Development of Novel and Standardized Cytotoxicity Tests for Additively Manufactured Biomedical Devices

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Abstract — **The popularity of additive manufacturing (AM) in biomedical engineering is steadily increasing as it offers a rapid fabrication route for patient-specific implants and other biomedical devices. While the use of AM for these applications is taking off, standardized cytotoxicity testing procedures for irregular, complex device shapes are not keeping up. This project aims to create a standardized toxicity test for complex AM implants. Using material extrusion, the team printed a carbon fiber polycarbonate knee implant as the test sample as it is made up of many curves, edges, and intricate details. The team experimentally determined the feasibility of methods for cell seeding and adhesion (direct contact) and identified a filter diffusion test (indirect contact) as the preliminary cytotoxicity test.**

Index Terms – Additive manufacturing (AM), material extrusion, cellular adhesion, cytotoxicity.

I. Introduction

Additive manufacturing (AM) is a processing technique in which an object is created using computer aided design, input into a machine as a standard tessellation language file, and built in layers. It is becoming more commonly used for biomedical implants due to its potential for patient specificity, rapid processing, and ability to create small, detailed structures.

Biocompatibility is the ability of a material/device to perform with a biological system. Biocompatibility testing determines the fitness of a material/device for human use, evaluating any potential harmful effects *in vivo* and *in vitro*. Within biocompatibility testing is cytotoxicity testing, which is performed *in vitro* to determine if the device causes cell malformation or cell lysis. Cell adhesion is important for biocompatibility testing because a cell's ability to adhere to a material affects signaling that regulates cell differentiation, the cell cycle, cell migration, and cell survival [1].

The problem associated with AM and current cytotoxicity testing standards (e.g., ISO 10993-12) is that current standardized tests require the sample to have a flat surface [2] and do not account for complex shapes. This constraint often limits testing on samples in their normal shape and may require samples with altered features.

The project goal was to design a rapid cytotoxicity test to evaluate the feasibility of standardized procedures for assessing AM samples. These samples will be designed with complex shapes and macro-level structural differences.

II. Design Process

The goal of the project is to overcome limitations of current standardized tests by testing irregular device shapes. The testing procedure(s) must meet the following design objectives: 1) cost effective, 2) time efficient (completed within 48 hours), 3) reproducible, 4) performed *in vitro*, and 5) appropriate for testing complex geometries.

The AM process chosen to fabricate the sample was material extrusion because it could process polymers, was accessible to the team, and would not leave behind toxic residue. Polycarbonate was chosen as the material due to its biocompatibility, accessibility, ability to be sterilized, and compatibility with material extrusion printing.

Two approaches were developed to determine appropriate procedure for seeding cells and assessing cell adhesion for subsequent cytotoxicity tests.

III. Methods

A. Sample Shape

The team initially considered a stent or part of a hip implant for complex shapes. A partial knee implant (Fig. 1) was chosen because the knee implant has more curves and edges compared to the other shapes.

Fig.1 Knee implant sample used in this project.

B. Drop Test

The drop test determines how well cells adhere to a sample under various conditions to establish a cell seeding method for direct contact tests. A 50-µL drop of NIH 3T3 mouse fibroblast cells (5000 cells) was placed on the surface of each sterilized sample in a 6 well plate. The positive control group was an empty well, and the negative control was a 17.5-µL drop of dimethyl-sulfoxide prior to the cell suspension drop. Samples were incubated for 4-6 hours to allow cells to attach but not divide, after which the samples were turned on

their sides and 1 mL of media was pipetted against the sample to dislodge any loose cells. A cell count was performed to determine the number of cells that were attached to the sample relative to initial seeding. Prior to cell studies, acellular tests with liquid were performed on a flat block at different elevated angles and on the knee implant sample.

B. Motion Test

The motion test also focused on cellular adhesion under dynamic conditions. Samples were sterilized as in the drop test and the same coating controls were used.

A volume of NIH 3T3 cells, totaling 100,000 cells, was added to twelve 100mm tissue culture plates containing samples. Plates were either stored on a platform rocker in the incubator (motion) or directly on a shelf in the incubator (stationary). After 24 hours, samples were removed and trypsinization protocol was followed to perform a cell count of the cells remaining in each well and determine the percentage of adherent cells relative to the initial seeded cells.

C. Filter Diffusion Test

This indirect contact method can be used to determine how well cytotoxicity can be measured from samples of complex shapes without a flat surface, per ISO 10993-12, and whether dynamic conditions affect the outcome. Samples would be sterilized. Dimethyl-sulfoxide would be used as the negative control group, compared to wells with no samples as positive controls.

For the test, NIH 3T3 cells would be grown to form a monolayer over a Millipore filter and placed cell-side-down on top of agar layers in two 6 well plates under dynamic (rocker) and stationary conditions. After 52 hours, the filters would be stained with trypan blue and imaged with a light microscope, then assessed using the reactivity grade table (TABLE 1) to determine cytotoxicity.

TABLE 1: REACTIVITY GRADE TABLE

Grade	Reactivity	Conditions
0	None	No malformed cells, 100% viability
1	Slight	Few malformed cells under specimen, 90% viability
2	Mild	Malformed cells limited to the zone under the specimen, 80% viability
3	Moderate	Malformed cells extend the zone up to 1cm, 70% viability
4	Severe	Zone of malformed cells extend past 1cm, 60% viability

IV. RESULTS

A. Drop Test

The drop test's positive control group, which was expected to have adhered cells, instead contained rounded cells. This reaction may be due to not having enough media in the well. This indicates that the drop test may not be a good method of determining cytotoxicity; however, more testing is necessary to confirm this.

B. Motion Test

The cell count from the trypsinized samples showed nearly zero cells. The plates were imaged, and cells were counted using ImageJ because of a limited supply of trypsin. The tables below (TABLE 2, TABLE 3, TABLE 4) show the cell count for each sample. The numbers in the table indicate the number of cells in the 563.2x422.4 µm image. The cell counts were abnormally high for the number of cells seeded.

TABLE 2: CELL COUNT FOR ALGINATE COATED SAMPLES (+)

	Dynamic	Static
Cell count, sample 1	227	310
Cell count, sample 2	52	249
Mean	139.5	279.5
Standard Deviation	123.7	43.1

TABLE 3: CELL COUNT FOR GELATIN COATED SAMPLES (-)

	Dynamic	Static
Cell count, sample 1	24	40
Cell count, sample 2	26	19
Mean	25	29.5
Standard Deviation	1.4	14.8

TABLE 4: CELL COUNT FOR EXPERIMENTAL SAMPLE

	Dynamic	Static
Cell count, sample 1	11	30
Cell count, sample 2	35	45
Mean	23	37.5
Standard Deviation	17.0	10.6

Two-tailed unpaired t tests were performed for each pair of dynamic and static samples. There was no difference in adhesion between the dynamic and static samples for all groups. Another t test was performed to test if there was a statistical difference between the alginate and gelatin coatings. There was no difference in adhesion between both coatings.

V. DISCUSSION

The drop test and motion test fulfilled Objectives 1, 2, and 4 as they are cost-effective, time-efficient, and performed *in vitro*. The drop test did not completely fulfill Objective 5 as it requires a relatively flat area on the sample. The motion test fulfilled Objective 5 and can test many devices of complex geometries. None of the tests fulfilled Objective 3. The tests are not known to be reproducible yet, as the drop test and motion test were only performed once, and the filter diffusion test was not performed. The filter diffusion test only met Objective 4 since the procedure is *in vitro*.

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