3D Printing of Biological Materials for Drug Delivery and Tissue Engineering Applications

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1. Introduction

Ink-jet technology relies on incorporation of data-driven, non-contact techniques that enable precise, picoliter volumes of material to be deposited with high speed and accuracy at target sites. We have used various approaches common to ink-jet technology to dispense biological materials three-dimensionally, the resulting structures having applications in drug delivery and tissue engineering.

2. Materials and Methods

General Setup

A standard JetLab® system routinely used to manufacture drug delivery systems is detailed schematically in Figure 1. Briefly, the system includes: 1) microdispensing hardware represented by one or multiple printing piezoelectric devices to allow for single- or multiple fluid dispensing or for scaling-up single fluid dispensing; 2) three-axis motion system for sample printing. This typically includes 3 positioning stages with optical encoders (encoder resolution 1.0 μm); 3) optics system represented by two black and white CCD cameras and a frame grabber allowing alignment of motion stage and printing substrate, quality check of the jetting process, and inspection of the final jetted products; 4) drive-electronics for generating an electrical pulse to the piezoelectric device (the characteristics of this pulse depend on the fluid characteristics and construction of the microdispensing devices); 5) software (in-house developed) allowing selection of the: printing device/fluid, characteristics of the electric pulse applied to the printing device, number of drops dispensed per location and printing pattern.

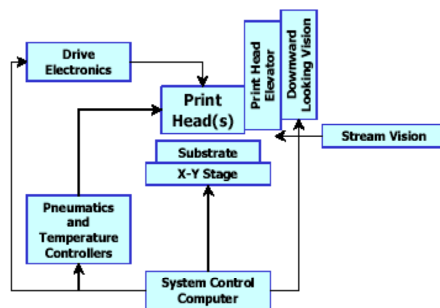

Fig. 1: JetLab® system block diagram.

Microsphere Manufacturing and Testing

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-dichorethane (Aldrich Chemical Co., Milwaukee, MI) and the resulting mixture was jetted in 0.1% (w:v) polyvinyl alcohol (PVA, Aldrich Chemical Co.) using either pressure-assisted drop-on-demand or simply drop-on-demand mode (no pressure applied to the printing device). After jetting, the solvent was removed by evaporation through continuous stirring, and the microspheres were rinsed with de-ionized water and lyophilized. The images of the jetted microspheres were transferred to a computer using the Pixera Viewfinder 2.0 software and average size and standard size deviation were estimated with the NIH ImageJ 1.25s software. The shape and texture of the surface were analyzed by scanning electron microscopy (SEM). The drug loading efficiency and the chemical quality of the jetted, encapsulated drug were evaluated by high-pressure liquid chromatography (HPLC) using a reversed-phase Water's Symmetry column (150 x 2.1,Waters Corp., Milford, MA), as previously described (1, 2). The ability of the jetted microspheres to release the drug over an extended period of time was tested *in vitro* and the bioactivity of the drug extracted from microspheres was evaluated in tissue culture, using the FaDu squamous cell carcinoma cell line (American Type Culture Collection, Rockville, MD).

Fig. 2: Desktop JetLab® system attached to a tissue culture hood for sterile manufacturing.

Nerve Conduit Manufacturing and Material Testing

Set-up: The standard set-up was adjusted to accommodate the nerve conduit manufacturing process. Such adjustments included: i) a desktop version of the JetLab® system was attached to a sterile, tissue culture hood; ii) the size of the motion stages and metal fixtures was scaled to allow unimpeded airflow within the hood; iii) a thermocouple coupled in a feed-back loop to a temperature controller was introduced in the electronic circuit to control the temperature of the printing substrate; iv) a rotational axis was added to allow rotation of the mandrel during the printing process (Figure 2).

Material selection: Initial selection of the conduit material was done based on four criteria: 1) ability of the material to biodegrade in the peripheral nervous tissue at the end of the wound healing process; 2) ability of the material to sustain cell viability and attachment; 3) ability of the material to sustain proper cell functioning (i.e. nerve growth factor expression); 4) ability of the material to be jetted at high concentrations and for extended time period of time (few hours). Poly(D,L-lactic acid), and various copolymers of poly(lactic acid) and poly(ε-caprolactone) were shown (3, 4, 5) to biodegrade in a period of time long enough to act as a support for neurite growth and extension during the nerve regeneration process but short enough to be absorbed by the body at the end of this process (criteria # 1).

Thus, these polymers were screened *in vitro* for their ability to sustain cell growth and attachment (criteria # 2). In the cell culture experiments, solutions of commercially available poly (D,L-lactic acid) (PLA) and commercially available copolymers 80/20, 25/75 and 40/60 of poly(D,L-lactic acid) and poly-ε-caprolactone, respectively (PLA/PCL, Absorbable Polymers International, Pelham, AL) dissolved in cellosolve acetate (Sigma-Aldrich Corp., St. Louis, MO) were prepared and used for coating 50 x 15 mm Pyrex Petri dishes (VWR International, West Chester, PA). After solvent evaporation, the dishes were sterilized by overnight incubation with Dulbecco's Modified Eagle's Medium (DMEM) and 10x antibiotics/antimycotics at 37^0C. The expression vector pIND-hNGF was transfected into EcR-293 cells (Invitrogen Corp., Carlsband, CA) using LipofectAMINE™ 2000 (Invitrogen), according to the instructions provided by the manufacturer. The transfected cells were plated on the polymer-coated dishes at a concentration of $5x10^4$ cells per plate. All plates were incubated at 37^0C and 5% humidity. Media was changed after every alternate day and cells were assessed microscopically each week. The ability of the cells to express NGF (criteria # 3) was tested using with an Emax NGF ELISA kit according to the manufacturer's instructions (Promega Corp., Madison, WI). NGF bioactivity was assessed using PC-12 cells cultured as previously described (6, 7). All the experiments were carried out in triplicate.

The polymers that successfully passed criteria 1), 2) and 3) were dissolved in various solvents (ex.: cellosolve acetate,

Fig. 3: PLGA paclitaxel-loaded jetted microspheres (100 mm after jetting, 50 mm after curing).

chlorinated organic compounds) in concentrations ranging from 1 % to10 % w/v and tested for jettability (criteria # 4). A solution was considered "jettable" when the jet of fluid

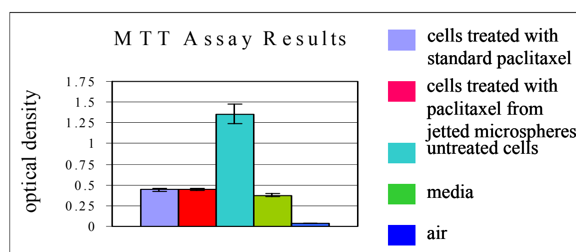

Fig. 4: Results of the MTT assay showing efficacy of paclitaxel extracted from jetted microspheres.

dispensed (polymer plus solvent) was stable for at least 10 minutes (the equivalent of the printing time for one layer). In this step, the electrical parameters (characteristics of the signal applied to the microdispensing device) that lead to a stable jet were identified and used in the fabrication process. The polymers that passed all the criteria were then used in the conduit fabrication process. The printing pattern, printing speed and printing substrate temperature had to be chosen so that the evaporation time of the solvent and the total printing time were minimized.

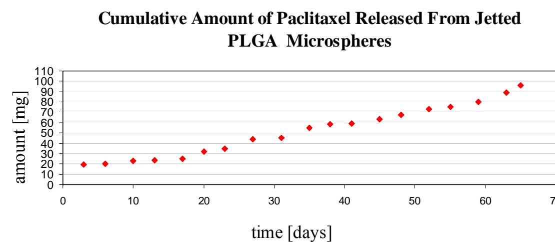

Fig. 5: Release kinetic profile of paclitaxel from jetted microspheres.

Cardiovascular Stent Coating and Testing

Using a similar set-up and approach, cardiovascular stents were coated with fenofibrate (model drug) and proprietary polymers and drugs. Printing was done in drop-on-demand mode jetting, i.e. the fluid was maintained at ambient pressure and the piezoelectric transducer was used to create

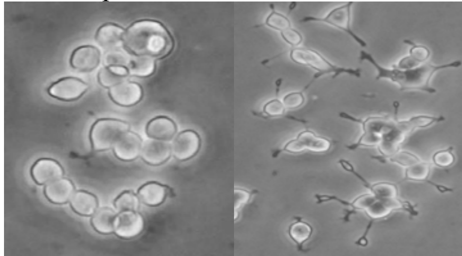

Fig. 6: Bioassay for the NGF expressed by hNGF-EcR-293. Left: control=PC12 cells cultured in the absence of NGF; Right: PC12 cells cultured on 1.5% w:v 80/20 PLA/PCL.

a drop only when needed. The motion controller was programmed to dispense a target amount of 100 µg drug only on the outer stent surface. The drug loading efficiency of the stents, the quality of the drug after jetting and the release kinetic profile of the drug were determined by conventional UV-spectrophotometry and confirmed by HPLC.

3. Results and Discussion

Drug-Loaded Microspheres.

Paclitaxel-loaded monodispersed microspheres of narrow size distribution (standard deviation ≤ 1µm) were fabricated using various approaches common to ink-jet technology such as drop-on-demand and continuous mode jetting (Figure 3). The drug-loading efficiency as determined by HPLC was at least 68%. The HPLC analysis showed that the manufacturing process did not affect the drug's molecule, while the MTT assay on the FaDu carcinoma cells confirmed that the drug retained its pharmacological efficiency (Figure 4). *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 (Figure 5). Thus, ink-jet technology can be one of the methods of choice for the fabrication of monodispersed microspheres of good pharmacological properties. In contrast to the methods currently described in the literature, it switches the microsphere fabrication procedure from a thermodynamically governed mechanism to a mechano-electrical driven one, hence easier to control.

Nerve Conduits.

Films made of 1.5% w/v of the 80/20 copolymer of poly (D,L-lactic acid) and poly-ε-caprolactone dissolved in cellosolve acetate promoted both cell attachment and growing without interfering with the NGF expression capability of these cells (Figure 6). Moreover, the expressed NGF was fully bioactive when this material was used as cell substrate, as confirmed by PC12 cell culture (Figure 7). Thus, the 80/20 PLA/PCL copolymer was a good candidate for being used in conduit manufacturing. The resulting conduits are shown in Figure 8. The layer-by-layer printing process was automated using a repetitive combination of the

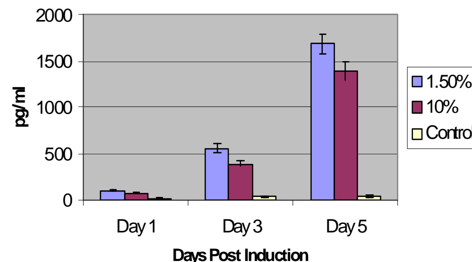

Fig. 7: NGF expression by hNGF-EcR-293 cells cultured on 1.5% w:v and 10% w:v 80/20 PLA/PCL copolymer. Control=cells cultured in the absence of the induction agent, PonA.

so-called "layer printing" subroutine and "cleaning" subroutine. The latter performs automatic cleaning the dispensing device after deposition of each layer, preventing possible clogging of the device due to particle contamination or polymer solidification.

Fig. 8: Nerve conduits manufactured by valve-jet technology.

Stent Coating.

Programmed target deliveries of 100 µg into spectrophotometric cuvettes gave a standard deviation of dose of 0.6 µg. Jetting on coated, uncut stent tubes exhibited 100% capture efficiency with a 1.8 µg standard deviation for a 137 µg dose.