA. Decrease in degradation rate due to increase in lipophilicity on increasing the molecular weight is responsible for this [23,24]. Besides lipophilicity, effect of particle size and entrapment efficiency on the release rate cannot be ignored. Particle size is an important parameter that could affect the degradation of the polymer matrix. With the increase in particle size, surface area/volume ratio decreases leading to decreased buffer penetration and slower release of the drug. This may be another reason for slower release of the drug from PLGA nanoparticles with comparatively high molecular weight. Also, difference in the release rate can be attributed to the difference in the entrapment efficiencies. In case of 45,000 Da molecular weight PLGA nanoparticles, the entrapment efficiency was less (~43.5%) compared to formulation with lowest molecular weight (14,500 Da) PLGA nanoparticles (~51.3%). Thus to obtain the same amount of drug (1 mg) more quantity of nanoparticles had to be used. This proportionate increase in the polymer may have been reflected in sustaining the release to a greater extent in the formulation with 45,000 Da molecular weight PLGA nanoparticles.

On the other hand, high molecular weight (85,000 and 213,000 Da) PLGA nanoparticles showed best fit into Higuchi's square root release model with R^2 values of 0.9894 and 0.9882 respectively. In case of high molecular weight PLGA nanoparticles, release is mainly mediated through the diffusion process (complies with the Higuchi's model) with very little contribution from degradation which also decreases further on increasing the molecular weight. More sustained release was observed in case of high molecular weight PLGA (85,000 and 213,000 Da) nanoparticles as compared to low molecular weight (14,500 and 45,000 Da) PLGA nanoparticles. Increase in lipophilicity on increasing the molecular weight (also responsible for shifting the release mechanism from a combined diffusion and degradation in case of low molecular weights to diffusion alone in high molecular weights) is the main contributing factor for this. Within the high molecular weights also, PLGA with highest molecular weight (213,000 Da) showed far more sustained release as compared to PLGA with relatively low molecular weight (85,000 Da). The release rate of the drugs from biodegradable particles also depends on the affinity of the drug with polymer matrices [19,25]. Due to extremely high affinity of the drug with the polymer in case of highest molecular weight PLGA nanoparticles, drug is very slowly diffusing out of the polymer matrices and showing a much sustained release.

The degradation behavior of nanoparticulate system also depends on hydrophilic/lipophilic ratio of the polymer apart from the molecular weight. The more hydrophilic is the polymer, the more rapid will be its degradation. The hydrophilicity

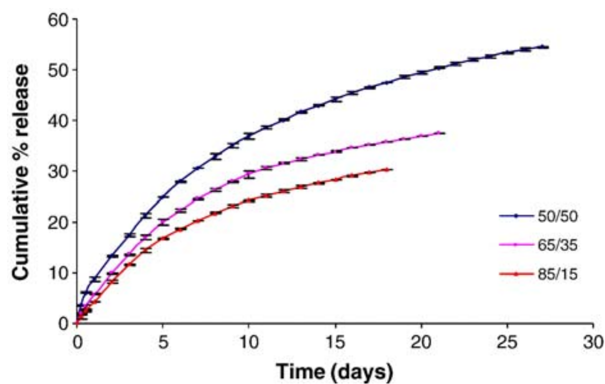

Fig. 2. *In vitro* release profiles of estradiol loaded PLGA nanoparticles of different copolymer compositions with DMAB as stabilizer in pH 7.4 phosphate buffer. Each data point is a mean of three values.

of the polymer is influenced by the ratio of crystalline to amorphous regions, which in turn is determined by copolymer composition. PLGA copolymer prepared from L-PLA and PGA are crystalline copolymers, while those from D,L-PLA and PGA are amorphous in nature. Lactic acid, being more hydrophobic than glycolic acid, makes lactide-rich PLGA copolymers more hydrophobic and subsequently slows down the degradation process. Therefore, by adjusting the lactide/glycolide ratio, the desired degradation rate and release pattern of the drug can be achieved [16].

PLGA nanoparticles prepared with different copolymer compositions; PLGA (50:50, MW-85,000 Da; 65:35; MW-97,000 Da and 85:15, MW-87,000 Da) showed decrease in release rate with increase in the lactide content. PLGA 50:50, 65:35 and 85:15 showed only 54.5, 37.3 and 30.2% release of total drug in 27, 21 and 18 days respectively (Fig. 2), after which drug levels went below detectable limits. All the three copolymer compositions got best fit into Higuchi's square root release model with R^2 values of 0.9894, 0.9800 and 0.9891 respectively. Higuchi's model describes the release mechanism largely on the basis of diffusion only. Thus in case of all three copolymer compositions, release is mainly mediated through the diffusion process with very little or no involvement of degradation within the studied period. Decrease in release rate on increasing the lactide content can be attributed to subsequent increase in hydrophobicity which further contributes to the decrease in degradation rate and increase in the affinity of the drug with the polymer [26,27].

Most of the drug loaded particulate formulations show a biphasic release pattern wherein there is an initial burst followed by a sustained release [28,29]. The high initial release may be due to the presence of free and weakly bound drug on the surface of particulate carriers. However, no such initial burst release was observed in the present case, possibly suggesting the absence of any unbound drug associated with the particles. It is difficult to design an *in vitro* release study for nanoparticle formulations because of a number of practical problems associated with it. Sink conditions are difficult to attain for a lipophilic drug during the release experiment, because sensitivity of the analytical method usually does not allow for sufficient dilution in commonly used release media. Even after keeping the volume of the release media as low as 5 ml (which is minimally required to completely dip the bags of dialysis membrane in the release media), most of the formulations did not show complete release and after certain period of release drug concentrations were below the detectable limits.

Table 3
Pharmacokinetic parameters of E2 after intravenous and oral administration of pure drug and oral administration of PLGA nanoparticles of different molecular weights and copolymer compositions

Formulations	C_{max} (ng/ml)	T_{max} (h)	$AUC_{0\text{-inf.}}$ (ng h/ml)
Pure drug i.v. solution	–	–	1185.27±48.16
Pure drug oral suspension	37.08±1.82	2.0±0.0	190.51±11.17
PLGA 50:50 (MW 14,500 Da) NP	56.43±2.91*	12.0±0.0	1735.29±346.21***
PLGA 50:50 (MW 45,000 Da) NP	43.09±5.20*	24.0±0.0	1936.79±116.26***
PLGA 50:50 (MW 213,000 Da) NP	28.55±5.38	32.0±13.8	1388.89±166.12***
PLGA 65:35 (MW 97,000 Da) NP	24.69±0.86	32.0±13.8	1734.17±273.84***

Data presented as mean±standard deviation (n=3).
NP: nanoparticles, C_{max}: maximum plasma concentration, T_{max}: time taken to attain C_{max}, AUC: area under the plasma concentration–time curve.
*p<0.05 vs. all other nanoparticulate formulations.
***p<0.001 vs. estradiol administered alone orally.

3.3. In vivo drug release studies

Estradiol loaded PLGA nanoparticles were designed to improve the oral bioavailability of the drug. To our knowledge, there was no comprehensive study reported in the literature demonstrating the *in vivo* performance of nanoparticles prepared of different molecular weight and copolymer composition. We believe that no single parameter can alone influence the fate of the formulations *in vivo*; rather it is a collective influence of many parameters. Blood levels after intravenous and oral administration of pure drug were compared with those

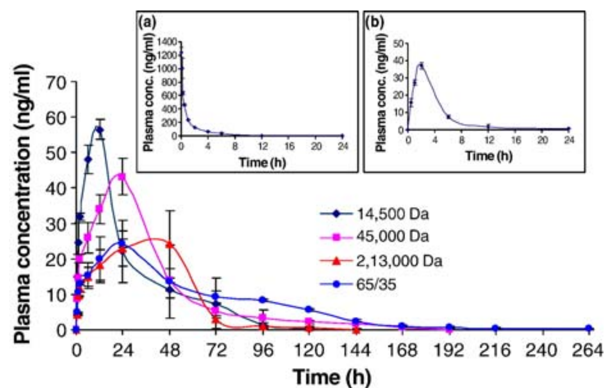

Fig. 3. Comparative *in vivo* profiles of PLGA nanoparticles (equivalent to 1 mg estradiol/rat) of different molecular weights and copolymer compositions on oral administration. Blood levels of the estradiol were determined using ELISA kit. Each data point represents the mean±standard deviation (n=3). Inserts show *in vivo* plasma concentration vs. time profiles of estradiol pure drug (1 mg/rat) on (a) intravenous and (b) oral administration (n=3).

after oral administration of different nanoparticulate formulations using ELISA kit. The significant pharmacokinetic parameters obtained are listed in Table 3. The mean plasma levels of estradiol after oral and intravenous administration of pure drug and of orally administered nanoparticulate formulations are shown in Fig. 3.

Plasma drug profile of all the PLGA nanoparticulate formulations showed that increase in molecular weight and lactide content of PLGA resulted in more sustained release. From the graphs obtained by plotting plasma drug concentration vs. time for all the formulations, it was observed that low molecular weight (14,500 and 45,000 Da) PLGA nanoparticles showed the release for 5 and 8 days with C_{max} of 56.43 ± 2.91 and 43.09 ± 5.20 ng/ml at T_{max} of 12 and 24 h, respectively whereas 213,000 Da molecular weight and 65:35 composition PLGA nanoparticles released the drug over the periods of 6 and 11 days with a C_{max} of 28.55 ± 5.38 and 24.69 ± 0.86 ng/ml, respectively at T_{max} of 32 h each. From the above data, it is evident that increase in molecular weight or lactide content in PLGA led to significant decrease ($p < 0.05$) in C_{max}, suggesting a strong correlation between the polymer molecular weight, copolymer composition and release behavior. Furthermore, particle size was also found to strongly influence the performance *in vivo*. In case of particles made of 213,000 Da PLGA (particle size 155.4 ± 3.9 nm), it was anticipated that because of its very high molecular weight, it would show a much sustained release, but contrary to this, release was observed only for 6 days, with a sudden fall in blood levels after 2 days. On the other hand, 11 days release profile shown by lactide rich PLGA 65:35 nanoparticles (particle size 126.0 ± 2.6 nm) clearly indicates the role of particle size in dictating the fate of nanoparticles *in vivo* through biodegradation, tissue deposition or elimination of these nanoparticles, all of which need to be investigated to help in further understanding the release behavior. Oral and i.v. administration of pure drug exhibited only 1 day plasma profile, where oral pure drug suspension showed a C_{max} of 37.08 ± 1.82 ng/ml at T_{max} of 2 h. A significant increase ($p < 0.001$) in the AUC values of all the nanoparticulate formulations in comparison to that of oral pure drug distinctly indicates the improved bioavailability with the nanoparticulate systems. A much lower AUC value for the orally administered estradiol suspension specifies the problems associated with conventional oral formulations of estradiol.

In vitro–in vivo correlations (IVIVC) are generally developed to explore the relationships between *in vitro* dissolution/release and *in vivo* absorption profiles. This relationship facilitates the rational development and evaluation of immediate/extended-release dosage forms as a tool for formulation screening, in setting dissolution specifications and as a surrogate for bioequivalence testing. However, ground rules for developing IVIVC for novel delivery systems such as nanoparticles, liposomes, implants etc. are still unclear today. Several studies [30,31] showed that *in vitro* release rate of these systems cannot be directly extrapolated to predict their behavior *in vivo*, possibly because of enzymatic reactions and biological effects in the animal body. Therefore, a more appropriate biorelevent release medium should be designed to establish a well *in vitro–in vivo* correlation.

3.4. Histopathological studies

Biocompatibility of the particulate matter refers to host response to them. Particulate matter is generally captured by macrophages or phagocytes in the body. Larger particles are known to be readily taken up by the reticuloendothelial system (RES) cells (cells of liver and spleen) in the body [32,33]. RES recognize the particles after adsorption of proteins and opsonins (mainly complement factors and immunoglobulins) on the surface of the particles. This process seems to be influenced by the surface curvature of the carrier systems, where smaller carriers lead to reduced adsorption of proteins and opsonins and therefore, are less susceptible to be attacked by RES [34,35]. Assessment of tissue compatibility of nanoparticles is essential because they are phagocytosed by the cells followed by inflammatory and tissue response. Histocompatibility is vital in oral, transmucosal, and regional administration. The morphology of the cells gives a clear picture about the inflammatory condition after administration of polymeric colloidal carriers. Therefore, from the toxicological point of view, it was important to study the interaction between tissues and nanoparticles.

Histopathological examination of the liver, spleen, and the intestinal segments (duodenum, jejunum, and ileum) was carried out to check the presence of any inflammation after the administration of estradiol loaded PLGA nanoparticles. Only the groups that received high molecular weight (213,000 Da) and high lactide content (PLGA 65:35) nanoparticulate formulations were

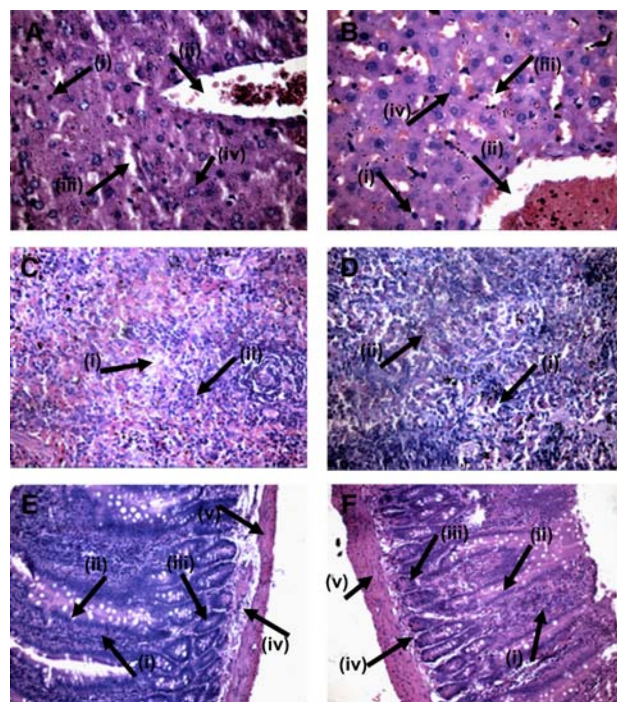

Fig. 4. Microscopic structures (11 days after dosing) of sections of liver (630×) of (A) control and (B) treated animals, showing (i) Kupffer's cells, (ii) central vein, (iii) sinusoids, and (iv) rounded nucleus. Sections of spleen (400×) of (C) control and (D) treated animals, showing (i) sinusoids, and (ii) lymphoid tissue and sections of jejunum (200×) of (E) control and (F) treated animals, showing (i) lymphocytes, (ii) villi, (iii) crypts, (iv) submucosa and (v) muscularis externa. The arrows highlight the absence of any inflammatory response.

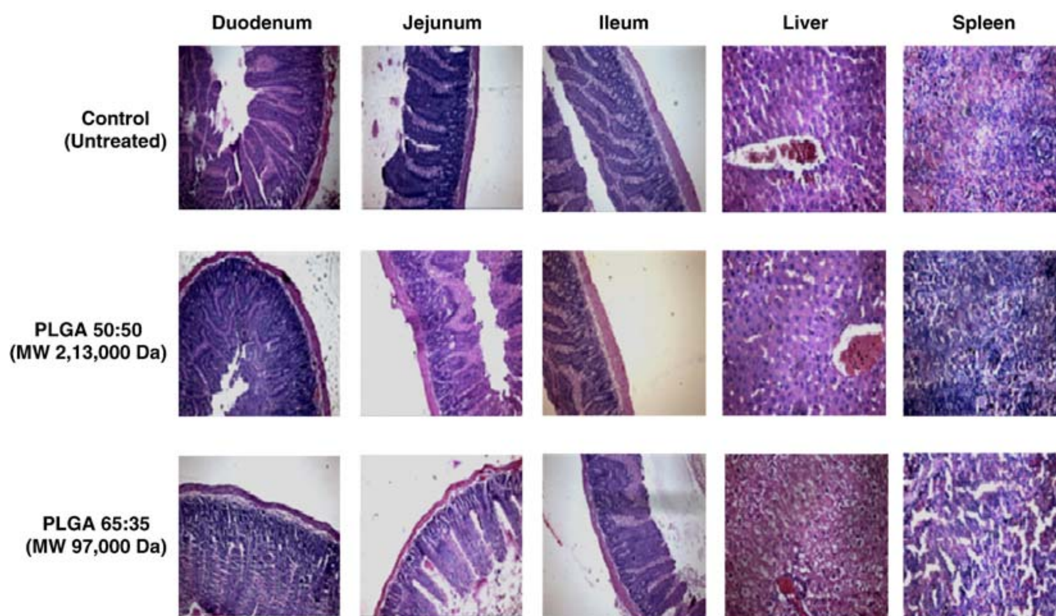

Fig. 5. Microscopic images of tissue sections of control (untreated) and animals treated with estradiol loaded PLGA nanoparticles. All the treated animals were sacrificed 11 days after dosing. Comparison between control and treated animals clearly indicates the absence of any histopathological changes.

taken further for histopathological examination as our previous findings have already shown the biocompatibility of low molecular weight estradiol loaded PLGA nanoparticles [13]. In comparison to the normal tissue, the liver in treated animals did not show any difference in number, arrangement, or appearance of Kupffer's cells, hepatocytes (amount of cytoplasm and shape of nucleus), and sinusoids (Fig. 4A and B). Spleen histology of treated animals also showed no difference from untreated ones in terms of lymphoid tissue mass, the sinusoids, and the number and appearance of RBCs (Fig. 4C and D).

Histopathological condition in the intestine is generally marked by the number or distribution of lymphocytes (inflammatory cells), which are always present in significant numbers, as a part of body's defense mechanism, in the lamina propria region of intestinal mucosal layer, because the gastrointestinal tract is frequently encountered by the foreign particles, food, and microorganisms. The treated animals did not show any change in number or distribution of these cells with respect to control. Inflammation in the intestine can also be evidenced by examining the histological layers i.e. mucosa, submucosa and muscularis externa of intestine, all of which were found to be intact without any signs of degeneration, indicating that nanoparticles did not induce damage or initiate any inflammatory response in these tissues (Fig. 4E and F). A comparison of healthy tissue from untreated animals with that of treated animals showed the absence of any inflammation (Fig. 5). Thus, high molecular weight and high lactide content estradiol loaded PLGA nanoparticles are as biocompatible as low molecular weight PLGA nanoparticles, under the studied conditions.

4. Conclusions

The present investigation suggests that drug release can be tailored significantly by varying the polymer molecular weight and copolymer composition. Furthermore, polymeric nanoparticles have substantial role in enhancing the delivery aspects of potent molecules like estradiol. Change in molecular weight and copolymer composition of PLGA produced different release behaviors from both *in vitro* and more importantly, *in vivo* perspective. *In vivo* data showed that with all the PLGA nanoparticulate formulations, same dose (1 mg estradiol/rat) produced detectable blood levels for 5–11 days, depending on the molecular weight, copolymer composition and resultant particle size, compared to 1 day profile shown by pure drug. Thus, estradiol loaded PLGA nanoparticles can be effective in improving the oral bioavailability and decreasing the dosing frequency, thereby minimizing the dose dependent adverse effects and maximizing the patients' compliance.

Acknowledgments

G.M. and D.K. acknowledge NIPER Fellowships during 2004–2006. V.B. is grateful to NIPER for providing PhD Fellowship. Start-up funds from NIPER and Research grant from Department of Biotechnology (BT/PR5097/BRB/10369/2004), Govt. of India, to M.N.V.R.K. are gratefully acknowledged. We sincerely acknowledge Orion Pharma (Finland) for providing estradiol as gift sample. Thanks are due to Rahul and Dinesh for providing the technical assistance to this project.

References

[1] K. Pazol, J.R. Kaplan, D. Abbott, S.E. Appt, M.E. Wilson, Practical measurement of total and bioavailable estradiol in female macaques, Clin. Chim. Acta 340 (2004) 117–126.
[2] R. Sitruk-Ware, Estrogen therapy during menopause practical treatment recommendations, Drugs 39 (1990) 203–217.
[3] N. Tanna, Hormone replacement therapy: (1) an overview, Pharm. J. 271 (2003) 615–617.

[4] A.M. Al-Ghananeem, A.A. Traboulsi, L.W. Dittert, A.A. Hussain, Targeted brain delivery of 17β-estradiol via nasally administered water soluble prodrugs, AAPS PharmSciTech 3 (2002) 1–8.
[5] N. Bodor, P. Buchwald, Barriers to remember: brain-targeting chemical delivery systems and Alzheimer's disease, Drug Discov. Today 7 (2002) 766–774.
[6] J.W. Yoo, C.H. Lee, Drug delivery systems for hormone therapy, J. Control. Release 112 (2006) 1–14.
[7] J. Deady, Clinical monograph: hormone replacement therapy, J. Manag. Care Pharm. 10 (2004) 544–557.
[8] P.U. Jani, D.E. McCarthy, A.T. Florence, Nanosphere and microsphere uptake via Peyer's patches: observation of the rate of uptake in rat after a single oral dose, Int. J. Pharm. 86 (1992) 239–246.
[9] M.P. Desai, V. Labhasetwar, G.L. Amidon, R.J. Levy, Gastrointestinal uptake of biodegradable microparticles: effect of particle size, Pharm. Res. 13 (1996) 1838–1845.
[10] A.T. Florence, The oral absorption of micro- and nanoparticulates: neither exceptional nor unusual, Pharm. Res. 14 (1997) 259–266.
[11] I. Bala, S. Hariharan, M.N.V.R. Kumar, PLGA nanoparticles in drug delivery: the state of the art, Crit. Rev. Ther. Drug Carr. Syst. 21 (2004) 387–422.
[12] V. Bhardwaj, S. Hariharan, I. Bala, A. Lamprecht, N. Kumar, R. Panchagnula, M.N.V.R. Kumar, Pharmaceutical aspects of polymeric nanoparticles for oral delivery, J. Biomed. Nanotechnol. 1 (2005) 235–258.
[13] S. Hariharan, V. Bhardwaj, I. Bala, J. Sitterberg, U. Bakowasky, M.N.V.R. Kumar, Design of estradiol loaded PLGA nanoparticulate formulations: a potential oral delivery system for hormone therapy, Pharm. Res. 23 (2006) 184–195.
[14] V.V. Ranade, Drug delivery systems: 3a. Role of polymers in drug delivery, J. Clin. Pharmacol. 30 (1990) 10–23.
[15] H. Okada, One- and three-month release injectable microspheres of the LH–RH superagonist leuprorelin acetate, Adv. Drug Deliv. Rev. 28 (1997) 43–70.
[16] R.A. Jain, The manufacturing techniques of various drug loaded biodegradable poly(lactide-co-glycolide) (PLGA) devices, Biomaterials 21 (2000) 2475–2490.
[17] A. Gopferich, Mechanisms of polymer degradation and erosion, Biomaterials 17 (1996) 103–114.
[18] M.L.T. Zweers, G.H.M. Engbers, D.W. Grijpma, J. Feijen, In vitro degradation of nanoparticles prepared from polymers based on DL-lactide, glycolide and poly(ethylene oxide), J. Control. Release 100 (2004) 347–356.
[19] T. Mogi, N. Ohtake, M. Yoshida, R. Chimura, Y. Kamaga, S. Ando, T. Tsukamoto, T. Nakajima, H. Uenodan, M. Otsuka, Y. Matsuda, H. Ohshima, K. Makino, Sustained release of 17β-estradiol from poly (lactide-co-glycolide) microspheres in vitro and in vivo, Colloids Surf., B Biointerfaces 17 (2000) 153–165.
[20] J. Panyam, M.M. Dali, S.K. Sahoo, W. Ma, S.S. Chakravarthi, G.L. Amidon, R.J. Levy, V. Labhasetwar, Polymer degradation and in vitro release of a model protein from poly(D,L-lactide-co-glycolide) nano- and microparticles, J. Control. Release 92 (2003) 173–187.
[21] P. Costa, J.M.S. Lobo, Modeling and comparison of dissolution profiles, Eur. J. Pharm. Sci. 13 (2001) 123–133.
[22] T.G. Park, Degradation of poly(D,L-lactic acid) microspheres: effect of molecular weight, J. Control. Release 30 (1994) 161–173.
[23] J. Braunecker, M. Baba, G.E. Milroy, R.E. Cameron, The effects of molecular weight and porosity on the degradation and drug release from polyglycolide, Int. J. Pharm. 282 (2004) 19–34.
[24] R. Jalil, J.R. Nixon, Microencapsulation using poly(DL-lactic acid) III: effect of polymer molecular weight on release kinetics, J. Microencapsul. 7 (1990) 357–374.
[25] K. Makino, T. Mogi, N. Ohtake, M. Yoshida, S. Ando, T. Nakajima, H. Ohshima, Pulsatile drug release from poly (lactide-co-glycolide) microspheres: how does composition of the polymer matrix affect the time interval between the initial burst and the pulsatile release of drugs? Colloids Surf., B Biointerfaces 19 (2000) 173–179.
[26] T.G. Park, Degradation of poly(lactic-co-glycolic acid) microspheres: effect of copolymer composition, Biomaterials 16 (1995) 1123–1130.
[27] W.K. Lee, J.Y. Park, E.H. Yang, H. Suh, S.H. Kim, D.S. Chung, K. Choi, C.W. Yang, J.S. Park, Investigation of the factors influencing the release rates of cyclosporin A-loaded micro- and nanoparticles prepared by high-pressure homogenizer, J. Control. Release 84 (2002) 115–123.
[28] D.T. Birnbaum, J.D. Kosmala, D.B. Henthorn, L. Brannon-Peppas, Controlled release of β-estradiol from PLAGA microparticles: the effect of organic phase solvent on encapsulation and release, J. Control. Release 65 (2000) 375–387.
[29] M. Otsuka, H. Udenodan, Y. Matsuda, T. Mogi, H. Ohsima, K. Makino, Therapeutic effect of in vivo sustained estradiol release from poly (lactide-co-glycolide) microspheres on bone mineral density of osteoporosis rats, Biomed. Mater. Eng. 12 (2002) 157–167.
[30] M. Sandor, J. Harris, E. Mathiowitz, A novel polyethylene depot device for the study of PLGA and P(FASA) microspheres in vitro and in vivo, Biomaterials 23 (2002) 4413–4423.
[31] Q. Cai, G. Shi, J. Bei, S. Wang, Enzymatic degradation behavior and mechanism of poly(lactide-co-glycolide) foams by trypsin, Biomaterials 24 (2003) 629–638.
[32] M.P. Desai, V. Labhasetwar, E. Walter, R.J. Levy, G.L. Amidon, The mechanism of uptake of biodegradable microparticles in Caco-2 cells is size dependent, Pharm. Res. 14 (1997) 1568–1573.
[33] I. Gutierro, R.M. Hernandez, A.R. Igartua, A.R. Gascon, J.L. Pedraz, Size dependent immune response after subcutaneous, oral and intranasal administration of BSA loaded nanosphere, Vaccine 21 (2002) 67–77.
[34] H. Harashima, K. Sakata, K. Funato, H. Kiwada, Enhanced hepatic uptake of liposomes through complement activation depending on the size of liposomes, Pharm. Res. 11 (1994) 402–406.
[35] K. Langer, S. Balthasar, V. Vogel, N. Dinauer, H.V. Briesen, D. Schubert, Optimization of the preparation process for human serum albumin (HSA) nanoparticles, Int. J. Pharm. 257 (2003) 169–180.