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Feasibility of localized immunosuppression: 2. PLA microspheres for the sustained local delivery of a soft immunosuppressant

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While biohybrid therapy shows promise, their further development into an "artificial pancreatic" system in diabetics also requires the management of the related immuneresponse triggered by such cellular therapies. Ideally this should be on a local level within the biohybrid device. This study relates to the design of sustained release formulations of the glucocorticoid soft drug loteprednol etabonate (LE) that are intended to locally suppress the immune response within the biohybrid devices, thereby warranting high local activity and reduced systemic side effects. Poly(D,L-lactic) acid (PLA) and poly(D,L-lactic glycolic acid (PLGA) microspheres of the soft corticosteroid loteprednol etabonate (LE) were prepared by solvent evaporation. A range of particles differing in particle size, nature of the polymer, emulsification method, and emulsifier were prepared and characterized. These results showed that the approach is able to customize slow release particles with predictable release characteristics over a period of days to month. Preliminary studies were performed with particles of a drug loading of 3.9 (±0.2) %, and a mean particle diameter of 5 μm. *In-vitro* release studies indicated that these particles released drug over a period of three months. *In vitro* cell toxicity studies suggested that at higher concentrations (>1 μM), unencapsulated LE showed some effect on the viability of the MIN-6 insuloma cell line, while the sustained release microspheres showed no cytotoxicity. The ability of these microspheres to provide localized immunosuppression has been evaluated in a set of early exploratory experiments with diabetic rats receiving islet transplantation. Animals treated using a biohybrid device loaded with microspheres showed improved results compared to those treated by delivery in solution form with an osmotic mini-pump. These results show the promise of localized glucocorticoid treatment by sustained release microspheres as a possible form of localized immunosuppression regimen. However, further confirmation is required before use in cell or organ transplantation.

1. Introduction

As outlined in the "accompanying" paper within this issue Buchwald et al. 2010 Type 1 diabetes mellitus is an autoimmune disease in which the insulin producing β cells are permanently destroyed. The development of a bioartificial pancreas for islet transplantation could represent a possible solution (Juang et al. 1996; Sakurai et al. 2003; Narang and Mahato 2006; Fort et al. 2008). Successful application, however, needs to control the observed immune response of the host. Immune isolating devices have been explored for a long time, but because of limited access to nutrients and oxygen (Buchwald 2009), the prospect of long-term survival and function is often limited. One possible alternative is the use of a device that provides mechanical protection in combination with a safe, localized immunosuppressive regimen (see accompanying paper).

Current immunosuppressive treatments in organ and cell transplantation depress the entire immune response beyond that required to prevent rejection and are associated with other severe side effects (Platz et al. 1994; Gruber et al. 1997; Brown et al. 1998; Perales et al. 2007). Similar to targeted inhalation approaches employed in asthma therapy, a more localized, sustained delivery (within the bioartificial pancreas) would be therapeutically beneficial since it would reduce systemic side effects (Bocca et al. 2008). Along these lines, the use of soft drugs could provide further benefits since they are specifically designed to provide local activity, but no systemic side effects by breaking down into predicted inactive metabolites after exerting their desired therapeutic effect(s) (Bodor and Buchwald 2000; Bocca et al. 2008). This paper reports on the development of a sustained release local formulation for the soft glucocorticoid loteprednol etabonate (LE), an FDA-approved safe and highly active corticosteroid (Druzgala et al. 1991; Buchwald and Bodor 2004, 2006).

There are various commercially available drug delivery systems (DDS) that focus on sustained localized delivery. They include infusion pumps (Harbaugh et al. 1988), monolithic devices (Brem and Gabikian 2001; Weinberg et al. 2007) and biodegradable microspheres (Hyon 2000). PLGA and PLA microspheres have been used as DDS for many years (Hyon 2000) because they can encapsulate and provide sustained release of both hydrophilic and lipophilic drugs (Bala et al. 2004). The polymer

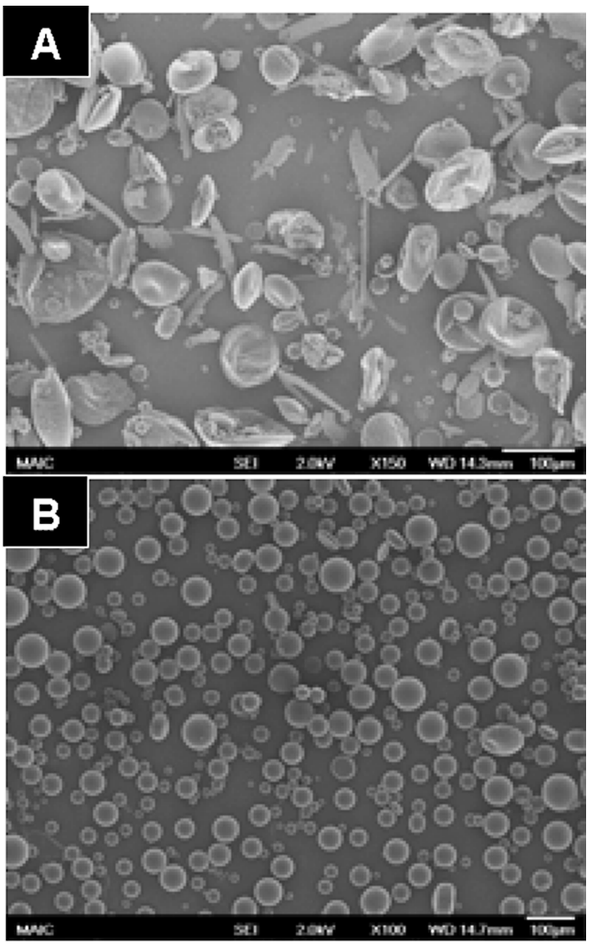
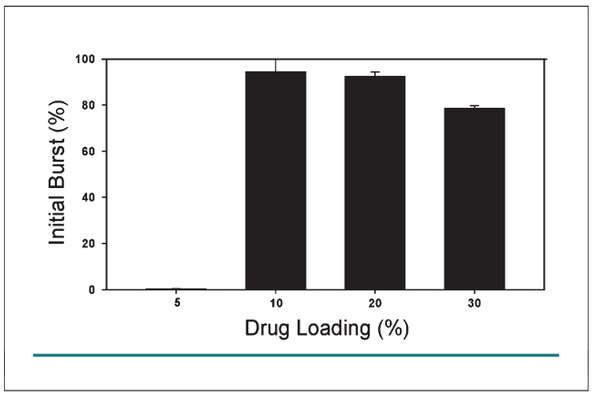
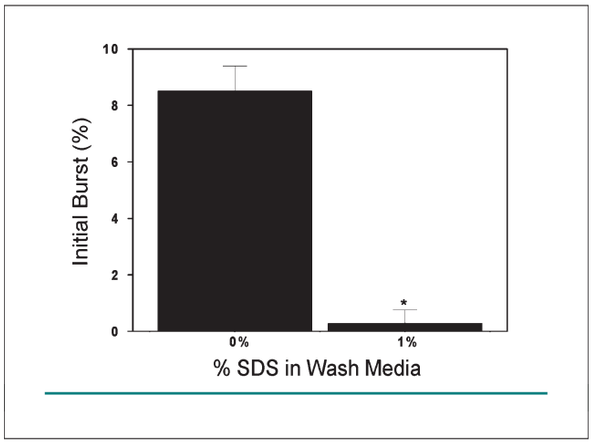


Fig. 1: Scanning electron microscope (SEM) pictures showing the surface morphology of LE-PLGA microspheres prepared by (A) sonication and (B) infusion (drop-by-drop addition) methods



(A)



(B)

Fig. 2: (A) Initial burst (% of total LE content in final formulation) for PLA microspheres prepared with 5 (5% drug, 95% polymer), 10 (10% drug, 90% polymer), 20 (20% drug, 80% polymer), and 30% (30% drug, 70% polymer) loteprednol etabonate (LE) in formulation ($n = 3$). (B) Initial burst of 5% LE-PLA microspheres (drug content 5%, 95 % polymer) prepared with 0% or 1% SDS in the wash media ($n = 3$). Prepared using the sonicator method

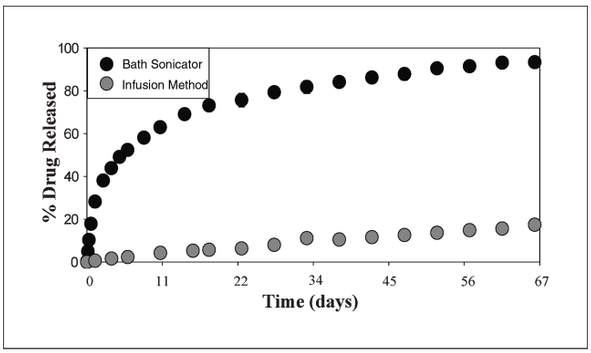
is biodegradable and biocompatible *in vivo*, where it degrades by hydrolysis of its ester linkages to CO_2 and H_2O (Dunne et al. 2000). Previously, sustained release PLA/PLGA microspheres have been made using solvent evaporation and exhibited drug release durations of over a month (Hickey et al. 2002; Kompella, et al. 2003; Liggins and Burt 2004; Zhang et al. 2007). This manuscript presents the design and preliminary results obtained with sustained release loteprednol etabonate PLGA/PLA microspheres intended for use in islet transplantation.

2. Investigations and results

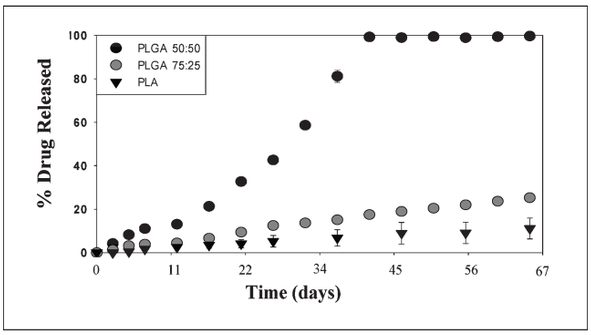
2.1. LE-PLA microspheres

Various process parameters were modified while developing the LE microspheres. The type of emulsification method used influenced the morphology of the microspheres produced. Emulsification using sonication (Fig. 1A) produced a polydispersed formulation of deformed/deflated microspheres. Emulsification by infusion of the organic phase into the aqueous phase stirred at a set speed (Fig. 1B) formed smoother, more monodispersed microspheres. As shown for PLA particles, the use of sodium dodecyl sulfate (SDS) in the wash media significantly reduced the initial drug burst observed during drug release studies as compared to the formulation washed with double distilled water (DDW) resulting in particles with an LE content of 3.9 ± 0.2 % (Fig. 2). The drug loading of SDS washed LE-PLA microspheres was also assessed for formulations that differed in the ratio of drug to polymer content. There was a dramatic difference in the initial burst (Fig. 2) for the four formulations as shown for particles. Whereas, the 5% LE-loaded microspheres had a very low initial burst of 0.2% ($\pm$0.4%), the 10%–30% LE-loaded microspheres had an initial bursts ranging from 78.5% to 94.4%. Use of the infusion method allowed the preparation of larger particles (diameter of around 30–40 µm; Fig. 1) with slower release kinetics (Fig. 3A). Changing the PLA/PLGA ratio (Fig. 3B) as well as the polyvinyl alcohol content in these formulations (Fig. 3C) had significant effects on the drug release rate suggesting that these parameters can be used to custom-tailor the release profiles.

LE-PLA microspheres with a low initial burst, prepared through sonication and with a drug loading of 3.9 ± 0.2%, were chosen for the preliminary pharmacological tests and, thus, were further characterized. The duration of the *in vitro* drug release was around three months with a slowly decreasing rate through this period (Fig. 3A). The particle size distribution of these microspheres was characterized by a mean diameter of 5.0 µm, a median diameter of 3.4 µm, and a standard deviation of 6.8 µm. A corresponding powder X-ray diffraction (PXRD) is shown in Fig. 4. There were small crystalline peaks at the 16, 17, 19, and 24 2θ angles for the 5% LE and blank PLA microsphere mixture that correlated to the prominent peaks for the unencapsulated drug at the same angles. The blank PLA microspheres



(A)



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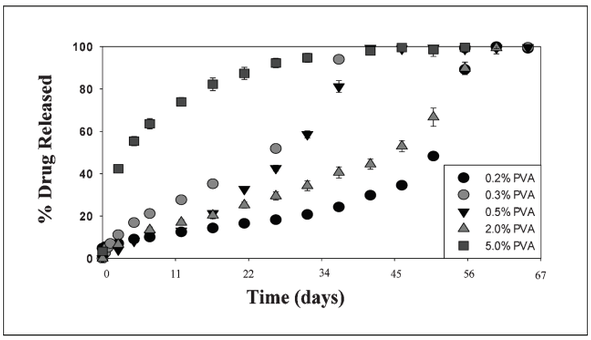

(C)

Fig. 3: (A) *In vitro* drug release profile of the LE microsphere formulation prepared via bath sonicator and infusion methods, using 0.3% PVA and PLA as polymer. Microspheres ($\phi = 5$ μm) prepared using a bath sonicator had a drug loading of 3.9%. Particles prepared according to the Infusion method had a diameter of 40 μm and a drug loading of 4.8%. (B) Effect of polymer on *in vitro* drug release profile of the LE microsphere formulation prepared by the infusion method and 4.8% drug loading and 0.2% PVA concenration. Microspheres had an average diameter of 40 μm. (C) Effect of PVA content on *in vitro* drug release profile of the LE microsphere formulation using the infusion method a drug content of 4.8 % and a ratio of PLGA/PLA co-polymer of 50:50. Microspheres had a mean diameter of 40 μm

were amorphous; hence, there were no crystalline peaks. However, with the LE-PLA microsphere formulation containing 5% LE, no crystalline peaks were observed. Changes in the particle morphology of an LE-PLA microsphere formulation during *in vitro* drug release have been monitored by scanning electron microscopy (SEM) up to 12 months (Fig. 5). The infusion method resulted in larger particles (30–40 μm) and slower drug release kinetics (Fig. 3A). The release characteristics could be further controlled by changing the polymer composition (pure PLGA; 50% PLGA/PLA mixture; pure PLA; Fig. 3B). Increase of the polyvinylalcohol content resulted in formulations with faster release characteristics (Fig. 3C). Thus formulations have been described that will allow the custom-tailored release of drug. Subsequent studies were performed with the 5% sonicator based particles.

2.2. MIN-6 cell viability

In vitro cytotoxicity evaluations with MIN-6 cells were performed to ensure that the LE microsphere formulation is not toxic to the islets. First, cells were exposed to increasing concentrations of LE (0.01, 0.1, 1, 10, and 100 μM). At the highest concentrations, LE showed evidence of cytotoxicity with a more pronounced effect after 4 days and an estimated median inhibitory concentration (IC_{50}) of around 20 μM (Fig. 6A). This is in agreement with previous results suggesting that LE concentrations up to 500 nM (0.5 μM) should not affect the viability of pancreatic islets (Bocca et al. 2008). Blank PLA microspheres at 0.1, 1, 10, 100, and 1000 mg/mL concentrations were exposed to the MIN-6 cells for 1 and 4 days. There was no decrease in the cell viability in either of these tests indicating no β-cell cytotoxicity (Fig. 6B). Interestingly, there was an increase in the cell viability with a threshold concentration of 100 mg/mL at day 1 and 1000 mg/mL at day 4. Finally, LE-PLA [LE content 5%] microspheres were also tested, and showed no cytotoxicity for concentrations up to 1 mg/mL at day 1 or 4 (Fig. 6C).

2.3. Islet transplantation

In an exploratory islet transplantation study with a 40-day prevascularized subcutaneous biohybrid device, the survival of the allogenic islet grafts in diabetic rats maintained on local immunosuppression only was increased with the use of the microsphere sustained-release formulation. The rate of survival was increased when compared to both the control group (no local immunosuppression) and the group receiving LE in solution form with the use of an osmotic minipump (accompanying paper). The streptozotocin-induced diabetic rats received two to three weeks of systemic immunosuppression following allogenic islet transplantation into the biohybrid device in conjunction with local immunosuppressant therapies, which was then maintained after the gradual tapering of the systemic administration. The LE-PLA microspheres formulation was inserted into the device at a loading dose of 4.5 mg at the time of the islet implant. They increased the average survival time from 9.3 (±3.6) days to 20.0 (±6.6) days (calculated starting after the cessation of the systemic treatment) and provided even some prolongation compared to the osmotic mini-pump-delivered LE formulation (Buchwald et al. 2010).

3. Discussion

The slow release delivery of a glucocorticoid soft drug was hypothesized to be able to provide local immune response without induction of systemic side effects. LE was selected as it provides high local targeting. We present several formulations that release LE with a wide range of release characteristics, depending on the formulation method, as well as on the polyvinyl alcohol (PVA) nature of the polymer and drug content. The presented range of formulations using different sizes, formulation methods and polymers will allow the synthesis of formulations with the desired release kinetics.

For unencapsulated LE, MIN-6 cytotoxicity was observed at concentrations higher than 1 μM. This is well above its K_d for the glucocorticoid receptor (≈5 nM) (Druzgala et al. 1991; Bocca et al. 2008), but it is in agreement with the fact that glucocorticoids can induce apoptosis in β-cells by the mitochondrial apoptotic pathway (Ranta et al. 2006). Glucocorticoids can also slow-down cell growth (Steffen et al. 1988). Dexamethasone

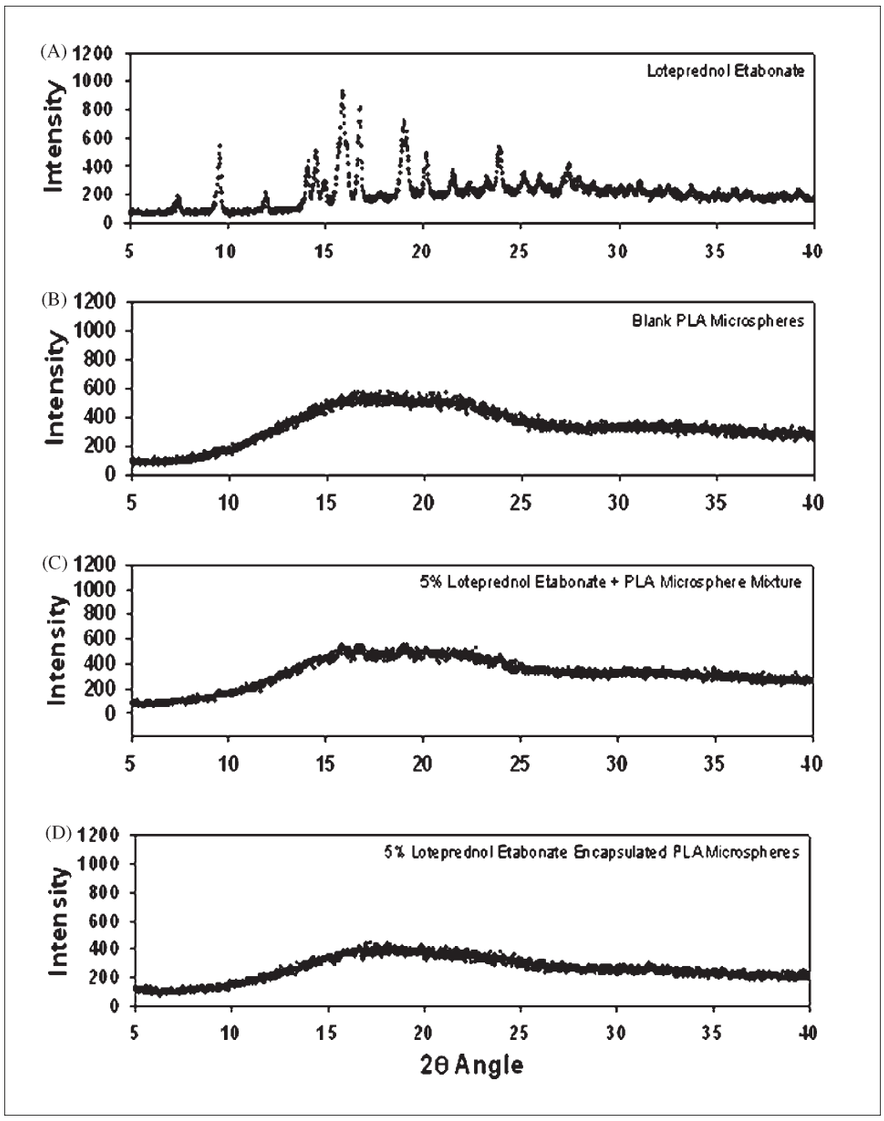

Fig. 4: PXRD patterns of LE (A), blank PLA microspheres (B), their physical mixture (C), and LE-PLA microspheres (D) for particles prepared according to the sonicator method

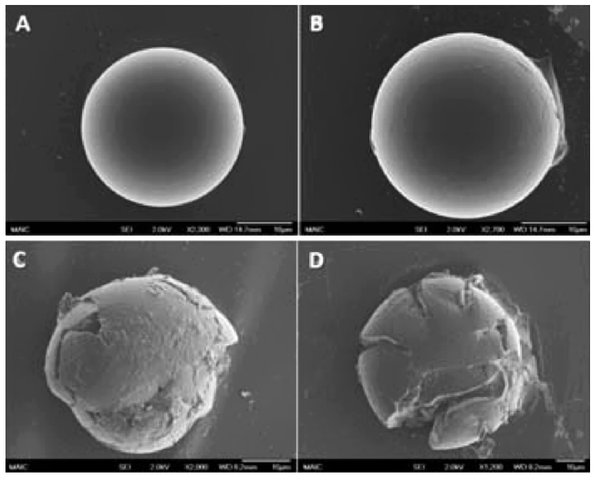

Fig. 5: Scanning electron microscope pictures showing the particle morphology of LE-PLA microspheres during *in vitro* drug release studies at (A) 0, (B) 4, (C) 8, and (D) 12 months

was shown to diminish cell proliferation by arresting the G_1 cell cycle (Tchekneva and Serafin 1994) and down-regulating growth-promoting factors (Rhee et al. 1995). These effects were not seen with blank PLA microspheres, which, in fact, seemed to have caused a slight increase in cell viability at 100 mg/mL at day 1 and 1000 mg/mL at day 4 (Fig. 6B). One possible explanation for this could be that the degradation product of the PLA polymer (lactic acid) fed the citrate cycle by producing pyruvate through the lactate dehydrogenase reaction (Ignatius and Claes 1996). The citrate cycle (Krebs cycle) is a metabolic pathway that produces energy in the mitochondria of living cells. The MTT assay used for viability assessment is based on cell metabolism by quantifying the formazan production from active mitochondria (only found in living cells). The higher concentration of lactic acid, from a higher concentration of the PLA polymer, could have produced more pyruvate. A higher concentration of pyruvate could then enhance the mitochondrial activity producing more formazan and resulting in an increase in the cell viability as assessed by this assay. Ignatius and co-workers noticed an increase in succinate dehydrogenase activity, a mitochondrial enzyme used in the citrate cycle when exposing clone L929 mouse fibroblast cells to degradation products of PLGA (70:30)

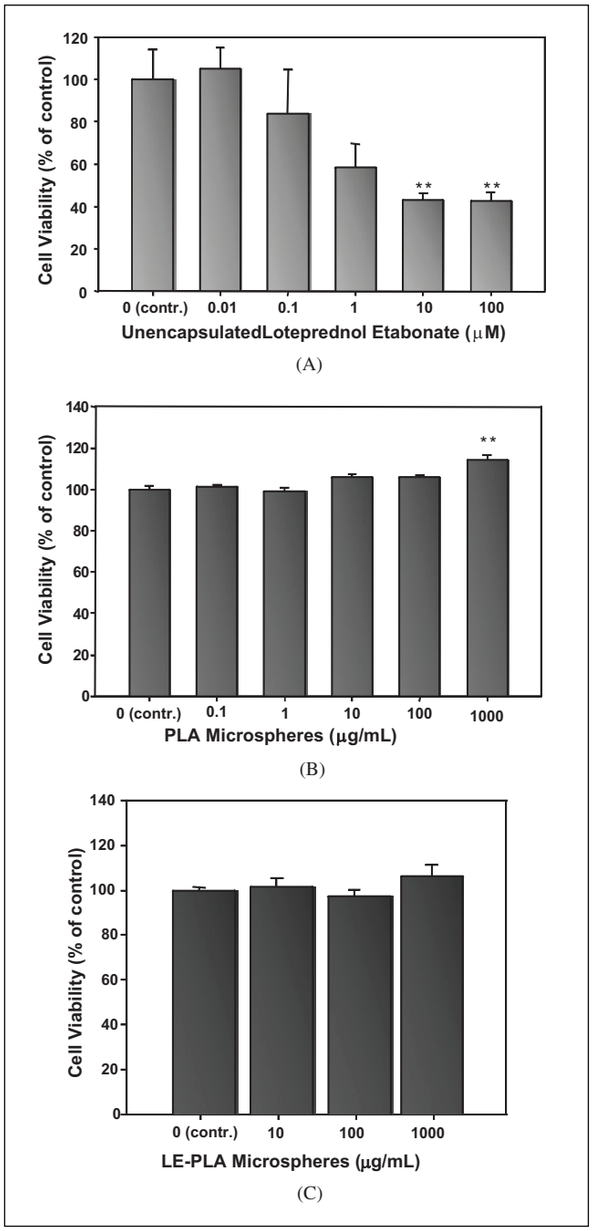

Fig. 6: Effect of (A) unencapsulated LE, (B) empty PLA microspheres, and (C) LE-PLA microspheres on MIN-6 cell viability after four days of incubation ($n = 4$)

and PLGA (90:10) (Ignatius and Claes 1996) suggesting that PLA could increase mitochondria activity.

LE-microspheres did not show the cytotoxic effects of LE probably because these particles retained LE in the microspheres and provided lower free drug concentrations during the cell culture experiments. Some of the very small effects seen on cell viability (Fig. 6C) could be due to the minimal crystalline drug present on the surface of the PLA microspheres and the initial burst of the tested formulation. The PXRD plots exhibit small crystalline peaks for the 5% LE and blank PLA microspheres (Fig. 4). However, with the LE-PLA microsphere formulation containing 5% LE, no crystalline peaks were observed indicating that the drug was incorporated within the microspheres and not residing on the surface. Due to the initial drug-release burst of the tested PLA formulation (around 1.7% at $t = 0$ h), the cells might be exposed to a somewhat elevated LE concentration in the beginning; however, this was still low enough to not affect cell viability (Fig. 6C). With higher bursts this can be a problem. For example, PLGA (50:50) microspheres containing hot saline antigenic extract showed cytotoxicity on the macrophage cell line J774.2 (cell viability of $24.4 \pm 6.3\%$) due to their high initial burst ($40.3 \pm 2.7\%$) thus exposing the cells to higher doses of the toxic dose (Murillo et al. 2002).

The LE-PLA microspheres showed some efficacy in local immunosuppression by delaying the rejection of islet transplantation in a rodent biohybrid device model (accompanying paper). However, the *in vivo* effect only lasted about 5–6 weeks (including the 2–3 week systemic immunosuppression period) – somewhat less than the *in vitro* estimated three month drug release duration. Possible reasons could be, *(i)* a faster *in vivo* drug release rate than that estimated in the current *in vitro* study, *(ii)* the washing away of smaller microsphere particles in the newly forming vasculature within the biohybrid device, which is already prevascularized at its borders, or *(iii)* the use of an inadequate dose in these early, exploratory studies. Dose estimates were made so as to ensure an approximate delivery rate of at least 1 nmol/day (0.5 μg/day) (Bocca et al. 2008); however, since LE is a soft drug subject to extrahepatic metabolism (Bodor et al. 1995; Bocca et al. 2008), this might not have been sufficient.

In conclusion, LE-PLA microspheres that can provide localized and sustained delivery of the soft corticosteroid loteprednol etabonate (LE) have been prepared and evaluated. Future studies need to identify the most optimal release characteristics for a safe local immune suppression system. Microspheres prepared by solvent evaporation and having a drug loading of 3.9%, mean particle diameter of 5.0 μm, and an *in vitro* drug release duration of approximately three months were evaluated for *in vitro* cell toxicity and *in vivo* efficacy. They showed no cytotoxicity in MIN-6 cells and showed some efficacy in prolonging the survival of islet allografts transplanted in a prevascularized subcutaneous biohybrid device in a rat model.

4. Experimental

4.1. Materials

Poly(D,L-lactic) acid (PLA) (0.68 dL/g inherent viscosity in chloroform at 30 °C) was purchased from DURECT Corporation (Pelham, AL, USA). The polyvinyl alcohol (MW_{avg} 30,000–70,000, 87–90% hydrolyzed) and Tween 80 was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Loteprednol etabonate (LE) was obtained from Dr. Nicholas Bodor. The MIN-6 mouse insulinoma cell line was obtained from Dr. Sihong Song. Dulbecco's modified Eagle's medium, trypsin EDTA, phosphate buffer saline solution (1x PBS), fetal bovine serum, penicillin and streptomycin solution were purchased from Cellgro (Manassas, VA, USA). MTT ((4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was purchased from Calbiochem (EMD Chemicals, Inc., Gibbstown, NJ, USA). The remaining chemicals were of analytical grade and purchased from Fisher Scientific Inc. (Suwannee, GA, USA).

4.2. Methods

4.2.1. Solvent evaporation

The drug LE and polymer PLA were added to the organic solvent dichloromethane (DCM) and sonicated until both were solubilized. The organic solution was added to the aqueous solution containing 0.3% emulsifier poly-vinyl alcohol (PVA) and emulsified either by (a) sonication in a bath sonicator for 5 min or (b) infusion of the organic phase into the stirred aqueous phase. The emulsion was constantly stirred overnight using a Bellco Multistir 9 to allow the DCM to evaporate and form a suspension. The suspension was centrifuged at 5000 rpm for 15 min using the 50 mL polypropylene tubes, Beckman centrifuge (Model # J2-21), and Beckman rotor (Model # JA-20). The supernatant was collected and the residue re-suspended in the wash solution. The collected microspheres were washed three more times. The residue was re-suspended in the minimal volume of double distilled water (DDW) and frozen at –80 °C for at least 2 h. The

samples were then lyophilized using Labconco Freeze Dry System 4.5 (Kansas City, MO) for three days and stored at 4 °C in a desiccator until use.

4.2.2. Drug loading

A known amount of the LE-PLA microspheres was dissolved in 2 mL of DCM by bath sonication for 15 min. The DCM was removed by vacuum centrifugation. The dried residue was redissolved in 2 mL mobile phase and 100 μL of (10x or 100x) dilution was injected into a reverse-phase Waters C_{18} 150 × 4.6 mm 5 micron HPLC column. The mobile phase consisted of acetonitrile:DDW:glacial acetic acid (60:40:0.4). The flow rate used was 0.8 mL/min and the monitoring wavelength was 254 nm. From a 100 μg/mL stock in acetonitrile, calibration samples of 30, 20, 10, 5, 1, 0.5, and 0.1 μg/mL were made with the mobile phase. The drug loading efficiency was calculated as follows:

$$Loading\ Efficiency = \frac{Experimental\ Drug\ Loading}{Theoretical\ Drug\ Loading} \cdot 100\% = \frac{\frac{x_{\exp}}{x_m}}{\frac{x_{LE}}{(x_{LE} + x_{PLA})}} 100\%$$

where $x_{\exp}$ is the amount (mg) of drug quantified by HPLC analysis for a given amount of the formulation, x_m is the amount (mg) of formulation analyzed, x_{LE} is the total amount (mg) of drug added in preparing the formulation, x_{PLA} is the total amount (mg) of polymer added in preparing the formulation.

4.2.3. Particle size analysis

A known amount of the LE-PLA microspheres was dispersed in deionized water and sonicated for 60 s to break apart aggregates. The suspension was then subjected to analysis using a Coulter LS13320 (Beckman Coulter, Fullerton, CA) laser diffraction particle size analyzer, which can analyze particles in the size range of 400 nm to 2 mm. The refractive index was set at 1.46 (0.01*i*) and run time at 60 s. The runs were done in triplicate per sample.

4.2.4. Scanning electron microscope

The LE-PLA microspheres formulations were analyzed using a JEOL (Model 6335F) scanning electron microscope (SEM) to obtain the size, shape, and surface morphology. The microspheres were mounted on aluminum SEM stubs with double stick carbon tape. A thin layer of carbon, approximately 10 to 15 nm thick, was evaporated onto the surface of the particles prior to SEM analysis. Characterization was performed at 2–5 keV under vacuum.

4.2.5. Powder X-ray diffraction (PXRD)

A known amount of LE-PLA microspheres were fixed to a microscope slide using double sided tape, and a Philips APD *X*-ray diffractometer was used. The taped samples were exposed to Cu radiation (40 kV, 20 mA) and scanned from 5° to 40°, 2θ at a step size of 0.02° and step time of 1 s.

4.2.6. In vitro drug release

The *in vitro* drug release studies were performed at 37 °C using 200 mL dissolution media in a capped 250 mL Erlenmeyer flask and continuously stirred. The dissolution media consisted of 1x phosphate buffer saline (PBS) (pH 7.4), 0.025% sodium azide, and 1% sodium dodecyl sulfate (SDS). Sodium azide was used as a preservative. SDS was used to enhance the stability of the drug and to keep the *in vitro* drug release assay under sink conditions. To assess the drug release, the "sample and separate" technique was used with a stir rate of 30 rpm.

A predetermined amount of LE-PLA microspheres was added to the bulk media. The amount of sample used was adjusted to keep the total released drug concentration in the dissolution media below 15% of the drug's saturation solubility (126 μg/mL). At defined time intervals, 0.5 mL of the bulk media was removed and centrifuged at 13,200 rpm for 10 min. The supernatant (100 μL) was injected into a reverse-phase Waters C_{18} 150 × 4.6 mm 5 micron HPLC column. The mobile phase consisted of acetonitrile: DDW: glacial acetic acid (60:40:0.4). The flow rate used was 0.8 mL/min and the monitoring wavelength 254 nm. Previous stability studies (data not shown) of LE showed that in aqueous media it degrades only into Δ^1-cortienic acid etabonate (AE) and not Δ^1-cortienic acid (CA) indicating no need for detecting CA. Calibration samples of 20, 10, 5, 1, 0.5, 0.1, and 0.05 μg/mL were prepared by diluting a 100 μg/mL LE and AE stock (in 100% acetonitrile) in dissolution media. Quality control samples consisted of 10, 1, and 0.1 μg/mL (LE and AE). The calibration curve was plotted in MS Excel using peak heights of the absorbance of the standard solutions, and the trendline was used to calculate the sample concentrations.

The LE-PLA microspheres remaining at the end of the study were dissolved in dichloromethane to extract the drug. This study was done in order to determine the amount of drug remaining within the microspheres and the stability of the drug within the microspheres. The supernatant was dried off, reconstituted in mobile phase, and analyzed using HPLC with the method described above. The initial burst was determined to be the % of LE and AE released into the dissolution media (supernatant) immediately after adding the LE-PLA microspheres to the media. The drug release profile was determined by plotting the % of LE and AE released at the time points corresponding to when the samples were taken.

4.2.7. Cell assays (MIN-6)

MIN-6 cells were cultured in 10 cm plates in 4.5 g/L glucose Dulbecco's modified Eagle's medium (DMEM) containing 15% fetal bovine serum (FBS) and 1% penicillin and streptomycin. The cells were incubated at 37 °C under 5% CO_2. The medium was changed every 2 to 3 days and subcultured when plate was confluent (∼weekly).

A standard MTT assay was used to estimate cell viability. MIN-6 cells (10,000 cells/well) were seeded in a 96-well flat-bottom plate (Corning Inc., Corning, NY, USA) in 4.5 g/L glucose DMEM for 24 h. The medium was removed and replaced with DMEM containing unencapsulated drug as well as PLA or LE-PLA microspheres at 100, 10, 1, 0.1, and 0.01 μM LE concentrations. A blank having 0 μM LE in the DMEM media was used as control. After incubation for 1 and 4 days, the medium was removed and replaced with filtered MTT (100 μL, 0.5 mg/mL in DMEM) and incubated for 3 h at 37 °C. The cells were treated with 100 μL isopropyl alcohol containing 0.04 mol/L HCl for 30 min under dark at room temperature. The absorbance was measured using the Dynex Technologies microplate spectrophotometer model MRX^{TM} (Chantilly, VA, USA) at a wavelength of 550 nm. The percent cell viability was calculated as:

$$\%Cell\ Viability = \frac{Absorbance\ of\ Sample}{Avg.\ Absorbance\ of\ Control} \cdot 100\%$$

4.2.8. Statistical analysis

Percent cell viability of the samples are shown as mean ± SE for $n = 4$ determinations. Statistical differences were determined using Student *t*-test in SigmaPlot 10.0 (Systat Software, Inc., San Jose, CA, USA). Curve fitting of the *in vitro* drug release profile was performed with SigmaPlot 10.0 and MS Excel.

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