Uniform Paclitaxel-Loaded Biodegradable Microspheres Manufactured by Ink-Jet Technology

1Delia Radulescu, 2Nathan Schwade, 2Debra Wawro
1MicroFab Technologies, Inc., Plano, TX 75074, U.S.A.
dradulescu@microfab.com
2Department of Otolaryngology Head and Neck Surgery, University of Texas Southwestern Medical Center at Dallas, Dallas, TX 75390, U.S.A.

ABSTRACT SUMMARY:
We have obtained paclitaxel-loaded monodispersed microspheres by using various approaches common to ink-jet technology. The drug-loading efficiency as determined by HPLC was at least 68%. The HPLC analysis showed that the manufacturing process did not affect paclitaxel molecule while the MTT assay on the FaDu carcinoma cells confirmed that the drug retained its pharmacological efficiency. In vitro testing demonstrated that paclitaxel was slowly released from the microspheres for a period of approximate 50 days, with over 80% of the drug being released during this time.

EXPERIMENTAL METHODS:
1. Experimental setup and manufacturing

Set-up. Paclitaxel-loaded PLGA microspheres were manufactured using the JetLab® system (MicroFab Technologies, Inc. Plano, TX) shown in Figure 1. Briefly, the system includes printing piezoelectric device(s) PC controlled through the JetLab® 3.05 software and the drive electronics box electronics controller II (MicroFab Technologies, Inc). A vertical CCD camera checks the stability of the jet while an horizontal camera inspects the jetted microspheres. The jetting process and the final microspheres can be followed on two monitors and the images can be captured and digitally stored in the computer. Control of the jet functioning and characteristics as well as inspection of the final microspheres can be automatically performed by special subroutines included in the JetLab® software. The backpressure on the printing device and the eventual applied “positive” pressure are controlled through a pneumatic console. HEPA filters are mounted in the top of the system and air duct under the system’s frame. The entire work area is enclosed. A block-diagram of the JetLab® components is presented in Figure 2.

Manufacturing. Microspheres were prepared by a solvent evaporation method. Namely, 1.5% (w:v)
paclitaxel (Sigma Chemical Co., St. Louis, MO) was added to 3% (w:v) poly(lactic-co-glycolic acid) (PLGA, Sigma Chemical Co., 85:15 PLA to PGA ratio) in 1,2-dichlorethane (Aldrich Chemical Co., Milwaukee, WI) and the resulting mixture was jetted in 0.1% (w:v) polyvinyl alcohol (PVA, Aldrich Chemical Co.) using either pressure-assisted drop-on-demand (Figure 3a-3c) or simply drop-on-demand mode (without any pressure applied to the printing device, Figure 3d-3f). During this process, we first tested the stability of the jet in air (Figure 3a and 3d). The jet of microspheres in PVA is shown in Figure 3b and 3e and the final microspheres in Figures 3c and 3f. Curing. After jetting, the solvent was removed by evaporation through continuous stirring at 150 rpm, for 1 hour and 30 minutes, and at room temperature. After being washed three times with deionized water to remove PVA residue and any free drug, the microspheres were collected by centrifugation at 1,500 rpm for 5 min. and gradually frozen in liquid nitrogen for 6h. They were lyophilized at –57°C and at 10-20 mtorr for 24 hours. The images of the jetted microspheres before and after curing were transferred to computer through Pixera Viewfinder 2.0 software and average size and standard size deviation were estimated with the NIH ImageJ 1.25s software.

2. Process characterization

The effect of processing parameters on the final drug-loaded microspheres was assessed by determination of microsphere size and size distribution (before and after curing), surface morphology, paclitaxel analysis via HPLC (retention time) and in vitro release kinetics.

Scanning electron microscopy (SEM) analysis: The shape and surface of the microspheres were observed by SEM. The microspheres in the form of free-flowing powder were coated with gold-palladium using a Denton DV-5A ion coater (Denton Vacuum, Mooresstown, NJ). The surface morphology was examined with a JEOL scanning electron microscope (SEM) (model JSM-840A) at 10kV accelerating voltage. The results of size and size distribution were further confirmed by SEM.

Encapsulation efficiency: An extraction procedure of the paclitaxel from the PLGA microspheres was developed. A quantity of 3 mg of microspheres was dissolved in 1ml of 1,1,1,3,3,3 hexafluoro-2-propanol (HFP, Oakwood Products, West Columbia, SC). A known quantity of methanol (Sigma Chemical Co.) was added until the polymer precipitated out of the solution. The solution was centrifuged at 2000 rpm for 5 minutes. The supernatant containing paclitaxel was removed and evaporated under a constant stream of nitrogen while gently heated at 40°C. Paclitaxel was reconstituted in 1 ml of HPLC mobile phase. The measurement of paclitaxel content in the microspheres was carried out in duplicate using HPLC according to a modification of published methods (3,4). For HPLC analysis, a reversed-phase Water's Symmetry (150 x 2.1,Waters Corp., Milford, MA) column was used. The column temperature was controlled at 40°C. The mobile phase consisting of a mixture of 35 mM of ammonium acetate at a pH of 5.0, acetonitrile, and tetrahydrofuran (50:45:5) was delivered at a flow rate of 0.3 ml/minute with an integrated pump (Dionex Corp., Sunnyvale, CA). A 25 µl aliquot with an auto injector (Dionex Corp.) in the column effluent was detected at 227 nm with an ultraviolet detector (Dionex Corp.). The area of each eluted peak was integrated with Peak Net software (Dionex Corp.) and used for paclitaxel quantitation. External standards of paclitaxel were obtained from a commercial source (Sigma Chemical Co.) and dissolved in methanol.

Release kinetics and SEM analysis after release: Paclitaxel-loaded microspheres were jetted and post-processed as previously described. Four mg of dried (free-flowing) paclitaxel-loaded microspheres were incubated in 13 ml of phosphate buffer saline (PBS, pH 7.4) under continuous shaking. Aliquots of 2 ml PBS were removed every three days, for 8 weeks. Equal volumes (2 ml) of PBS were added to the dissolution media to maintain a constant volume. Five ml of diethylether was added to each removed aliquot. The mixture was vortexed and the supernatant was removed and evaporated under a constant stream of nitrogen while gently heated at 40°C. Paclitaxel was reconstituted in 1ml of HPLC mobile phase, as described above.

Bioassay: The FaDu squamous cell carcinoma cell line (American Type Culture Collection, Rockville, MD) was grown in Eagle’s minimum essential medium with Earle’s BSS nonessential amino acids, and 10% (vol/vol) fetal bovine serum. The cells were incubated in 25 cm² cell culture flasks, at 37°C and 5% CO₂ environment. After 48 hours, the cells were harvested by trypsinization and counted with the hemocytometer. Four thousand cells per well were plated in a 96 well culture plate (or 4,000 cells in 100 µl media). One hundred µl paclitaxel extracted from the jetted microspheres as previously...
described was added to each well. Wells containing 100 µl cells treated with 200 µl of commercial standard paclitaxel (Sigma Chemical Co.) and wells containing 100 µl untreated cells were used as positive and negative controls, respectively. Cell viability was estimated with the MTT assay. Namely, after 96 hours, 20 µl of MTS/PMS (Promega Corp., Madison, WI) was added to each well. The plate was incubated for 2 hours and the optical density was read at 460 nm with a plate reader (Dynex Technologies, Chantilly, VA). Analysis of variance was performed with the multiple comparison Tukey-Kramer test using the JMP 4.0.4 software (SAS Institute, Cary, NC). Positive values for pairs of optical density means were considered significantly different at a P value of ≤0.05.

RESULTS AND DISCUSSION:
Paclitaxel is one of the best chemotherapeutic drugs found in nature during the past years (3). However, several obstacles have to be overcome in order to achieve a successful paclitaxel chemotherapy. They mainly consist of side effects due to the use of toxic surfactants to increase the solubility and its limited availability (5). Therefore, attention has been focused on developing new delivery systems with improved efficiency and reduced toxicity. Localized delivery via injectable paclitaxel-loaded microspheres is such a delivery system. PLGA microspheres were prepared using a traditional single emulsion solvent evaporation recipe. A high drug loading level (33%, w:w) was used in this process, a value that is reported to be advantageous in the therapy of cancer, when PLGA is used as a carrier. Control over the manufacturing procedure, in particular over size and size distribution, was achieved by using approaches common to ink-jet printing technology, such as drop-on-demand jetting or a pressure-assisted drop-on-demand mode jetting.

Ink-jet technology relies on dispensing small amounts of fluids in a controlled fashion via a piezoelectric device (PZT). Such a device consists of a glass tube surrounded by an annular PZT element.

![Fig. 3: Drop-on-demand mode jetting](image)

In drop-on-demand mode, the drops are generated only when needed. When a fluid (at atmospheric pressure or at a slightly higher pressure in the case of pressure-assisted drop-on-demand mode) is loaded in the glass tube and an electric pulse is applied, the PZT deforms and generates acoustic waves that propagate towards the orifice, where the drop develops. This principle is schematically shown in Figure 3. The drop-on-demand jetting mode, in particular, offers more control over jetting process than the traditional vibratory methods described in the literature (6,7). The jetting process and the resulting microspheres are shown in Figure 4. After jetting, the microspheres were cured by evaporating the solvent under continuous stirring, at low speeds (150 rpm) and for at least one hour. These curing parameters were important for preserving the uniformity achieved during jetting. The final microspheres had a narrow size distribution (± 1 µm for an average diameter of 60 µm) as determined by the optical microscope analysis and confirmed by the SEM micro-
graphs (Figure 5). Higher and lower diameters (from 15 to 100 µm) could be obtained by varying different mechanical or electrical parameters, such as PZT orifice diameters (20 to 80 µm), device geometry, and jetting frequencies (up to 20,000 Hz).

The manufacturing procedure did not affect the structure of paclitaxel molecule, as indicated by the HPLC chromatogram comparing the external standard with the extracted drug. This further suggests retention of pharmacological efficacy. Indeed, the MTT assay showed that, while the treated (extracted: 0.45±0.02, standard paclitaxel: 0.45±0.01) and untreated cells (1.36±0.12) were statistically different, there was no statistical difference between the effect of standard and extracted paclitaxel on the cancer cells (Figure 6).

As expected, the manufacturing conditions influenced the drug loading efficiencies (expressed as the ratio between the amount of extracted paclitaxel from the microspheres and the amount of loaded paclitaxel). The encapsulation efficiency was at least 67% for spheres fabricated by pressure-assisted drop-on-demand procedure and at least 46% for the spheres fabricated by drop-on-demand mode, as determined by HPLC. The calibration curve was linear over a range from 12.5-200 µg/ml (standard concentration of paclitaxel) with a correlation coefficient of $r^2 = 0.9998$. The loss of mass associated with the extraction procedure could be explained by two possible mechanisms. First, as it is well known, paclitaxel will bind to plastic and glass tubes (3). Secondly, paclitaxel may remain in the polymer after the precipitation stage. Each of these possibilities deserves further investigation.

One of the desired characteristics of a drug delivery vehicle is to provide a sustained release of the encapsulated drug, a property correlated with improved pharmacokinetics and efficacy (8). The microspheres exhibited a slow, sustained release for approximately 50 days, with over 80% of the drug released during this time. The jetted monodispersed microspheres of 60 µm in average diameter displayed a sustained release for approximately 50 days, with over 80% of the drug being released during this time.

**CONCLUSION:**

In conclusion, ink-jet technology can be one of the methods of choice for the fabrication of monodispersed microspheres with good pharmacological properties. The drop-on-demand mode jetting could be useful in research applications where small quantities of expensive drugs have to be encapsulated while pressure-assisted drop-on-demand mode could be more attractive for applications requiring jetting of high quantities of fluid in a short period of time. Both fabrication methods can be scaled up by using several PZT devices simultaneously or multi-channel array print-heads. The manufacturing process can be performed semi-automatically in the Jet-Lab® aseptic environment. The PZT devices can also be autoclaved without affecting their operational parameters.

Jet technology, besides being a more simple method than most of those currently described in the literature, offers the advantage of a controlled manufacturing process. Microsphere formation is, by nature, a thermodynamically driven process. It is determined by a multitude of parameters with no or very little control over. By contrast, jetting technology switches the fabrication procedure from a thermodynamically governed mechanism to a mechano-electrical driven one, hence easier to control.
REFERENCES: