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

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Contents

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Abstract

Additive manufacturing (AM) in biomedical engineering is becoming more popular as it offers rapid fabrication for patient-specific implants and devices. Though the use of AM for these applications is increasing, standardized cytotoxicity testing procedures for complex device shapes is not keeping up. We planned a series of cellular adhesion tests and a cytotoxicity test and determined the feasibility of these methods with the use of a polycarbonate printed knee implant sample. The drop test promotes cellular interaction with the sample by placing a drop of cell suspension onto the surface, and the motion test is a direct contact test where the sample is stored in motion in complete medium containing cells. The filter diffusion test is an indirect contact test where the cells and samples are separated by a filter and stored in motion to measure cytotoxicity. Circumstances prevented us from completing replicates of the tests, but initial feasibility tests provided insight into future improvements that can be made to refine and validate our approach.

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Table 1: A list of frequently used acronyms throughout this paper.

Acronym	**Full term**
AM	Additive manufacturing
BJ	Binder Jetting
BME	Biomedical Engineering
CAD	Computer aided design
CFDA	China Food and Drug Administration
DED	Direct energy deposition
DMSO	Dimethyl sulfoxide
EBM	Electron beam manufacturing
ISO	International Organization for Standardization
KFDA	Korean Food and Drug Administration
LOM	Laminated object manufacturing
L-PBF	Laser powder bed fusion
ME	Material extrusion
MJ	Material jetting
PBF	Powder bed fusion
PCL	Polycaprolactone
PDLLA	Poly-d,l-lactide
PEU	Polyester urethane
PGA	Polyglycolide
PLA	Polylactide
PLLA	Poly-l-lactide
PU	Polyurethane
SBF	Simulated body fluid
SLA	Stereolithography
STL	Standard tessellation language

Chapter 1: Introduction

The modern accepted definition of biocompatibility is "the ability of a material to perform with an appropriate host response in a specific application" [1]. This means that for a material to be biocompatible, it must be functional in its intended use, trigger an appropriate response in the body, and can safely interact with the body with no risks of significant harm. Biocompatible materials are also known as biomaterials or biomedical materials. A bioresorbable, often called biodegradable, biomaterial is one that can be degraded by physiological processes without causing harm to a living system [2]. They must have a controlled degradation rate such that they do not degrade faster or slower than the body's healing process. Biomaterials, degradable or nondegradable, may be implants, drug delivery systems, imaging agents, or tissue scaffolds.

One manufacturing process used to fabricate biomaterials is additive manufacturing (AM). AM refers to the process in which parts are created using computer aided design (CAD) and input into a machine as a standard tessellation language (STL) file that is sliced into layers. These layers are deposited in a layer-by-layer process to fabricate the part. An example CAD model of a biomedical device can be seen in Figure 1. There are seven major AM process categories, which include vat photopolymerization, binder jetting, material extrusion, material jetting, powder bed fusion, direct energy deposition, and laminated object manufacturing. A variety of materials can be used depending on the technique, but polymers and metals are the most common materials used in these processes. The ability of these materials to perform an appropriate response regarding a biological system makes them biocompatible. Biocompatibility testing involves determining the fitness of the material/device for human use and evaluates any potential harmful effects.

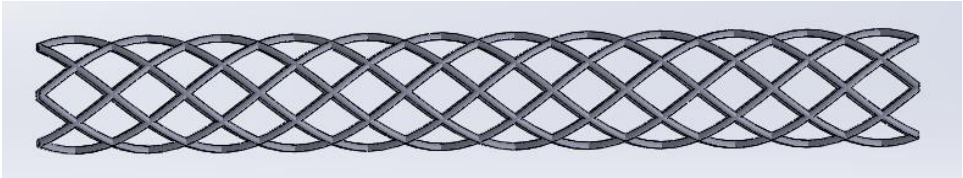


Figure 1: An example CAD model of a stent.

Because AM can produce small, complex-shaped devices, the technique is suitable for use in the medical field [4]. Testing new additively manufactured materials is important because a biological system can become nonfunctional due to systemic toxicity. However, current standardized cytotoxicity tests, a type of biocompatibility test that focuses on cellular properties, require a flat sample of the material, which does not match up with the unique, complex features of additively manufactured biomedical devices and implants. Thus, there are no standardized cytotoxicity tests that fully account for the non-standard shapes seen in many types of additively manufactured parts that might not already have a flat side. The level of detail that AM provides is very promising for the medical field, but without new standardized cytotoxicity tests, a device will have to be tested as a flat-surfaced sample, which might result in the loss of some of its original features.

There is a need for a standardized *in vitro* toxicity test for additively manufactured biomedical devices. The goal of this project was to design a rapid *in vitro* cytotoxicity test to

evaluate the feasibility of various toxicity testing procedures on additively manufactured biomedical devices with non-standard shapes. This involved identifying an appropriate AM process, selecting a material, designing a representative sample of a complex shape found in an existing biomedical device, developing tests for adhesion and cytotoxicity, and identifying appropriate control groups for adhesion and cytotoxicity tests. We were to design and validate a series of cytotoxicity and adhesion testing procedures to a) correlate cellular responses with macro level structural differences (e.g., shape) of additively manufactured biomedical devices and b) determine the effect of sample shape on the outcome of the procedures.

Chapter 2: Literature Review

AM can be used in a variety of different biomedical applications, including tissue engineering scaffolds, implants, and drug delivery devices. This section describes these applications, types of AM processes and biocompatibility testing, and biocompatible polymers and metals used in AM.

2.1 Additive Manufacturing in Biomedical Engineering

One of the challenges presented in biomedical engineering is patient specificity. Even though all patients are human with human anatomy, each patient has their own physiological variations. For example, a heart valve fabricated for one patient may not function well in another because of variation in cardiac structure. AM allows for patient-specific design of biomedical devices. The applications of AM biomedical devices for use inside the body include tissue engineering scaffolds, implants, and drug delivery devices [5].

Tissue engineering scaffolds require incredibly detailed structures to optimize cell growth and attachment as a wound heals. Tissue scaffolds must be biodegradable and degrade at a rate that corresponds with the healing process [5]. Cell shape, function, and differentiation are all affected by the chemistry, surface roughness, porosity, and stiffness of the scaffold. AM allows one to finely adjust the porosity and surface roughness of tissue scaffolds at the microscale, optimizing performance depending on where in the body the scaffold will be [5]. Because AM allows such detailed adjustments, it is a very favorable technique for designing tissue scaffolds.

Implants are surgically inserted into the body, either fully or partially, and help carry out a particular physiological function. Implants may be intended for permanent use (e.g., joint replacement) or for temporary use (e.g., bone fracture fixation). Before research was conducted on biodegradable materials, nonbiodegradable implants would have to be surgically removed after fulfilling their purpose. Biodegradable materials eliminate the need for this second surgery. Just like tissue engineering scaffolds, implants benefit from the level of detail AM can provide. AM implants can be designed specifically for the patient, so that the implant fits perfectly into place inside the body [5]. Control of porosity and other surface properties also alters tissue adhesion, which may or may not be desired, depending on the application [5]. Drug delivery systems are a challenging field of study. When one takes a pill, the liver metabolizes most of that pill. It is also difficult to design a system that will release a drug at an appropriate rate, which is why many prescription medications are designed to be taken daily [5]. Although hydrogels are a major focus of drug delivery research, AM also has potential [5]. A drug can be enclosed inside an AM structure, which is designed specifically to release the drug at a certain rate. The structure also acts as a barrier between the drug and the liver, leading to less of the drug being prematurely metabolized [5].

2.2 Additive Manufacturing Processes

AM is the joining of material, typically layer by layer, to create an object out of a variety of materials for a variety of purposes. Table 2 below shows an overview of the seven different AM

processes and the materials that they can utilize to fabricate objects. Below is a more detailed explanation of the different techniques [3].

Table 2: Summary of different AM processes.

Process	Brief Description	Materials
Vat Photopolymerization	Photosensitive polymer is selectively polymerized with light	Polymer
Binder Jetting	Powder is bonded with liquid agent then cured	Metal/polymer/ceramics
Material Extrusion	Material is pushed through nozzle selectively	Polymer
Material Jetting	Droplets of a build material are deposited selectively	Polymer
Powder Bed Fusion	Powder is selectively fused by using an energy source	Metal/polymer
Directed Energy Deposition	Materials are fused as they are deposited using a nozzle or feeder	Metal
Laminated Object Manufacturing	Sheets of a material are joined together	Certain materials able to be processed into sheet form (ceramic, metals, polymers)

2.2.1 Vat Photopolymerization

Vat photopolymerization refers to the process in which a vat of photopolymer is selectively cured by a UV light to create a solid design. Because it is a liquid-based process, support structures are often needed. A blade is used to create a smooth layer of resin. Post processing is required to remove wet resin and to fully cure the polymer. One downside is that oftentimes, when being cured, the material shrinks, putting strain on previous layers. For this reason, some companies have made specific scanning patterns to avoid this issue [3].

Vat photopolymerization can create devices with a fine resolution and with high accuracy. It can also create devices out of multiple materials by draining out the vat and replacing the resin inside with the next material. The leftover resin is also able to be reused for future purposes, reducing waste. However, this process is more expensive than other techniques, such as material extrusion and laminated object manufacturing, in terms of obtaining both the material and the machine [3].

2.2.2 Binder Jetting

Binder jetting (BJ) involves spreading a metal, polymer, or ceramic powder and using a liquid binder to join the powders together. In this way, the object is built up layer by layer. It is then placed in a furnace to join the metals or ceramics together. Binder jetting requires much post processing in order to obtain a finished object. Devices made with BJ are not suitable for all applications, as the objects created can be porous due to post processing. However, it does not

need support structures like many other processes do. It also does not need fine powders, which can lower the overall cost of the process [6].

2.2.3 Material Extrusion

Material extrusion (ME) is a technique used for polymers. The process involves depositing a molten polymer in a specified area. The price of a machine varies depending on the size and properties of the machine, and the temperature needed varies depending on the material being used. The surface roughness of the final product depends on the number of layers and the height of each layer. Post processing is sometimes used to create a more polished surface finish. ME is also capable of creating parts of varied materials in cases where the device is equipped with more than one extruder. The accuracy depends on the size of the object being created. The bigger the object, the more accurate it is. Depending on the object shape, support layers may be needed [3].

2.2.4 Material Jetting

Material jetting (MJ) involves a photosensitive polymeric material being deposited in drops onto a platform. This technique has accurate positioning, little waste, and small droplet sizes. After being dropped, the material is cured with a UV light. Like material extrusion, it is possible to create devices with varied materials in one part. Also, like material extrusion, support layers are needed for overhangs. Material jetting can create fine resolution parts. As a result, little, if any, postprocessing is needed [3].

2.2.5 Powder Bed Fusion

Powder bed fusion (PBF) involves selectively melting a layer of powder with a laser or electron beam, after which a new layer of powder is added. The powder is polymer, ceramic, or metallic materials. The most common types of PBF are laser powder bed fusion (L-PBF), including selective laser melting (L-SLM) and selective laser sintering (L-SLS), and electron beam powder bed fusion (E-PBF). Support structures are required for overhanging structures when fabricating parts using PBF. Post processing is often used to create parts with finer surface roughness. PBF is able to replicate complex structure found in biological systems, making it a promising technology in the biomedical field. Powder can also be recycled, allowing for less waste [3].

2.2.6 Direct Energy Deposition

Direct energy deposition (DED) involves heating a wire or powder composed of metal and depositing the melted material in specified positions. The material solidifies almost immediately after it is deposited. It has the downside of having a low resolution on the vertical axis when compared to other AM processes [7].

2.2.7 Laminated object manufacturing

Laminated object manufacturing (LOM) involves material which is in thin sheet form being placed, cut, and attached to the other layers through pressure and heat application. Paper, metal, ceramics, and polymers can all be used if the material is able to be in sheet form. This process is generally fast, depending on the size of the shape, and can create large parts, as well as not needing support structures. However, it is unable to create objects that are hollow, have fine resolution, or have complex geometries [3].

2.3 Classification of Biocompatibility Tests

Biocompatibility tests are done to determine how well a device or material can perform with a host to ensure that there are no potentially harmful physiological effects. ISO 10993-1: 2018 has a section dedicated to the different biological testing methods. When testing, it should be done on the final medical device, or samples/materials processed in the same manner as the final medical device, which includes sterilization. ISO states that the following shall be considered:

- The intended use exposure to humans in means of nature, degree, duration, and conditions of exposure.
- The chemical and physical properties of the final medical device, as well as the topological activity of chemicals.
- Some biological tests are not justifiable where leachable materials have been excluded, or where chemicals have a known toxicity profile that is acceptable.
- Ratio of the device to recipient body size (ex. device miniaturization).
- Existing information stated in literature.

ISO also states that positive and negative controls shall be used where appropriate and that the test should be reproducible, repeatable, and robust [9].

2.3.1 In Vitro Methods

Cytotoxicity tests, the type of test which this project focuses on, are *in vitro* and use cell culture techniques to determine cell lysis, the inhibition of cell growth, and other effects on cells that are caused by the medical device, material, or extract. ISO 10993-5 goes into detail of how to conduct cytotoxicity tests [10]. The three different categories of cytotoxicity tests are direct contact tests, indirect contact tests, and extract tests, which are discussed later in Section 2.3.3. Other *in vitro* biocompatibility tests include hemocompatibility tests, material-mediated pyrogenicity tests, genotoxicity tests, and degradation tests.

Hemocompatibility tests, mainly *in vitro* but utilize further testing by *in vivo*, evaluate the effects of blood-contacting medical devices, materials, or their extracts, on blood/blood components. They can also be designed to simulate the geometry, contact conditions, and flow dynamic to determine blood to material to device interaction [9]. This kind of method is important

for devices that come into contact with blood to ensure that there are no toxicities that travel throughout the body. Material-mediated pyrogenicity tests are *in vitro* and are used to detect material-mediated pyrogenic reactions of the medical device, material, or extractions. No single test can differentiate the difference between reactions that are mediated versus those that are due to endotoxin contamination. This reaction is rare and has been observed in devices containing biologically derived materials [9]. Genotoxicity tests are used to assess potential of gene mutations, changes in chromosome structure and/or number, and any other gene toxicities caused by the medical device, material, or extract. A battery of *in vitro* tests is used initially. If any of the *in vitro* tests are positive, a follow up can include the reason for the positive result. A risk assessment is used to determine the genotoxic risks [9]. Both of the two testing procedures ensure that the material/device is suitable for human use.

Degradation information must be presented for any medical device, material, or extract that have potential for degradation in the human body. Tests shall be done if the material is absorbable or if the composition of the device indicates that toxic products might be released upon contact with the human body. *In vitro* tests shall be used to determine the rates of degradation and the release of any toxins. *In vivo* tests are used to assess degradation of a material [9]. Degradation can provide useful information for how the material/device will behave in contact with the human body.

2.3.2 In Vivo Methods

Sensitization tests, *in vivo*, are used to determine if medical devices, materials, or their extracts cause contact sensitization. These tests are important in determining allergic reactions from any harmful leachable, which is defined as any product that migrates out of the system over time [9]. Irritation tests, usually *in vivo*, estimate potential irritation of medical devices, materials, or the extracts. These tests can be used to assess the localized reaction of the tissue. This method may be used when dermal or mucosal tests are inappropriate, or when extractables are hydrophobic [9]. Irritation testing is also important at determining any allergic reactions.

Acute systemic toxicity tests, *in vivo*, are used to estimate potential harmful effects of single or multiple exposure of the medical device, material, or extract in an animal model. The period of exposure time is less than 24 hours. ISO states that if feasible, this test shall be combined with sub-acute and sub-chronic toxicity and implantation test protocols [9]. Sub-acute and sub-chronic toxicity, also *in vivo*, test the effects of single or multiple exposures of the medical device, material, or extract. The period is not less than 24 hours and not greater than 10% of the life span of the test animal, which in rats is up to 13 weeks. These tests shall be waived if there is sufficient data to show the effects without the need of the test. It is required to state the reason for waiving the test, and if possible, include implantation test protocols to evaluate systemic and local effects [9]. These tests are important in determining if there are any negative reactions *in vivo* for an extended period. Chronic toxicity tests are used to determine effects of single or multiple exposures of the medical device, material, or extract over a period of the life span of the testing animal, up to six months in rats. These tests are appropriate regarding route and duration of the contact of the device/material. If possible, this method should include implantation test protocols to evaluate

systemic and local effects [9]. This method is similar to the ones above, though it is for a much longer period of time. Implantation tests assess the local pathological, micro and macroscopical, effects of a sample material or final product that is surgically implanted in a tissue or implant site that is appropriate to the application. It is possible for implantation tests to evaluate both local and systemic effects to meet acute, sub-acute, sub-chronic, and chronic toxicity testing [9].

Carcinogenicity is addressed with a risk assessment including weight of evidence and mode of action information if available. Information should be appropriate for the route and duration of exposure to the toxicity. If carcinogenicity testing is needed, then lifetime studies or transgenic models may be required [9]. This method will address any carcinogenic effects of the material/device, and if they are appropriate. Reproductive and developmental tests are used to evaluate potential effects of the medical device, material, or extraction on reproductive function, embryonic development, and prenatal and early postnatal development. A risk assessment addresses the effects. This test shall only be done if the medical device has a potential impact on the reproductive potential on the patient, or if materials or devices are used during pregnancy [9]. If the material/device has impacts on the animal, it is not suitable for use. Immunotoxicology testing shall be considered based on the chemical composition of the materials of manufacturing data suggesting possible immuno-toxicological effects. Currently the only method is through *in vivo* studies [9].

Toxicokinetic studies are done to evaluate the absorption, distribution, metabolism, and excretion of a chemical. *In vivo* toxicokinetic studies shall be considered in the light of results from *in vitro* degradation studies. These studies shall be considered if the medical device is absorbable, a long-term implant with likely corrosion or degradation, likely to release a leachable or any toxins into the body, susceptible to substantial quantities of released nano-objects, or for any food and drug combination products [9].

2.3.3 Cytotoxicity Tests

In vitro cytotoxicity tests are used to observe cell growth, cell reproduction, and structural effects of biomedical devices. Tests have continuously been developed and from that, methods have changed from qualitative evaluation to quantitative evaluation [10]. Three *in vitro* cytotoxicity tests are listed in ISO 10993-5: 2009: Extract tests, direct contact tests, and indirect contact tests. Table 3 shows a comparison of each test in means of qualitative vs quantitative, candidate materials, duration of test procedure, and the cytotoxic measurement. To determine which test is used, the nature of the sample, the potential site of use, and the nature of the use of the device are evaluated. The choice of test determines "the details of the preparation of the samples to be tested, the preparation of the cultured cells, and the way in which the cells are exposed to the samples or their extracts [10]." Once exposure time reaches the end, the cytotoxic effect is evaluated. The categories of this evaluation are assessments of cell damage by morphological means, measurements of cell damage, measurements of cell growth, and measurements of aspects of cellular metabolism. ISO 10993-5 states that the investigator should be aware of the test categories, and where to fit techniques. Examples of quantitative tests are given in Annex A-D of ISO 10993-5 [10].

Table 3: Comparison of the three categories of current cytotoxic testing methods [8,9].

Name of Test	Qual./ Quant.	Candidate Material	Duration	Toxicity Measurement
Direct Contact Test	Quantitative and Qualitative	Materials that are not dense enough to damage cells	Up to 72 hours	• Malformation • Degradation • Lysis • Reactivity grade (0-4)
Extract Test	Quantitative and Qualitative	Soluble substances	72 hours	• Measures cell metabolism by absorbance
Indirect Contact Test	Qualitative	Materials that may cause damage to cells without a cushion layer	Up to 72 hours	• Malformation • Degradation • Lysis • Reactivity grade (0-4)

Cytotoxic effects must be determined by either qualitative or quantitative means. Qualitative evaluation requires the investigator to examine the cells microscopically with a cytochemical staining (if desired). Cell lysis, membrane integrity, vacuolization, detachment, and general morphology of the cells are assessed. Morphology can be described numerically or descriptively. In this case, morphology is defined as rounded, shrunken, unattached cells. Morphology is an important indication of toxicity, and these general shapes can be applied to other kinds of cells.

Quantitative evaluation measures cell death, cell growth, cell proliferation, or colony formation. This is the type of testing preferred by ISO. Reduction of cell viability of at least 30% is a cytotoxic effect [10]. An example of a procedure that measures these preferred qualities is an extract test, such as MTT assay. That method is an indirect procedure that measures cellular metabolism through exposure to the material via solution and examined by measuring absorbance when the cells are exposed to a chemical, diphenyl tetrazolium bromide.

For all cytotoxic testing methods, a positive control and negative control shall be included. Positive control will yield a known cytotoxic response, and negative control will yield a known non-cytotoxic response. A minimum of three replicates shall be used for test controls and test samples in each case of testing [10]. The direct contact, indirect contact, and extract tests are all rapid and simple [11]. Rapid is defined as within 72 hours, since these are standard procedures that take up to 72 hours from start to completion. Simple is defined as being relatively few and/or straight forward steps from start to completion.

Extract tests are most suitable for determining the toxicity of soluble substances leached from medical devices and remains consistent with results from animals' toxicity tests [10]. Extract tests allow for both qualitative and quantitative assessment of cytotoxicity. In this method, testing involves extracting the medical device or material into cell culture medium and exposing that fluid to cells [10]. Cytotoxicity is measured in accordance with the tables in ISO 10993-5, and cellular metabolism is measured by any quantifiable method. The major advantage of extract tests is that the results are not up to analysist interpretation and there is a throughput of multiple screenings at

once [11]. The disadvantages of this method are that the cause of cell death is unknown, and it may only show the end stages of death [11].

Direct contact tests are qualitative and quantitative. In this method, cells are cultured, and a medium solution is aliquoted onto the cells and a sample of the material is laid over the top to cover approximately one tenth of the cells' surface [10]. Chemicals and/or dyes are then added to determine the cytotoxic effects in accordance with the tables in ISO 10993-5. Advantages of direct contact tests are that they are sensitive, rapid, and simple procedures. The main disadvantage of this method is that lighter weight materials might float on top of the culture medium and not completely represent the true toxicity [11]. An example of a sample that would float would be when testing a lightweight sample of latex.

Indirect contact tests, which are only qualitative, include both agar diffusion and filter diffusion tests. Agar diffusion tests involves putting a culture medium with melted agar over the cells. A sample of the device or material is placed on top the agar medium and cytotoxic effects are determined by using a stain and observing the cell growth, death, proliferation, and other morphological effects [10]. When doing filter diffusion, a surfactant free filter with a pore size of 0.45 μm is placed in a vessel, followed by cultured cells. This is incubated and is then placed on a solidified layer of agar with the cells facing the agar. The material sample is then placed on top of the filter. A stain is used to determine the cytotoxic effects [10]. These methods are also rapid and simple procedures. The disadvantage of this method is that the leachable may not diffuse through the layer between the cells and the material being tested, or the leachable has the possibility of reacting with the layer between [11].

2.4 Biocompatible Materials in Additive Manufacturing

Polymers, also known as plastics, are composed of molecular chains connected to each other via secondary or covalent bonding. They are typically soft, have a low elastic modulus, and resemble biological tissues more so than metals and ceramics [5]. Metals have higher stiffness and higher strength than polymers, making them the material of choice for a variety of applications, such as bone implants, artificial joints, and other load bearing applications [12].

2.4.1 Biodegradable Polymers

Most biocompatible polymers used in AM are biodegradable polymers. Polymers derived from nature (e.g., cellulose and chitosan) are degradable by physiological processes, making them an appropriate choice of biodegradable materials [5]. Natural polymers can interact with cells by signaling, promoting cell attachment, and changing their structure in response to cells. However, it is difficult to control the shape and porosity of these polymers. What this means in terms of AM is that the dimensions of the final product will not be very accurate. Synthetic polymers are more controllable than natural polymers. By changing how they are processed, properties such as physical, chemical, mechanical, and biodegradation properties can be changed [5]. They are a more suitable candidate for AM than natural polymers.

Polylactide (PLA), polyglycolide (PGA), polycaprolactone (PCL), polyester urethanes (PEUs), polypropylene fumarate, and polycarbonate are all biocompatible polymers that can be

used in AM. PLA, PGA, PCL, PEUs, and polypropylene fumarate are all polyesters. Characteristics that all polyesters share is that they are biodegradable and degrade by bulk degradation via hydrolysis [5], which is the severing of the carbon-oxygen single bond in the ester group. The downside to this type of degradation is that it leads to acidic byproducts in the body. Despite this, various polyesters are used for their range of desirable properties. A list of these polymers along with their degradation rates, AM techniques, and biomedical applications can be seen in Table 4.

PLA is available as poly-l-lactide (PLLA), an optical isomer of PLA, and poly-d,l-lactide (PDLLA), a racemic mixture of the (d) and (l) PLA isomers [5]. Poly-d-lactide (PDLA) cannot be isolated, but the amount of PDLA relative to PLLA can be controlled. When a sample of PLA is more than 90% (l) isomer, the material is semicrystalline. The polymer becomes amorphous when the (l) isomer makes up less than 80% of the polymer. The glass transition temperature tends to decrease as the amount of (d) isomer increases, with the Tg of 100% PLLA ranging from 55 to 80°C. PLLA can be manufactured by material extrusion and laser powder bed fusion, and PDLLA can be manufactured by material extrusion [5]. One thing that makes PLA desirable is that it is a renewable material. It is made from lactic acid and degrades back into lactic acid. Because PLLA is more crystalline, and therefore more tightly packed together than PDLLA, it takes longer to degrade than PDLLA [5].

Other commonly used biodegradable polymers are PGA and PCL. PGA has a higher degradation rate than the PLAs despite being more crystalline than PLLA. This is due to PGA's hydrophilic nature, leading to hydrolysis occurring more rapidly than in the more hydrophobic PLAs [5]. It has good tensile strength, but it degrades too quickly for load-bearing applications. To improve the degradation properties of PGA, it can be copolymerized with PLA. PCL is the most widely used polymer in the world of biomedical AM [5]. It has a very low glass transition temperature, making it soft in the body. Advantages of PCL include easy and inexpensive production, controllable degradation, ability to blend with other polymers, and solubility in many solvents. PCL can be manufactured by material extrusion, laser powder bed fusion, and SE-AM, though the latter is a newer technique for PCL [5].

Polyurethanes are more advanced biomedical polymers in terms of structure and application. Their mechanical properties can be easily controlled by changing their structure with functional groups and changing AM processes [5]. For example, polyurethanes can be made biodegradable by adding a biodegradable group, such as ester, to the polymer backbone. Polyester urethanes (PEUs) have good tensile strength, wear resistance, and elasticity. They show a good resistance to hydrolysis, though like all polyesters they will eventually degrade via this mechanism. Short-term applications of PEUs are desired over long-term because if they are in the body for too long, they tend to be attacked by the immune system, crack at high-stress areas, and lose their mechanical properties [13]. Most AM techniques do not work well with biodegradable polyurethanes. SLA leaves behind unremovable toxins, and material extrusion and laser powder bed fusion require high temperatures that degrade them, leading to a loss of tensile strength and release of acidic byproducts. To make polyurethanes more suitable for AM, researchers have mixed polyurethane with other substances as well as changed the AM techniques [14].

Polypropylene fumarate contains a double bond in its mer structure, which makes the polymer easy to covalently crosslink. Its main application is in the restoration of hard tissues such

as bone, and it can be processed by photoreactive AM. However, polypropylene fumarate is difficult to polymerize, which is hindering its progression to the market [5].

Polycarbonate is a polymer that has a high melting point temperature of around 265°C. It also has high impact strength and is resistant to water. However, it will break down slowly with base materials [13]. Polycarbonates are also shown to have some rate of biodegradability [15].

Table 4: Biodegradable polymers and their degradation times, AM processes, and biomedical applications.

Polymer	Degradation Time	AM Processes	Biomedical Applications
Poly-l-lactide	2-5 years	ME L-PBF [5]	Sutures Suture reinforcements Suture anchors Tissue Regeneration Vascular Stents [5]
Poly-d,l-lactide	2-16 months	ME [5]	Drug delivery [5]
Polyglycolide	6 weeks [5]	No data available	Filler for tissue regeneration [5]
Polylactide-co-glycolide	Varies	Material extrusion [5]	Drug delivery (electrospun fibers) Tissue engineering (scaffolds) Injectable treatments Absorbable sutures Bioresorbable bone fracture fixation [5]
Polycaprolactone	Over 2 years	ME L-PBF CAWS SE-AM [5]	Soft and hard tissue scaffolds Wound healing Drug delivery Tissue engineering films [16]
Polyester urethanes	Varies	ME SE-AM bioprinting inkjet printing [5] [13]	Tissue engineering Drug delivery [13]
Polypropylene fumarate	Months	SLA SE-AM [5]	Hard tissue repair [5]
Polycarbonate	Unknown	Material Extrusion L-PBF [12]	Disposal devices; PCR; [1]

2.4.2 Biodegradable Metals

A biodegradable metal is a metal that is expected to corrode *in vivo* and later be absorbed or metabolized by bodily tissues. Currently, research has been done on three metals and their ability to be used as biodegradable implants: magnesium, zinc, iron, and their alloys [17]. Biodegradable metals would make a significant difference in orthopedic bone grafting, which

currently uses non-degradable metals that eventually need to be taken out, particularly for younger patients whose bones are still growing [17].

Magnesium is prevalent in bone material. It is a useful metal due to its good mechanical properties that closely mimic those of bones as a bulk metal. Magnesium ions have also been shown to promote bone regeneration. However, the degradation rate of magnesium alloys is too rapid for many clinical uses. Alloying magnesium with various metals can help slow the rate of degradation, though limited metals can be used due to biocompatibility requirements. Magnesium is the only biodegradable metal that has been clinically approved for use, including pure magnesium and two alloys [18].

Pure magnesium screws have been clinically approved for use by the China Food and Drug Administration (CFDA), and applications are being designed for the fractures in the upper leg and foot [17]. MgYREZr, which contains magnesium, yttrium, zirconium, and a rare earth metal, has been approved for use in Germany for the healing of mild foot deformity [17]. A MgCaZn alloy screw has been approved by the Korean Food and Drug Administration (KFDA) for the treatment of hand fractures. The alloy was approved in 2015, and more implants are being designed with this material [17].

Zinc is the newest biodegradable metal to be studied. It is the second most abundant transition metal in the human body. One of the strengths of zinc is that it has a slower degradation rate than magnesium, which degrades too quickly, but degrades faster than iron, which degrades too slowly. Of the three categories of metals, zinc shows the most promise for a degradation rate that is ideal for orthopedic uses. However, zinc is unable to bear high loads due to a low tensile strength, which requires it to be alloyed with other metals, such as Ag and Al, when used for certain applications [18].

Of the three metals, iron is the most easily manufactured metal and has the best mechanical properties. However, iron degrades slowly in the human body, and as such is unsuitable for many orthopedic applications. Attempts have been made to alloy iron with other biocompatible metals, but so far, the resulting alloys are not able to degrade as quickly as is needed. However, by increasing porosity, the degradation rate can be increased [1].

2.4.3 Biodegradable Metals in Additive Manufacturing

Currently, traditional manufacturing methods are being used to fashion biodegradable metal implants. However, traditional methods cannot control the pore size and geometry, which is often customized for each patient and needed for proper cell ingrowth. For this reason, AM is needed to be able to use biodegradable metals as orthopedic scaffolds. One major difficulty with AM of biodegradable metals is that tests are primarily done on bulk metals. There is little research done with AM biodegradable metals [17].

PBF is one of the most common methods used for orthopedics due to its ability to create fine, customized geometries with acceptable mechanical properties. Most porous scaffolds have been made using nondegradable metals, such as titanium and stainless steel. Powders used as a raw material in PBF processes are primarily made by two types of processes: water atomization and gas atomization. Water atomization creates powders that are often non-spherical and not uniform, as well as containing density defects, while gas atomization creates powders that are

spherical and uniform. For this reason, gas atomization powders tend to be the powders of choice for PBF [17].

One difficulty with AM of magnesium is its high combustibility under high heat and its tendency to form magnesium oxide in oxidated environments. Due to the small distance between its melting point and vaporization point, the laser power and laser speed drastically affect the porosity of the final product. At low and high temperatures, the magnesium evaporates and causes a lower density of the final product. However, under certain L-PBF conditions, magnesium can achieve a densification of 99.5% [18]. Due to the small powder size, the high surface energy of magnesium causes rapid oxidation. Because of this, magnesium is often printed in inert environments to avoid oxidation. Another common solution is alloying magnesium with different elements to reduce the oxidation rate. The most common elements include zinc, calcium, and manganese. However, zinc causes solidification cracks if the quantity of zinc is too high (>1%wt) [19]. Like magnesium, zinc tends to evaporate during the melting process in power bed fusion-based manufacturing techniques. For both metals a gas circulation system can be used when using powder bed fusion. This both prevents oxidation from occurring and blow away the evaporation fumes of the metal, allowing for a more densified part [18]. Iron is the easiest to process with AM, as it is similar to stainless steel, which has been used extensively in AM. Iron has a high evaporation point compared to its melting point, which prevents evaporation from occurring during the process. After optimization of porosity in iron-based objects, the strength of the object approached that of bone [17].

Other forms of AM that use metallic materials include BJ and DED. However, there is limited use of these technologies in the field. BJ can be used for the creation of degradable iron implants. Problems with porosity, as well as other issues, prevent it from being widely used in the field [17].

Chapter 3. Project Strategy

This section outlines the client statement, objectives, and functions, as well as describing ISO standards.

3.1 Initial and Revised Client Statement

The initial client statement of this project is as stated:

"Design and validate a rapid biocompatibility/toxicity test to correlate cellular responses with different micro-and/or macro level structural differences in additively manufactured metal samples used for biomedical applications."

Initially, the materials of focus were to be biodegradable metals and plastics. We had decided to include plastics in the hopes of creating a toxicity test applicable to all kinds of AM materials. After conducting research and analyzing the techniques available to us, it became clear that we would not be able to manufacture biodegradable metals. Because we wanted to use a biodegradable material, this left us with the remaining option, plastics. We also eliminated micro-level structural differences from the client statement, instead deciding to focus on the macroscale and device shape. ISO 10993-12 states that cytotoxicity tests require a flat surface [20], but a flat, block-shaped sample is not representative of the device *in vivo*. This led us to research the effect surface topography and device shape may have on the cytotoxicity of the device, but we did not find any research exploring the possibility. Since we did not find any research on cytotoxicity related to shape and topography, we incorporated the device shape into our testing.

The revised client statement is as follows:

"Design and validate a series of cytotoxicity and adherence testing procedures to correlate cellular responses with macro level structural differences of additively manufactured samples used for biomedical applications and determine the effect of sample shape on the outcome of the procedures."

3.2 Objectives and Constraints

The objectives for the cytotoxicity testing procedure were that it must be standardized, *in vitro*, accurate, rapid, and cost efficient. Standardization is a term that is used across cytotoxicity testing. This ensures reproducibility, performance, and consistency. Our test must be standardized for multiple samples to be tested at once and for a variety of AM biomaterials to be tested with the same technicalities. The test must also be able to be repeated to obtain the same or comparable results. *In vitro* studies use cells and biological molecules to study behavior outside of the normal physiological context. *In vitro* is often referred to "in test tube" experiments, since these studies and experiments are performed in labs with materials such as culture dishes, centrifuge tubes, and

other such materials. Accuracy is the degree to which a measurement is correct, meaning the results from the cytotoxicity test must be qualitatively and quantitatively accurate. Though one could argue this would fit into the category of standardized, we felt as if it was its own aspect that needed to be separate. The importance of accuracy comes from the limitations of current methods where the results are often determined by analyst interpretation. We defined rapid as being within 48 hours. This time frame was developed based on the current standards from ISO 10993-5, with all the current cytotoxicity methods being up to 48 hours.

Our constraints involved cell survival, specific adhesion testing methods, and sample dimensions. For the sample to be considered non-cytotoxic, 80% of cells must survive. Too many cells dying means that *in vivo*, the device will likely have a harmful effect on the body, making it unsuitable for use. We developed a Reactivity Grade Table (see Appendix A.8) to determine cytotoxicity; grade 2 and lower is considered not cytotoxic. For the adhesion testing methods, the cells must be able to maintain contact with the sample for a sustained amount of time to maximize adhesion. Procedures should be handled properly to ensure sterility is maintained to ensure there is no cross-contamination between samples and plates, and contamination of the entire incubator. Lastly, the samples must fit in the culture plates they are being tested in. The dimensions must be smaller than the height and diameter of the plate. If they are not, then the cover cannot be placed on the plate and the medium will become contaminated as soon as it is removed from the biosafety hood.

3.3 Functions and Means

The main functions of the procedures developed were to measure cytotoxicity using direct or indirect methods and to accommodate complex shapes. To directly measure cytotoxicity, we performed adhesion tests under various conditions. First, we performed a test in which we placed a drop of cell suspension directly on top of a flat surface on the sample. This type of interaction ensures that cells interact directly with the sample rather than migrating to another part of the plate. We also tested adhesion on static and dynamic samples by placing them directly in cell culture medium. We hypothesized that dynamic samples, that is, samples in constant motion, would allow for more cell-material interactions as the sample and the cells may bump into each other during the movement.

To indirectly measure cytotoxicity, we developed a filter diffusion test where the cells would grow on one side of the filter while the sample sits on the other side. The cells would interact with the material by reacting to any potential leachable that comes out of the material. This test would also be performed statically and dynamically, as we hypothesize that the motion may promote leaching of the sample. Cytotoxicity would be determined via cell staining. Using Trypan Blue, the number of dead cells relative to living cells can be assessed. The morphology of cells can also be observed using phalloidin staining with fluorescent microscopy to visualize F-actin [21] or using scanning electron microscopy on unstained samples to assess cell shape as an indicator of overall cell health. We developed a reactivity grade table, found in Appendix A.8, to reference to when determining the cytotoxicity of the sample. The table was designed using previous knowledge from Biomaterials Lab and Cellular Engineering Lab taken at WPI by two of the team members, in combination with Table 2 in ISO 10993-5 [10].

Our tests must accommodate complex shapes, as we wanted to account for the possibility of device shape influencing cytotoxicity. To do this, we had to choose an appropriate, representative implant model that had curves, edges, and flat surfaces. SolidWorks was used to design various non-flat samples, and we chose to use a knee implant as it contained the desired features. Polycarbonate was chosen as the material, and it was fabricated using a ME printer as it could provide the details necessary to print the sample. The sample had to be printed small enough to fit into the well plates for the testing procedures, but also not too small where the printing process would cause any distortions to the samples where they print out with different dimensions than the CAD model.

3.4 Management Approach

Each term we generated a Gantt chart to keep track of the work and deadlines related to hands-on project work and report preparation. The specific Gantt charts can be found in Appendix A.1. It is important to note that the deadlines were not all accurate, as the team had some changes in due dates that did not get updated on the respective Gantt charts. We divided up the work based on the area of expertise required for the specific task. Interim project reports were required at the end of each term leading to the final report at the end of the academic year. We managed meeting agendas, meeting minutes, and other materials related to this project via Microsoft Teams. We recorded all laboratory procedures and findings in a laboratory notebook and uploaded a scanned PDF version to Microsoft Teams for all team members and advisors to access.

3.5 Standards

Protocols set by the FDA and ISO were used in this project. FDA standard 21 CFR 58 outlines procedures for good nonclinical laboratory practices. Any testing of any samples followed this standard. We followed Subpart B: Organization and Personnel; all the procedures were performed in the lab, and we had performed similar procedures before, meeting the standard of having to be knowledgeable of the protocols. Also, we wore proper PPE, including a lab coat, lab safety goggles, proper lab attire, and gloves when performing experiments. We also met the standard of not going into the lab if sick. Subpart G: Protocol for and Conduct of a Nonclinical Laboratory Study, was met since this project did not involve any clinical trials and respective procedures for non-clinical studies were followed. Subpart J: Records and Reports, was followed by recording all laboratory experiments and information in a laboratory notebook, properly documented with titles, dates, and times.

International Organization for Standardization is an organization that publishes industrial and commercial standards. ISO standard 128 outlines procedures for the types of lines in mechanical drawings. These standards were followed when creating drawings of the geometries to ensure that there were clear, legible dimensions that were easy to follow and understandable so that others can make the same parts.

ISO standard 10993 outlines procedures for *in vitro* cytotoxicity testing. Information from this standard was used to evaluate current ISO standard 20391-1:2018: outlines procedures for cell counting. A cell count done prior to and after exposure to the materials was a way to determine

the effect of the materials. It was not a direct measure of cytotoxicity, but it provided some insight as how the cells reacted when they were exposed to the material. This is part of the proposed procedures cytotoxicity testing methods. The information that was utilized can be found in 2.2.1. ISO 10993-12 outlines preparing samples for testing methods. This information was used to determine the size and shape of the specimens that were used for testing. This standard goes into detail for the ratio of the specimen compared to surface area of the testing well, and specific size of testing sample for the methods was determined by this ratio. This information was also used to develop a method to test the geometrical shape of specimens to determine if there are gaps in the current standard. This ISO standard states that the testing specimens must have a flat side to ensure proper contact with the cells.

ISO standard 17665-1:2006: outlines procedures for autoclaving parts. Sterilization is an important part of biocompatibility. Autoclaving was a sterilization process for any materials used in the lab that could withstand 121°C, such as forceps and Pasteur pipettes that did not come sterilized already. ISO standard 24998:2008: outlines use of petri dishes to ensure sterility of the dishes and medium used for cell culture in our procedures. ISO 11737-2:2009: outlines sterilization of medical devices, which was taken into consideration when sterilizing the materials. These standards were used for this study when setting up the cell culture plates to ensure sterility throughout the process.

Chapter 4. Design Process

This chapter discusses aspects of the design process, such as needs analysis and proposed solutions to these needs. We will discuss the needs of the clients in detail, as well as the feasibility of fulfilling these needs. This section also outlines the various feasibility, adhesion, and cytotoxicity tests the team designed and the results of the tests that were performed.

As previously discussed, there is a current need for standardized *in vitro* cytotoxicity tests of additively manufactured biomedical materials. Because AM processing is a fast manufacturing process compared to other methods, the testing procedures should be rapid as well. The procedure should also account for a variety of geometries, such as complex designs that cannot be accommodated by traditional laboratory materials and procedures. The main goal of this project was to create a standardized *in vitro* cytotoxicity test for additively manufactured biomedical materials. The AM process, samples used, and cytotoxicity testing method were chosen carefully to fulfill certain criteria.

4.1 Needs Analysis for Cytotoxicity Testing Procedure

We used a pairwise comparison chart to rank each of the objectives to determine which is the most important, shown in Table 5. The most important objective was found to be standardization, followed by *in vitro*. The next ranked objective was accuracy, another important aspect of the test. This was followed by rapid, which is a word commonly used across literature for biocompatibility testing. We ranked this based on it being important since the amount of time should not be longer than current methods, but it is not as important as the above-mentioned criteria. The last ranked objective is cost effective. Though important, compared to the other objectives it ranked the lowest regarding the project goal all together. It is, however, still an important aspect due to the budget.

Table 5: Pairwise comparison chart for the ranking of the testing objectives.

Objective	Standardized	Accurate	Rapid	*In Vitro*	Cost Efficient	Novel	Total
Standardized	-	1	1	1	1	0	4
Accurate	0	-	1	0	1	1	3
Rapid	0	0	-	0	1	0	1
In Vitro	0	1	1	-	1	1	4
Cost Efficient	0	0	0	0	-	0	0

This generated the ranking for the Objectives. The objectives were converted to a scale of 1-5 based on the rank, shown in Table 6. Standardized and *in vitro* were determined as equally most important since both terms are stated directly in the project goal. We also decided that accuracy is significantly more importance than rapidness, resulting in accuracy having a weight of 4 and rapidness having a weight of 2.

Table 6: Objective versus weight for the testing method.

Objective	Weight
Standardized	5
In Vitro	5
Accurate	4
Rapid	2
Cost efficient	1

4.2 Needs Analysis for Additive Manufacturing Process

The criteria for the AM process were that it must not leave behind toxic residue, it could create complex geometries, it was accessible to the team, it was material efficient, it required minimal to no post-processing, and it was time effective. The first need for the AM process was that it must not leave behind permanent, toxic residues after it processes a part. If it does leave behind toxic residue, it is an unsuitable process for use in the biomedical field. The process must have been able to create complex geometries, as complex geometries were needed for a variety of applications in the medical field. One major advantage that AM brings was its ability to create such parts. Accessibility wawas a key factor for our project. We needed to use a process that is accessible to us in terms of cost and availability. From a cost perspective, material efficiency was also important. The amount of the material used, as well as the cost of the material itself, was important in the overall cost of the project. Minimal post processing was important because of the rapid nature of AM. By increasing post processing, additional time and money is spent. Additionally, post processing could often damage delicate parts. Finally, time efficiency was important so that the devices can be produced quickly, allowing them able to be produced as they are needed and ensuring enough are produced to fulfill the demand. We defined time efficient for this sized sample as 2 hours.

4.2.1 Ranking of Criteria

The criteria for the AM process were compared in a pairwise comparison chart, shown in Table 7. Using the chart, we determined that the most important criterion for the manufacturing method was not leaving behind any toxic residue. The second most important criterion was the ability of the method to create complex geometries, followed by accessibility, then material efficiency, then minimal post processing. The least important criterion was found to be time efficiency.

Table 7: Pairwise comparison chart for the selection of the AM process criteria.

	Accessible	Time Effective	Material Efficient	No toxic residue	Minimal post processing	Can create complex geometries	Total
Accessible	-	1	1	0	1	0	3
Time Effective	0	-	0	0	0	0	0
Material Efficient	0	1	-	0	0	0	1
No toxic residue	1	1	1	-	1	1	5
Minimal post processing	0	1	1	0	-	0	2
Can create complex geometries	1	1	1	0	1	-	4

The scores obtained in the pairwise comparison chart were converted to a base of 1 to 5, with a score of 5 being the most important and a score of 1 being the least important. This was illustrated in Table 8. Because there were six criteria, we decided that material efficient and minimal post processing should be the same weight.

Table 8: Criteria versus weight for the AM process.

Criterion	Weight
No toxic residue	5
Can create complex geometries	4
Accessible	3
Material efficient	2
Minimal post processing	2
Time effective	1

4.2.2 Selection of Method

The selection of AM process was determined based on the importance of the criteria. A Pugh matrix (Table 9) was used to compare different AM processes to a baseline, which we chose to be PBF. We chose PBF as the baseline because it is the most commonly used technique for complex biomedical devices.

Table 9: Pugh matrix for the selection of the AM process.

	Wt	PBF	BJ	ME	DED	LOM	MJ	SLA
No toxic residue	5	0	0	0	0	0	0	0
Can create complex geometries	4	0	0	0	0	-1	0	0
Accessible	3	0	-1	1	-1	-1	-1	0
Material Efficient	2	0	0	0	0	-1	0	0
Minimal post processing	2	0	0	0	0	-1	0	0
Time Effective	1	0	0	-1	-1	1	-1	-1
Total		0	-3	2	-4	-6	-4	-1

4.3 Needs Analysis for Material Sample

The criteria for the material samples were that it must be biocompatible, accessible, sterilizable, processable by AM, and clinically approved. First, the sample should be known to be biocompatible. This means it does not induce an adverse response in the body. Because the AM part was intended to be used in the body, the material being printed must be nontoxic. Using a known biocompatible material also allowed us to hypothesize the cellular response. Accessibility was another important criterion. A limited number of resources, such as sterilization and AM processes, were available to us. This is likely true for other researchers as well. Therefore, the material we used must have be sterilizable by means available to us, which includes autoclave, ethylene oxide sterilization, and UV sterilization. The material must also have been processable by AM, as the goal of this project was to create standardized tests for AM parts. Specifically, the material should have been processable by an AM method that is available to us, such as PBF, ME, and SLA. Clinical approval, the final need for the material sample, was not given to materials, but to implants utilizing materials. The material chosen should have been a material that has been used in a clinically approved device. The reasoning for this was that clinical approval for a device composed of this kind of material will take less time than clinical approval for a device made of new materials.

4.3.1 Ranking of criteria

The material criteria were ranked against each other using a pairwise comparison chart, seen in Table 10. Biocompatibility was deemed most important, with sterilizability and accessibility being the second and third most important. Processability by AM was of moderate importance compared to the other criteria, and clinical approval was found to be the least important compared to the higher-ranking criteria. As was done with the AM process, the criteria scores were

scaled for a base of 1 to 5, which is shown in Table 11. Accessibility and sterilizability were decided to be of equal importance.

Table 10: Pairwise comparison chart for the selection of the material criteria.

	Biocompatible	Processable by AM	Biodegradable	Clinically approved	Accessible	Sterilizable	Total
Biocompatible	-	1	1	1	1	1	5
Processable by AM	0	-	1	1	0	0	2
Clinically approved	0	0	1	-	0	0	1
Accessible	0	1	1	1	-	1	4
Sterilizable	0	1	1	1	0	-	3

Table 11: Criteria vs weight for the material used.

Criterion	Weight
Biocompatible	5
Accessible	4
Sterilizable	4
Processable by AM	3
Clinically approved	2

4.3.2 Selection of Material

The material to be used was selected based on how well it fulfilled the above criteria. Table 12 shows the Pugh matrix used to compare the baseline polymer, PLA, to other polymer contenders. The baseline was chosen to be PLA because of its wide use in biomedical materials as a biodegradable plastic. Polycarbonate scored the highest and was the only polymer more suitable than PLA. Polycarbonate, like the other polymer contenders, is biocompatible. It was more accessible to the team than other polymers, and it could be sterilized by high temperatures, which was the major sterilization method available to us. It could be processed by ME, the chosen AM process, and polycarbonate-containing medical devices are clinically approved.

Table 12: Pugh matrix for the selection of the polymeric material to be used.

	Wt	PLA	PGA	PGLA	PCL	PEUs	Polypropylene fumarate	Polycarbonate	Dental Resin
Biocompatible	5	0	0	0	0	0	0	0	0
Accessible	4	0	0	-1	-1	-1	-1	0	-1
Sterilizable	4	0	0	0	0	0	0	1	0
Processable by AM	3	0	-1	0	0	0	-1	0	0
Clinically approved	2	0	0	0	0	0	-1	0	0
Total		0	-3	-4	-4	-4	-9	4	-4

4.4 Materials Used for Testing

The materials used for testing included multiple AM samples of different shapes, cell culture medium, and 3T3 mouse fibroblast cells.

4.4.1 Sample Modeling

SolidWorks was used to model various designs for the sample parts. Figure 2 below shows the CAD model of the flat sample that was used for preliminary testing. The model was a rectangular prism fulfilling the size constrains as per the ISO standard for a 6-well plate. The sample was printed on a ME printer using PLA.

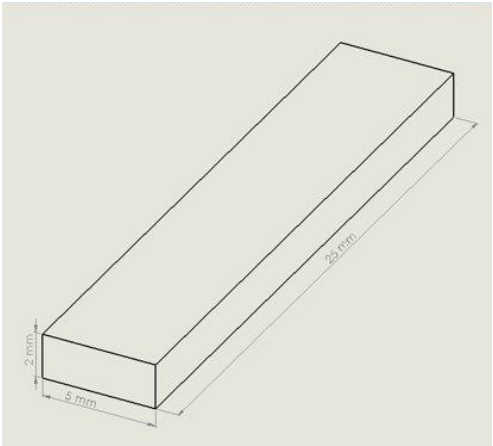


Figure 2: CAD model of the flat polymer sample.

We had four potential main samples that we could have used: a flat cylinder, a cup of a hip implant, a stent, and a knee implant. Three were eliminated. The flat cylinder was eliminated due to changes in the client statement, as we wanted a design with no accessible flat surfaces. The hip implant was eliminated because we wanted more geometric variety within the shape, and the stent was eliminated after we decided to use ME, as it was not printable with the process. Figure 3 shows the three eliminated designs.

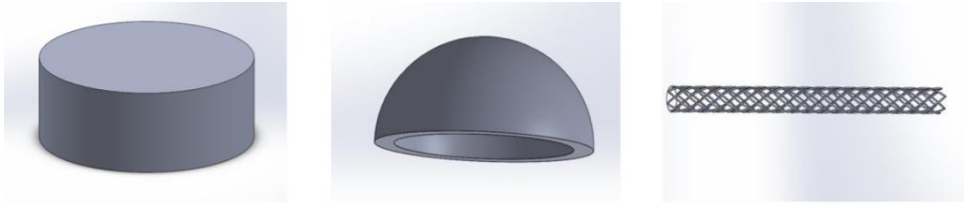


Figure 3: Other sample designs considered.

Figure 4 below shows a CAD model of an example of a portion of a knee implant, which was the geometry that was tested. The knee implant was chosen because it has many curved surfaces and a flat surface that was unreachable without modification of the shape. This means that it could not be tested with direct contact tests without changing the design.

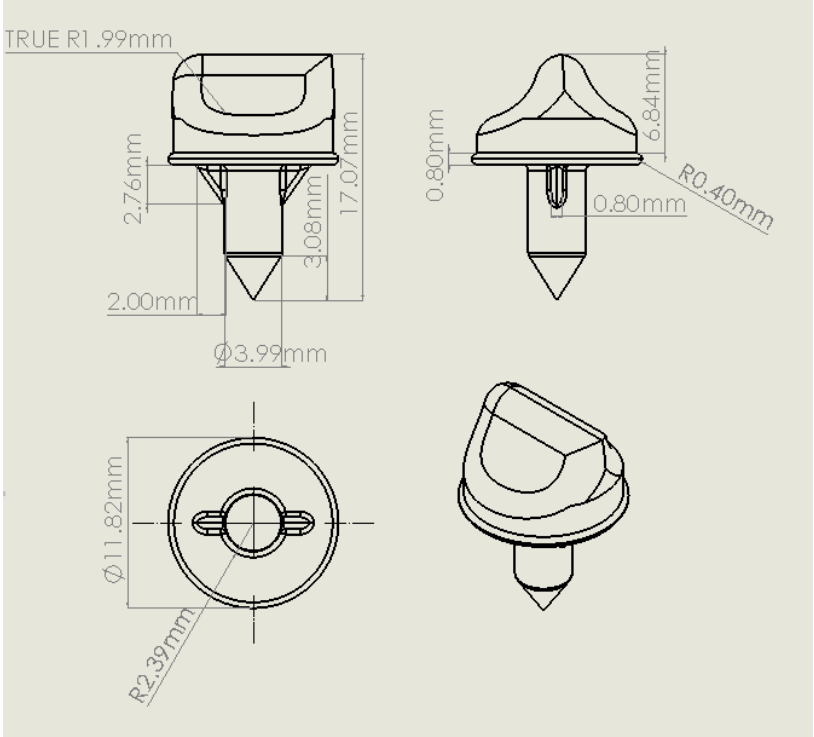


Figure 4: CAD drawings of a knee implant.

For acellular feasibility testing, three samples were created out of PLA. A texture was picked in SolidWorks based on the fineness of the details, which would translate to more ridges and a better representation of naturally rough surfaces. The texture that was chosen can be seen below in Figure 5. The smoothest sample was printed with no texturing, the moderately rough sample was printed with dark surfaces raised 0.12 mm, and the roughest sample was printed with 0.3 mm raised dark surfaces. The drawing of the samples can be found in Figure 6 below.

Figure 5: Texture selected.

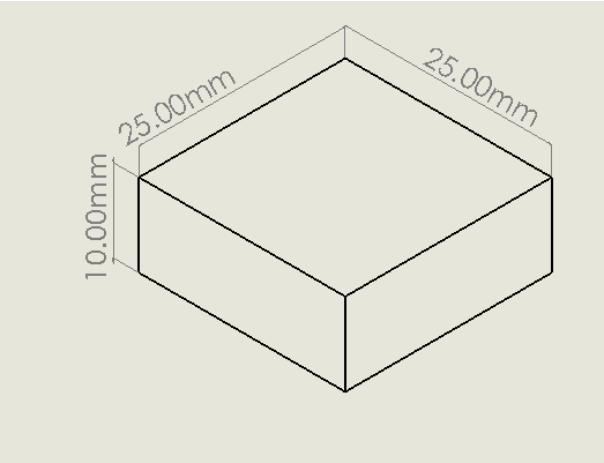


Figure 6: CAD drawing of the samples.

An altered version of the knee implant was created in order to obtain a flat, stable horizontal surface. A support was added to the model to keep the geometry while allowing the model to stay stable while in the culture plate. This can be seen in Figure 7 below.

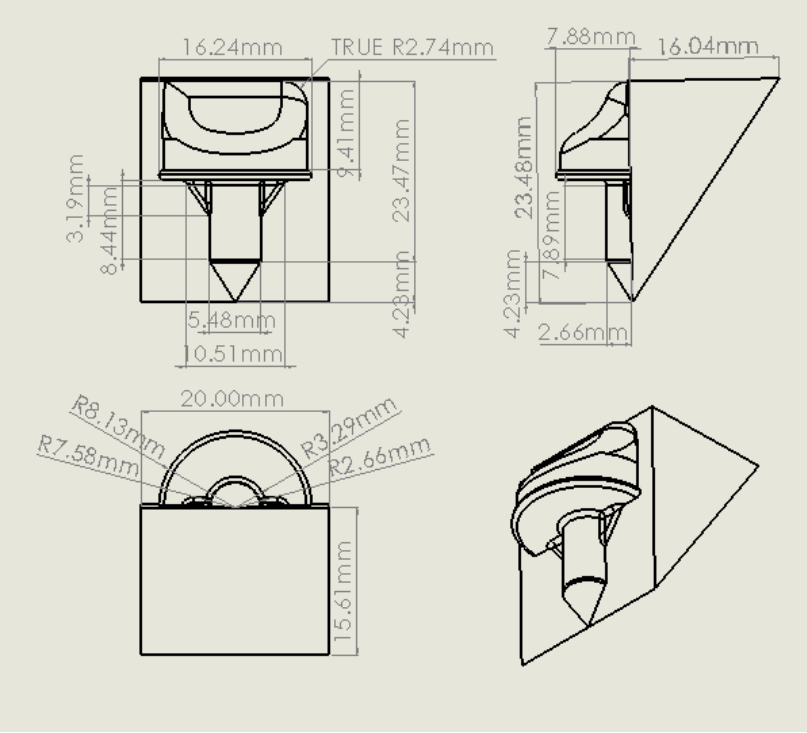


Figure 7: CAD drawing of the modified knee implant.

4.4.2 Cell Culture

For all procedures that use cell culture medium, the cell culture medium was made using 1X Dulbecco's Modification of Eagle Medium (DMEM) with 4.5 g/L glucose and sodium

pyruvate without L-glutamine. It contained 10% fetal bovine serum, 0.1% Glutamax, and 0.1% Penicillin/Streptomycin. For our experiments, we used a 100X Glutamax stock solution and a Penicillin/Streptomycin stock solution containing 10,000 U Pen. /mL and 10,000 ug Strep./mL. We made culture medium 50 mL at a time, which consisted of 5 mL of fetal bovine serum, 0.5 mL of Glutamax, 0.5 mL of Penicillin/Streptomycin, and 44 mL of Dulbecco's modified eagle medium. All components were sterile, and the medium was stored at 4˚C when not in use.

The cell type used for cellular testing was 3T3 mouse fibroblast cells. This cell line was chosen due to its accessibility and our prior experience with 3T3 cells. Many research studies also use the 3T3 line due to it being one of the most frequently used cell lines for studying material/cell interaction [22]. The cells were stored in a humidified incubator with a level of 5% CO_2, at 37°C.

4.5 Conceptual/Alternative Procedures

This section discusses the initial ideas we had for cytotoxicity testing that we either developed or eliminated in favor of other ideas. These ideas included an acellular and a cellular degradation test. Both tests utilize measuring effects of degradation by change in pH.

4.5.1 Acellular degradation test

One proposed method of determining cytotoxicity was performing an acellular degradation test to determine how the material would fare under physiological conditions. This test would measure the mass and pH before and after exposure to an electrolytic solution. Because this test was acellular, it was quicker, easier, and cheaper than performing a test involving cells. The detailed, step-by-step procedure can be found in Appendix A.2. Using various salts, a 7.4-pH solution of simulated body fluid was made. After weighing the 2 material samples, they were placed in two 15-mL centrifuge tubes, which were then filled with the solution. An empty third centrifuge tube was also filled with the solution, to act as the control. All three centrifuge tubes and the solution were stored at 4°C. After three days, the pH of the solutions in the three centrifuge tubes was measured. The liquid was aspirated, and the samples were then weighed.

4.5.2 Degradation and Leaching with pH and Cell Staining

This test evaluated cytotoxicity from degradation and leaching by measuring the pH of seeded cells in cell culture medium and by staining dead cells. If the material degraded or releases harmful molecules, the cells would become damaged or die and would not be able to maintain a constant pH of 7.4. A detailed procedure of this experiment can be found in Appendix A.3.

To conduct this experiment, the samples were weighed followed by autoclave sterilization. Each sample was then added to a well of a 6-well plate, and 10 mL of 10% complete medium is added on top of them. The pH was recorded. The plate was incubated for 3 days, after which the pH of the medium in each well was recorded again.

After 3 days, cells were cultured and counted. After centrifuging the cells, sample-exposed medium was added to the cell pellet and resuspended at 250,000 cells in each well of a 6-well plate in 5 mL of sample-exposed medium. Cell suspensions were incubated for 3 days. After 3 days, the

pH of the medium was recorded, were counted, and dead cells were observed from addition of Trypan Blue.

Some limitations of this approach are its length and complexity. Not including the cell preparation, this experiment lasts a week. Many of the same steps would have to be repeated for each cell passage, which will increase with the number of samples used. This test also does not account for long-term degradation that occurs over months and years.

4.6 Dimethyl Sulfoxide Experiments

Two different experiments were conducted to determine the dimethyl sulfoxide (DMSO) concentration necessary to elicit a cytotoxic response. The purpose of these experiments was to develop an appropriate cytotoxic control group containing XX% DMSO in complete medium. The first DMSO experiment used concentrations of 0%, 20%, 30%, 35%, 40%, and 50% DMSO in complete medium. A total of 500,000 cells were cultured in each well of a 6-well plate, each well containing a different concentration of DMSO. After about 16 hours of incubation, each well was imaged to qualitatively show cytotoxic effects.

The second DMSO experiment used varying amounts of DMSO concentrations and cell numbers. The DMSO concentrations were 0%, 10%, 20%, 30%, 40%, and 50%. Each DMSO concentration was tested with 25,000, 50,000, 75,000, and 100,000 cells. This was done between two 12-well plates. The two plates were incubated for 4-6 hours, after which each well was imaged to visually determine cytotoxic effects.

4.7 Control Coatings

The following protocols detail how the poly-l-lysine, alginate, and gelatin coatings were applied to the well plates and samples. Full procedures can be found in Appendix A.4.Poly-l-lysine and gelatin were picked as we hypothesized that cells would adhere to the coating, and alginate was picked as we hypothesized that cells would not adhere to the coating due to the lack of alginate-specific adhesion molecules on mammalian cells.

4.7.1 Poly-l-lysine preparation

The poly-l-lysine was already in solution form as 0.1% poly-l-lysine solution from Sigma. It was sterilized using vacuum filtration. To coat the wells of the plate, 1 mL of the sterilized solution was added to two wells, and the plate was incubated for one hour. The wells were aspirated after the hour and left to dry for 30 minutes in the biosafety cabinet. Once dry, the plate was wrapped in parafilm and stored at 4°C. The sterilized solution was stored in the biosafety cabinet, at room temperature.

4.7.2 Alginate culture plate preparation

To create the alginate coating in the 6-well plate, the plate was first stored overnight with two wells containing 5 mL of a sterilized 5-M $CaCl_2$ solution. The $CaCl_2$ was aspirated from the

wells, and 1.5 mL of a vacuum-filter-sterilized 1.5% alginate solution was added in its place. The plate was incubated at room temperature for 40 minutes and washed with DPBS (-). The plate was then wrapped in parafilm and stored at 4°C. The protocol for making the alginate solution is in Appendix A.4.1. A detailed protocol of the alginate culture plate coating can be found in Appendix A.4.3.

4.7.3 Gelatin culture plate preparation

A 2% gelatin solution was prepared by dissolving 1 gram of gelatin in 49 mL of water to obtain a 2% gelatin solution, which was sterilized in the autoclave for 20 minutes at 121°C. The plate was coated with 240 µL of gelatin solution and dried for two hours at room temperature under the biosafety cabinet. If not being used immediately, the plate would be wrapped in parafilm and stored at 4°C. The full procedure for preparing the gelatin solution can be found in Appendix A.4.2, and the full procedure for the coating of the well plate with gelatin can be found in Appendix A.4.4.

4.7.4 Alginate sample preparation

Four knee implant samples were placed in a 50-mL conical tube, and 5-M CaCl2 was added to the tube until it reached the top. This tube was left in the biosafety cabinet overnight. The next day, the solution was aspirated, the samples were transferred to a new 50-mL conical tube, and the tube was filled with alginate solution. The samples were left in the solution for 30 minutes, after which they were removed and washed with DPBS (-). They were then immediately used for cellular experimentation. A detailed protocol for coating the samples with alginate can be found in Appendix A.4.5.

4.7.5 Gelatin sample preparation

Four knee implant samples were placed in a 50-mL conical tube. A completely liquefied 2% gelatin solution was added to the tube until it reached the top. The samples were left in the solution for 30 minutes, after which they were removed and placed on a 6-well plate to dry for 2 hours. A detailed protocol for coating the samples with gelatin can be found in Appendix A.4.6.

4.8 Drop Test

The first of the cellular tests that were performed was the drop test for adhesion and possible cytotoxicity testing. In this test, a small drop of cell suspension is placed on top of the sample, forcing the cells to interact with the sample surface rather than sticking to the culture plate. It was intended to evaluate the degree of adhesion for direct contact testing and to measure the cytotoxicity of sample contains a flat surface.

4.8.1 Acellular Feasibility Testing: Various-sized Drops on Flat Surfaces of Varying Angles

For the first acellular drop test, three flat PLA samples of varying surface roughness were used. Sample roughness was changed to test how different designs may affect the potential of the drop test to be performed. Sample 1 contained the flattest surface, and Sample 3 had the roughest surface. On each surface, we dropped 10, 25, and 50 µL of nonsterile complete medium on each surface at three different angles: 5.7°, 11.4°, and 22.8°, shown in Figure 8, which were based off the supports that were available to us. To change the angle of the surface, supports of 1 mm in thickness were placed under the sample. The angle was determined using trigonometry.

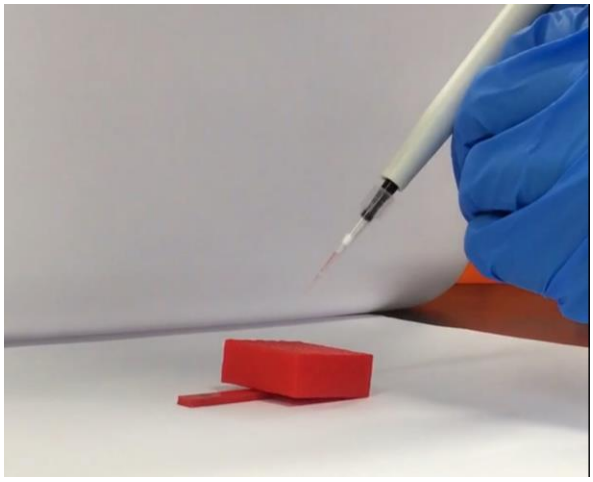
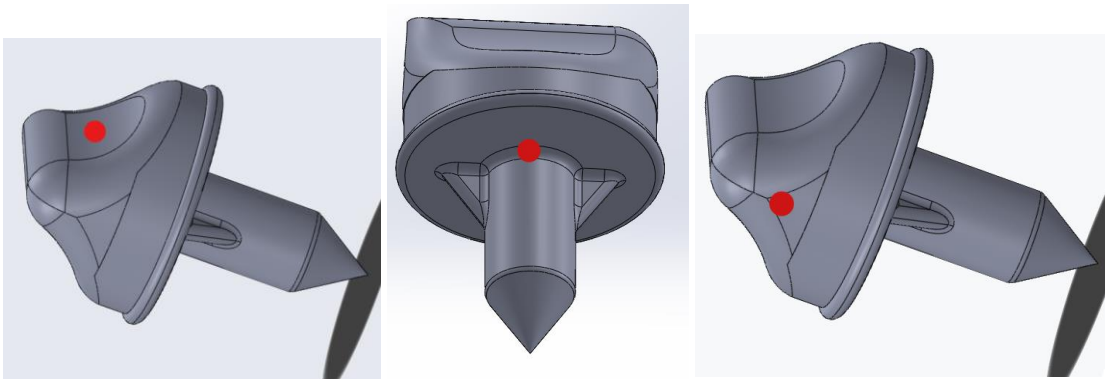


Figure 8: Acellular drop test on flat, angled surface.

4.8.2 Acellular Feasibility Testing: Drops on Knee Implant Sample

For the second acellular drop test, a polycarbonate knee implant model was used. Three different samples of the same size were placed in the wells of a 6-well plate. A 50-µL drop of cell culture medium was then placed in three different spots that drops could easily be placed on the three samples and represented different surface curvatures (Figure 9). The red dot represents the placement of the drop. The cover was put on the well plate, and the plate was placed on a shelf and then removed to simulate the movement of being put into and taken out of an incubator.



a. Sample A *b. Sample B* *c. Sample C*

Figure 9: Diagram showing where the drops were placed on the samples.

4.8.3 Qualitative Cellular Drop Test

In the first cellular drop test, which was qualitative, a modified version of the knee implant (Figure 7) was used. It allowed a drop of cell suspension to be placed on the sample surface without falling off. A detailed protocol of this test can be found in Appendix A.5. All samples and forceps were sterilized using an autoclave. Four samples were placed in four wells of a 6-well plate, with two wells left empty for non-cytotoxic control. A 17.5-µL drop was placed on two samples for cytotoxic control. A 50-µL drop containing 5,000 cells was placed on each of the four samples and in the two empty wells. The plate was incubated for about 16 hours. After incubation, each sample was turned on its side and gently rinsed with complete media to dislodge loose cells on the surface. Samples were removed and wells were imaged.

4.8.4 Quantitative Drop Test

After performing the first drop test, we developed a modified version of the drop test to address some issues with the first cellular drop test. This method tested adhesion instead of cytotoxicity and is quantitative instead of qualitative. Six samples, as well as forceps, were sterilized using an autoclave. Two samples were coated with gelatin as the adhesive control, and two samples were coated with alginate as the non-adhesive control. All samples were placed in a 6-well plate. A 50-µL drop of cell suspension, still containing 5,000 cells, was placed on the surface of each sample. The plate was incubated for 4-6 hours to allow time for the cells to adhere without letting the population double. After incubation, the plate was removed, samples were turned on their side, and each sample surface was gently rinsed with complete medium to dislodge loose cells. The cells from each well were transferred to a 1.5-mL microfuge tube, Trypan Blue was added, and 7-µL suspension samples were taken for cell count.

4.9 Motion Test

In addition to the drop test, we also developed a test to determine cytotoxicity by keeping the sample and culture medium in motion. The motion test intended to test whether cells can interact and adhere to a surface when placed in a cell suspension in constant motion. By keeping

the cells in motion, the cells may have been more likely to come in contact with the sample rather than sink to the bottom of the plate. To test this, we tested the adhesion to see if the cells would come into contact with the surface and stay attached, rather than remaining floating in the medium. Unlike the drop test, this test did not require any changes to be made to the CAD model of the implant. The culture plates were kept in motion by being stored on a rocker inside an incubator.

4.9.1 Acellular Testing with Culture Plates

Before testing with cells, it was necessary to test a) how fast the plate could be shaken and b) how much liquid the plate could hold without spilling. These parameters were tested with a 100 mm culture plate and a 6-well plate using water. First, we filled a 100 mm culture plate with 11 mL of water as this was the recommended volume of medium to use for cell culture in this type of plate [23]. We placed the plate on the rocker and set the speed to half of what the rocker was capable of. We counted an approximate RPM to get an idea of how fast the rocker was going. We repeated this with ¾ speed and full speed. We continued to do this for each mL of water added to the plate until reaching a total volume of 16 mL. We did not test any volume larger than 16 mL because this was the maximum recommended volume for cell culture [23]. We repeated the experiment using a 6-well plate, with 3 wells empty (sample-less) and 3 wells containing the knee implant sample. We started with 2 mL of water and went up to a total volume of 11 mL. We stopped at 11 mL because adding any more volume of water would cause the wells to overflow before being placed on the rocker.

4.9.2 Cellular Adhesion Testing

If it is impossible for cells to stick to a sample during the motion test, then direct contact cytotoxicity cannot be determined, and this test will not work. Therefore, feasibility tests must be performed to ensure that it is possible for cells to adhere to a surface while they are constantly in motion. Firstly, we must understand the behavior of the cells when kept in motion. This adhesion test compares cell adhesion while in motion to cell adhesion when kept stationary. A detailed protocol can be found in Appendix A.6. The adhesive control group for this experiment was four gelatin-coated polycarbonate knee implant samples, and the non-adhesive control was four alginate-coated knee implants. Four uncoated samples were used as the experimental group. Cells were seeded in twelve 100 mm tissue culture plates at 100,000 cells in 10 mL of complete medium. Two of each group were placed on the rocker at full speed, and the remaining six plates were stored stationary. After 4-6 hours, all plates were removed from the incubator. For each sample, the part of the sample exposed to the medium was placed in 3 mL of Trypsin in the well of a 6-well plate and incubated for 10 minutes, and cells in the wells were then counted. The cells remaining in the 100 mm plates were imaged and counted using ImageJ. Ideally, the number of cells adhered to sample plus the number of cells still in the plate would equal the number of cells seeded, which in this case was 100,000 cells.

4.10 Summary of Final Design Selection

The final material samples chosen to be used for this project was polycarbonate. This was due to its accessibility and ability to be sterilized with the techniques available to us. Material extrusion was the AM process picked to fabricate the material. Out of all the AM processes, material extrusion was the process that received the highest score on the Pugh matrix. The final testing methods that were pursued included the drop test and the motion test for adhesion and cytotoxicity filter diffusion test. The acellular degradation test and the cellular degradation test with cell staining were not chosen as a final design because our project goals focused on cytotoxicity and adhesion more than degradation.

Chapter 5. Final Design Verification

The experiments we were able to perform in the lab were the acellular degradation test, the two dimethyl sulfoxide experiments, the control coatings, the drop test feasibility testing, the qualitative drop test, the motion test feasibility testing, and the motion test itself. This chapter provides the results of all laboratory experiments.

5.1 Acellular Degradation Test: Results

Table 13 shows the results of pH measurement on days 0, 3, and 4; measurements were made on day 4 due to the pH meter on day 3 appearing to be broken. For both day 3 and day 4, the control was found to have a lower pH than the samples.

Table 13: pH values of the solutions on days 0, 3, and 4.

	Starting pH (Day 0)	pH (Day 3, pH meter #1)	pH (Day 3, pH strips)	pH (Day 4, pH meter #2)
Sample 1	7.4	7.2	6.7	6.46
Sample 2	7.4	7.4	6.7 – 7.0	6.27
Control	7.4	6.9	6.7	6.10

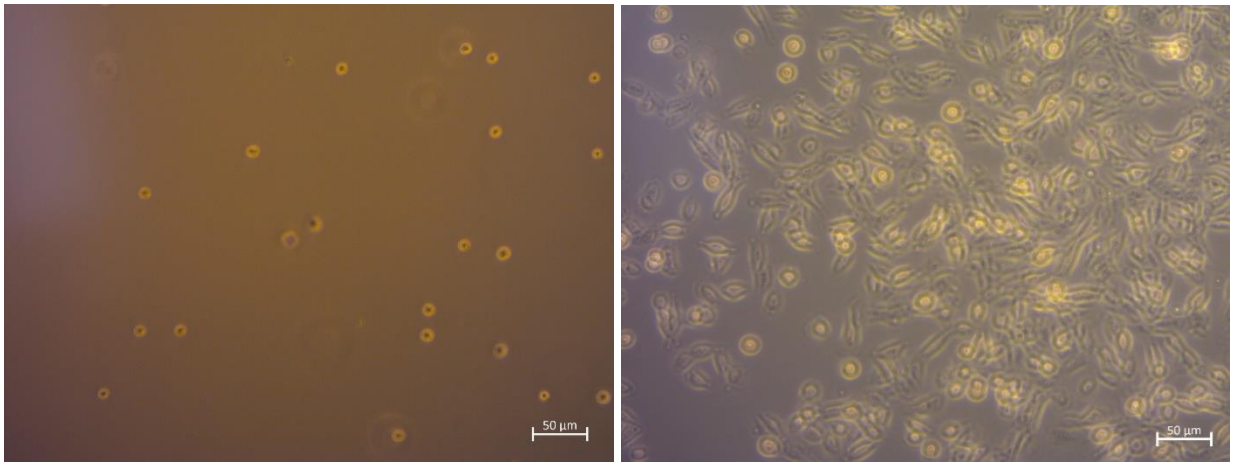
The masses of the samples on day 0 and day 4 are shown in Table 14. Sample 1 appeared to have gained 84 mg (a 32% difference), while sample 2 appeared to have lost 9 mg (a 3% difference). The pH showed a drop from the first day to the last. However, the pH meter gave different results than the pH strips and may not have been calibrated correctly, leading to potentially inaccurate results. On day 4 a different pH meter was used because we figured it would be calibrated and give us more accurate readings. However, we concluded that the pH meter and pH strips were not an accurate means of measuring pH in our case.

Table 14: Masses of the samples on days 0 and 4.

	Mass (g) on Day 0	Mass (g) on Day 4
Sample 1	0.2604	0.2688
Sample 2	0.2665	0.2646
Average	**0.2635**	**0.2667**

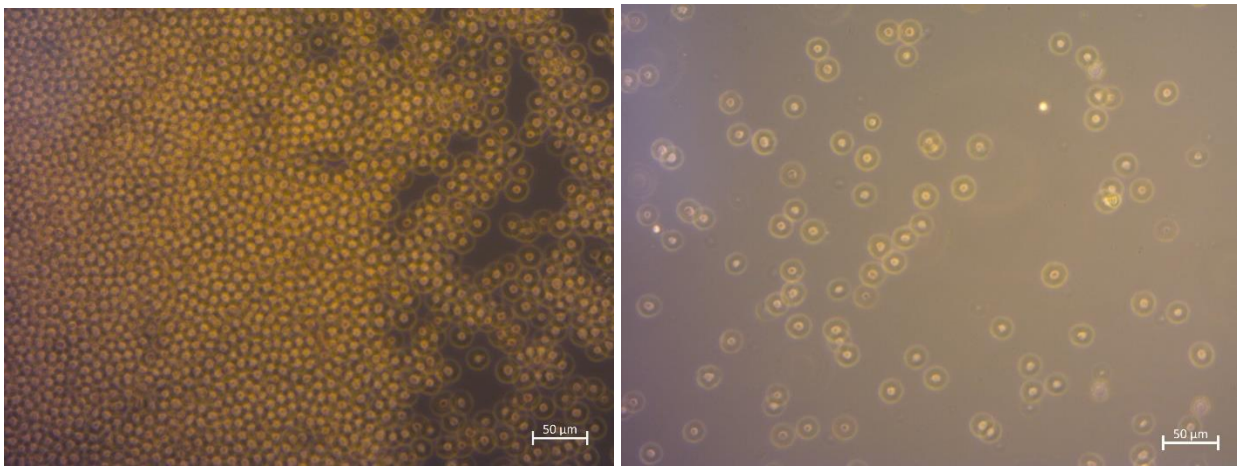
5.2 Dimethyl Sulfoxide Experiments: Results

For the first DMSO experiment, wells were imaged before and after incubation. However, it was difficult to observe the cells as the cell seeding number was too high. We redid the experiment with varying concentrations of DMSO and cell number. Figure 10 shows healthy, DMSO-less cells seeded at 25,000 before and after incubation. Figures 11 and 12 show the same number of cells before and after incubation when exposed to 10% and 50% DMSO. As seen in the Figures, 10% DMSO is enough for cells to be rounded and unhealthy. Figure 12 shows greater cytotoxicity at higher DMSO concentrations, as cells are smaller and more rounded. All images taken from the DMSO experiments can be found in Appendix A.9.



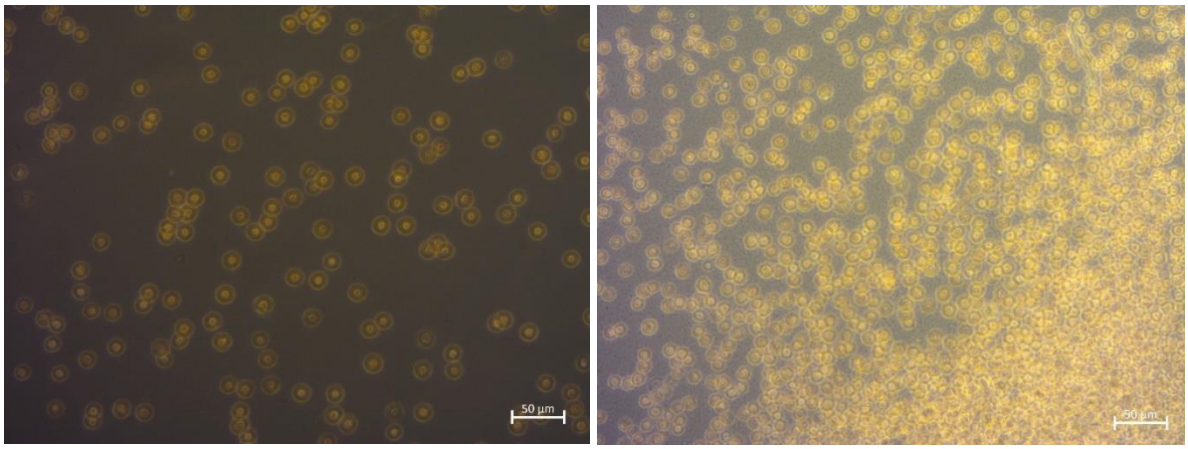
a. Before incubation *b. After incubation*

Figure 10: 25,000 cells at 0% DMSO concentration before (a) and after (b) incubation.



a. Before incubation *b. After incubation*

Figure 11: 25,000 cells at 10% DMSO concentration before (a) and after (b) incubation.



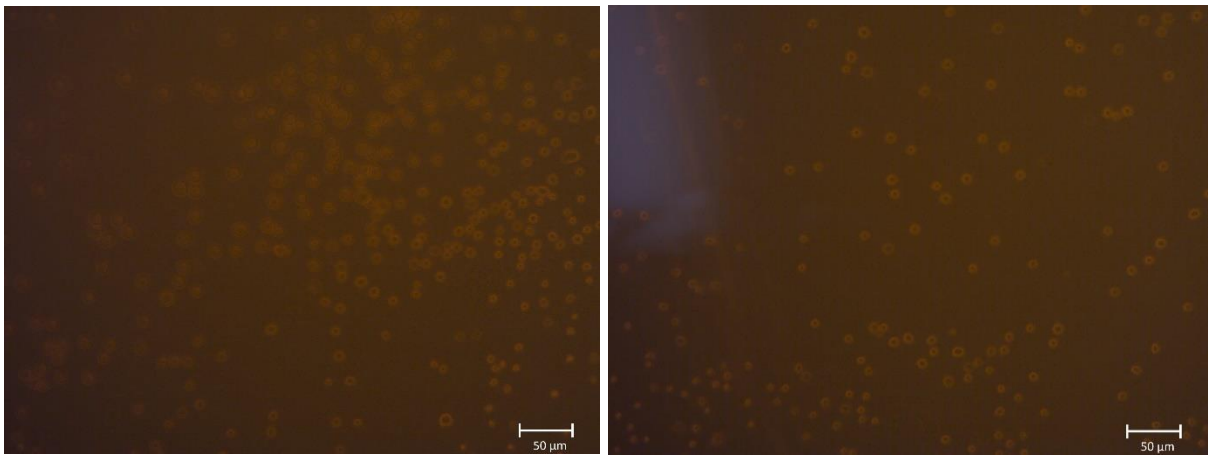
a. Before incubation *b. After incubation*

Figure 12: 25,000 cells at 50% DMSO concentration before (a) and after (b) incubation.

5.3 Control Coatings: Results

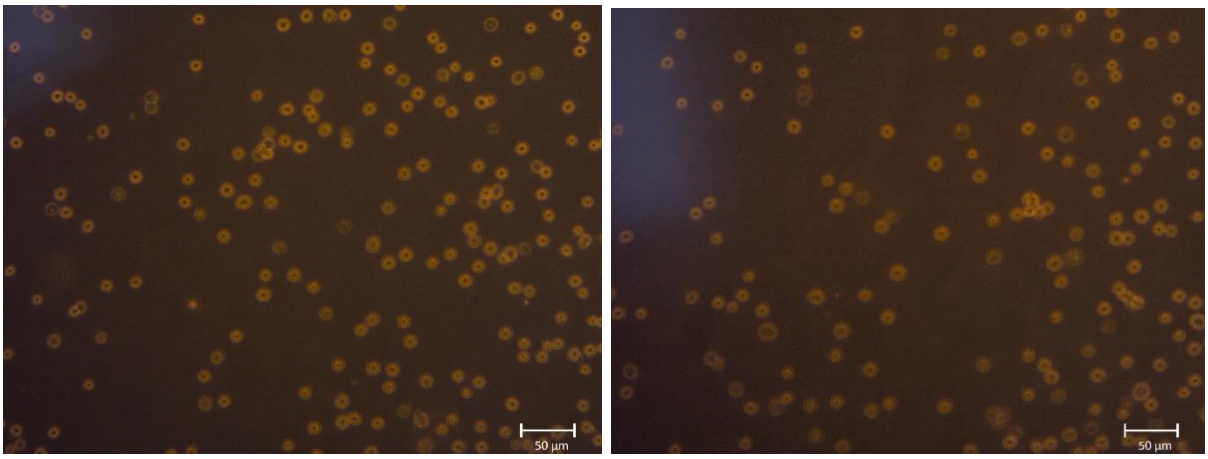
For feasibility testing, we tested how cells would react to different coatings. We coated cell plates with alginate, poly-l-lysine, and gelatin and imaged the cells before and after incubation. Figures 13, 14, and 15 show the alginate, poly-l-lysine, and uncoated plates after being seeded with cells but before incubation. The cells in the alginate-coated well plate (Figure 13) were on various plane because the coating was not smooth, making it difficult to take clear images. Before incubation, all plates looked relatively the same.

Figures 16, 17, and 18 show the plates after 4-6 hours of incubation. After incubation, the poly-l-lysine coated plates and alginate plates did not show adhesion, while the uncoated plates did show adhesion.



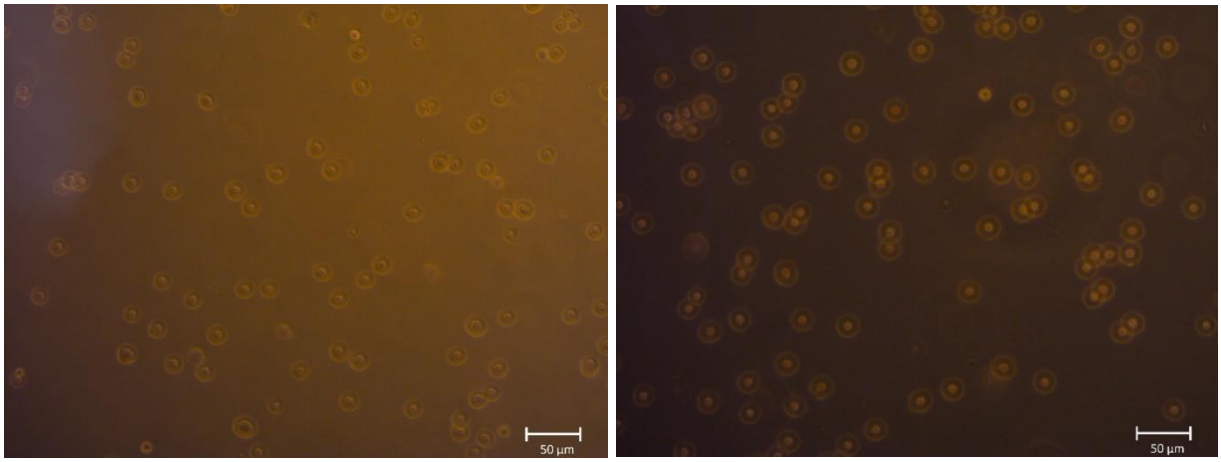
a. Sample A ***b. Sample B***

Figure 13: Alginate-coated wells before incubation.



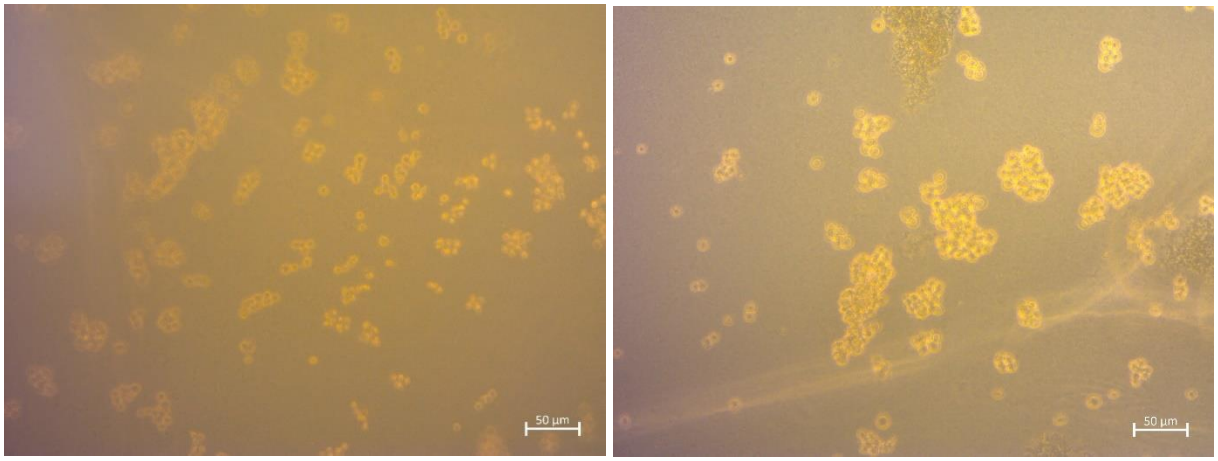
a. Sample A ***b. Sample B***

Figure 14: Poly-l-lysine-coated wells before incubation.



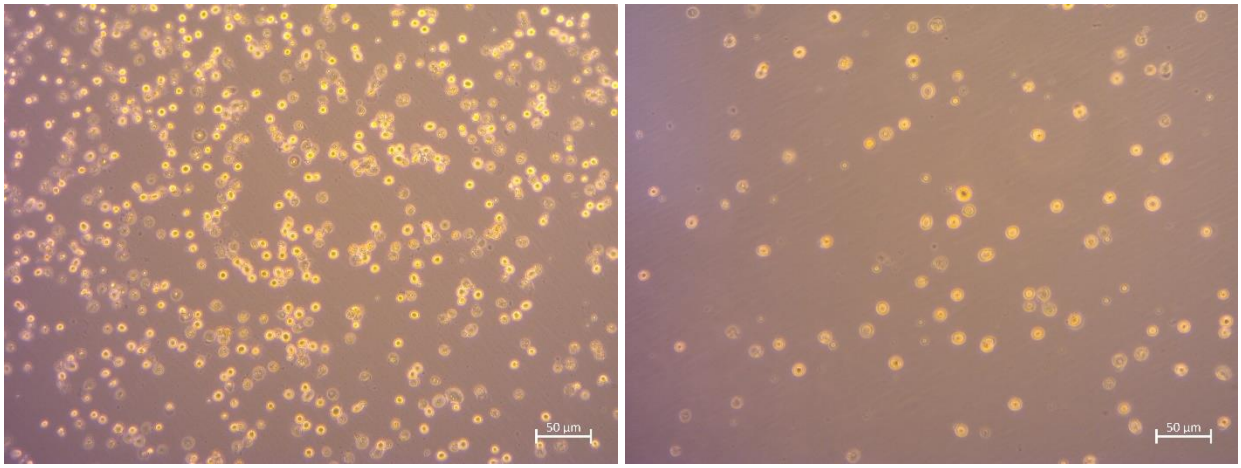
a. Sample A *b. Sample B*

Figure 15: Uncoated wells before incubation.



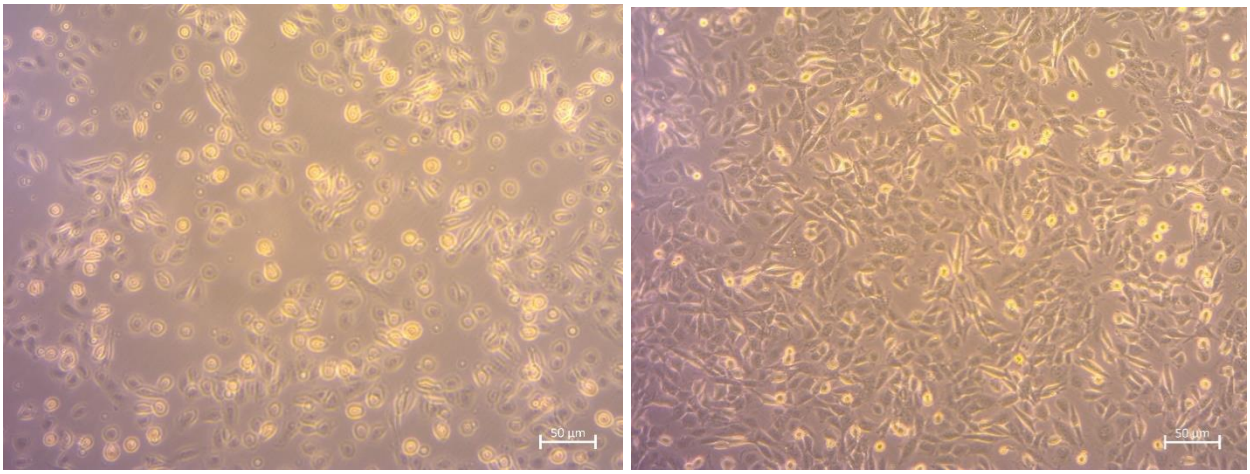
a. Sample A *b. Sample B*

Figure 16: Alginate-coated wells after incubation.



a. Sample A *b. Sample B*

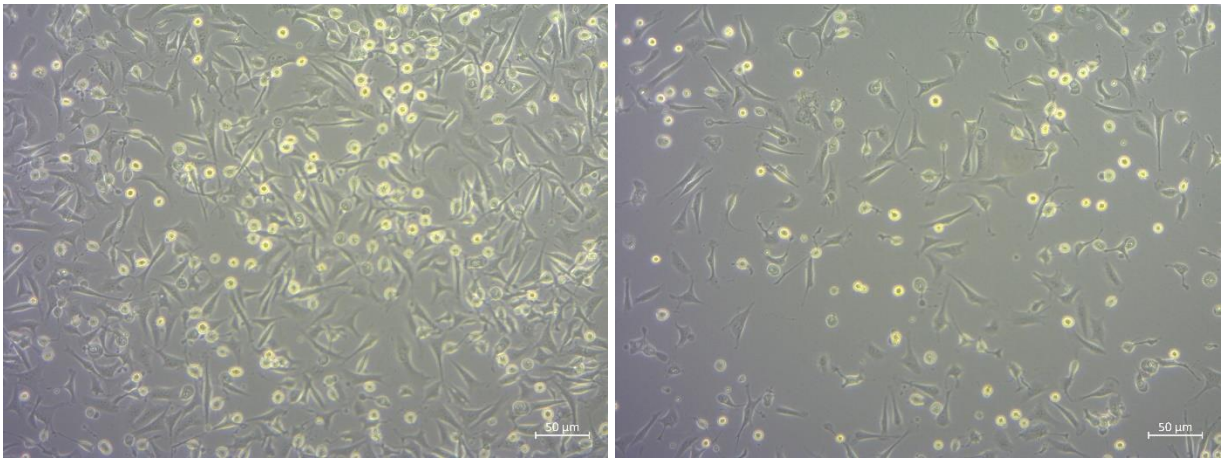
Figure 17: Poly-l-lysine-coated wells after incubation.



a. Sample A ***b. Sample B***

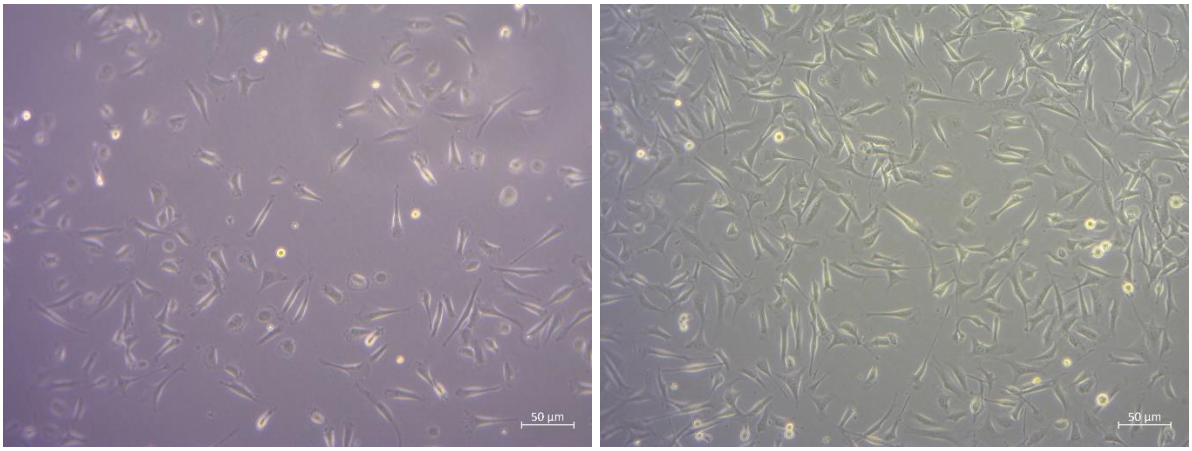
Figure 18: Uncoated wells after incubation.

Next, we tested how cells would react to a gelatin coating compared to no coating. The results are shown in Figures 19 and 20. The gelatin plates showed some adhesion, but less than the uncoated plates.



a. Sample A ***b. Sample B***

Figure 19: Gelatin-coated wells after incubation.



a. Sample A *b. Sample B*

Figure 20: Uncoated wells after incubation.

5.4 Drop Test: Acellular Feasibility Testing: Results

The tables below (Tables 15, 16, and 17) show our results for the first acellular drop test, which used flat surfaces of varying roughness at different angles. For every angle tested, none of the drops moved except for the 50-µL drop on sample 3, the roughest surface, at an angle of 22.8°.

Table 15: Movement of drops on sample 1 (smoothest surface).

Angle	10 µL	25 µL	50 µL
5.7°	No movement	No movement	No movement
11.4°	No movement	No movement	No movement
22.8°	No movement	No movement	No movement

Table 16: Movement of drops on sample 2.

Angle	10 µL	25 µL	50 µL
5.7°	No movement	No movement	No movement
11.4°	No movement	No movement	No movement
22.8°	No movement	No movement	No movement

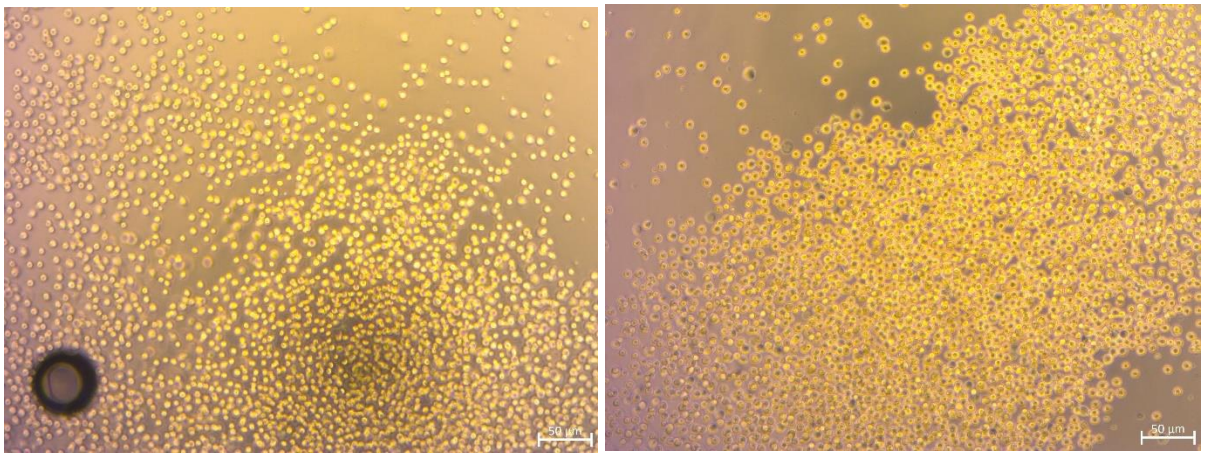
Table 17: Movement of drops on sample 3 (roughest surface).

Angle	10 µL	25 µL	50 µL
5.7°	No movement	No movement	No movement
11.4°	No movement	No movement	No movement
22.8°	No movement	No movement	Movement

The next acellular drop test was performed at various locations on the knee implant. The drop on sample C immediately rolled off the sample, while the drops on samples A and B remained in place. However, the drops on these two samples rolled off when the plate was being moved due to the samples moving inside the plate.

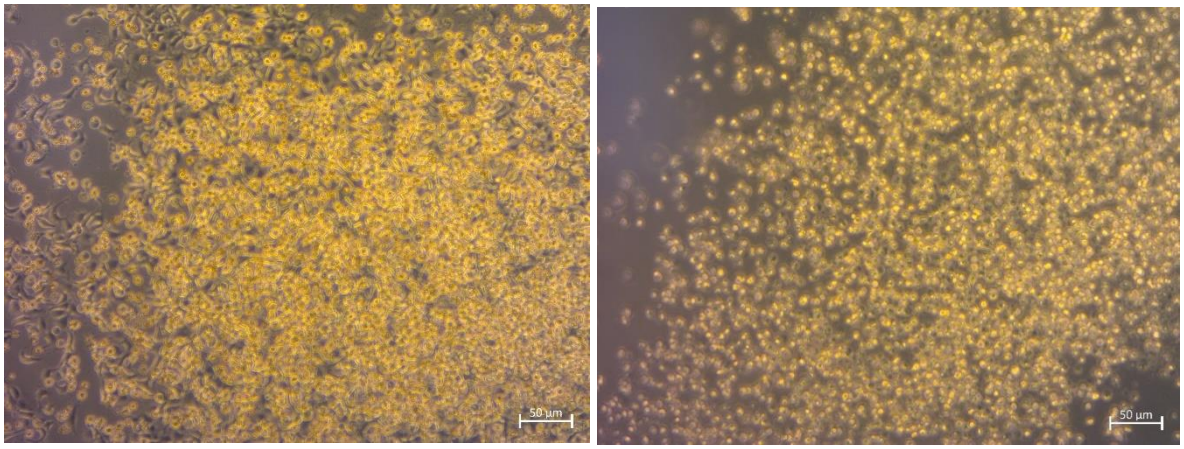
5.5 Qualitative Drop Test: Results

In this test, a 50-µL drop containing 5,000 cells was placed in 2 empty wells (noncytotoxic control), on top of a sample containing a drop of DMSO (cytotoxic control), and on top of an untreated sample. Images were taken of the noncytotoxic control wells immediately after seeding, seen in Figure 21. After ~16 hours, the noncytotoxic control was imaged and is seen below in Figure 22. The cytotoxic control and sample were rinsed with complete medium to dislodge loose cells on the surface, shown in Figure 23 and 24.



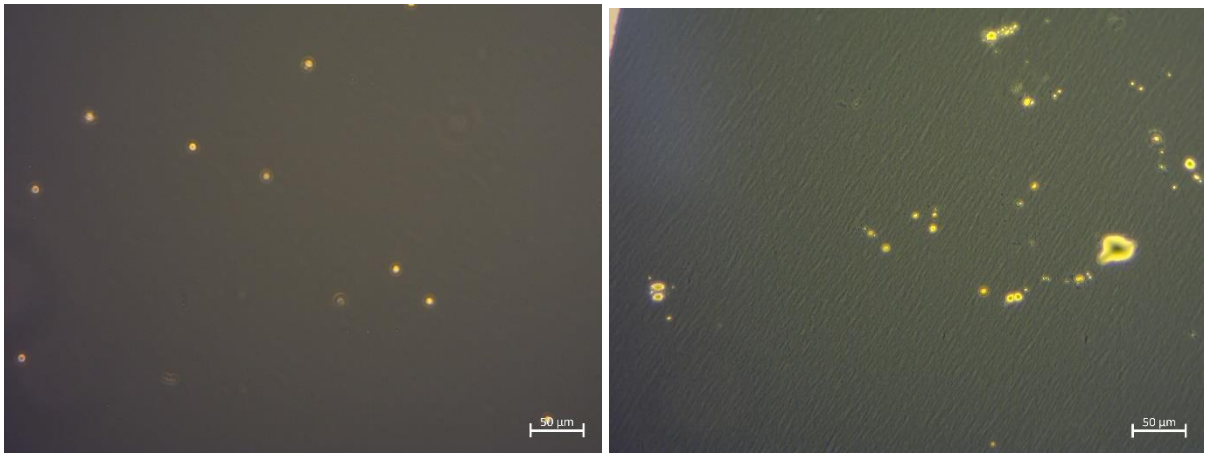
a. Sample 1 ***b. Sample 2***

Figure 21: Noncytotoxic control before incubation.



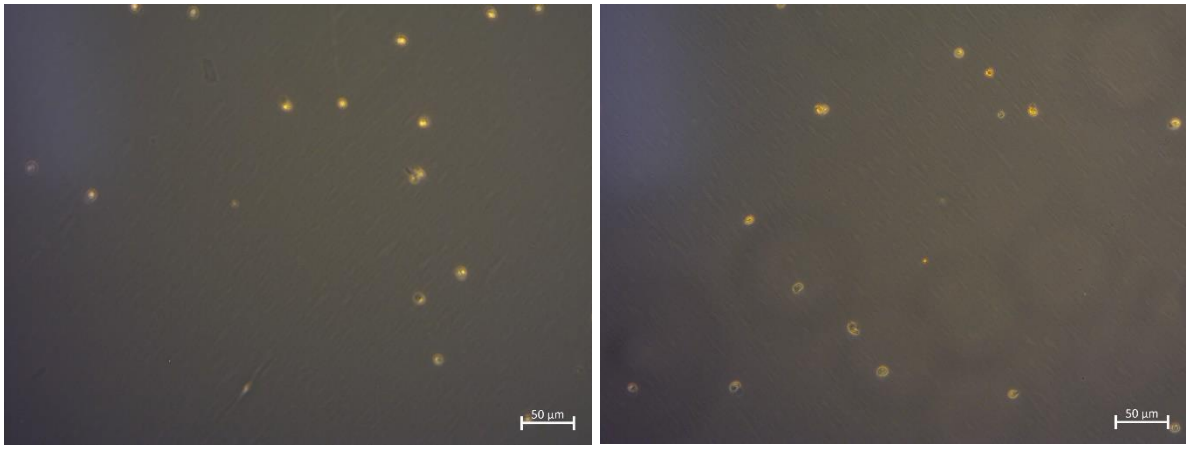
a. Sample 1 ***b. Sample 2***

Figure 22: Noncytotoxic control after incubation.



a. Sample 1 *b. Sample 2*

Figure 23: Cytotoxic control after incubation.



a. Sample 1 *b. Sample 2*

Figure 24: Experimental sample after incubation.

5.6 Motion Test: Acellular Feasibility Testing: Results

The tables below (Tables 18 and 19) show the results from this experiment. The rotations per minute (RPM) are not exact, but an estimate due to the rocker not having a setting to choose a speed. The only setting was a small knob indicating which way to turn. We estimated the RPM for each speed using a stopwatch and counting the rotations in a 30 second period.

Table 18: Spill data for the 100 mm cell plate at ½ speed (~30 RPM), ¾ speed (~50 RPM), and full speed (~80 RPM).

Volume	½ speed	¾ speed	Full speed
11 mL	No Spill	No Spill	No Spill
12 mL	No Spill	No Spill	No Spill
13 mL	No Spill	No Spill	No Spill
14 mL	No Spill	No Spill	No Spill
15 mL	No Spill	No Spill	No Spill
16 mL	No Spill	No Spill	No Spill

Table 19: Spill data for 6-well plate at ½ speed (~30 RPM), ¾ speed (~50 RPM), and full speed (~80 RPM).

Volume of Water	Plate 1 (no sample)	Plate 2 (no sample)	Plate 3 (no sample)	Plate 4 (sample)	Plate 5 (sample)	Plate 6 (sample)
2 mL	No spill	No spill	No spill	No spill	No spill	No spill
3 mL	No spill	No spill	No spill	No spill	No spill	No spill
4 mL	No spill	No spill	No spill	No spill	No spill	No spill
5 mL	No spill	No spill	No spill	No spill	No spill	No spill
6 mL	No spill	No spill	No spill	No spill	No spill	No spill
7 mL	No spill	No spill	No spill	No spill	No spill	No spill
8 mL	No spill	No spill	No spill	No spill	No spill	No spill
9 mL	No spill	No spill	No spill	No spill	No spill	No spill
10 mL	No spill	No spill	No spill	No spill	No spill	No spill
11 mL	No spill	No spill	No spill	No spill	No spill	No spill

5.7 Motion Test: Results

Initially, samples were placed into the incubator, half stored stationary and half on the rocker at full speed. After Trypsinization, a cell count was performed for the cells adhered to the samples using a hemocytometer. There were very few cells under the hemocytometer; the count ranged from zero to one. The total cell counts from this data is tabulated in Table 20 below. When one cell was counted under the hemocytometer, the total cell count totaled 12,500. The averages for most plates were 6,250, with the exceptions being the static alginate-coated sample (0 cells) and the static uncoated sample (12,500 cells).

Table 20: Cell counts for cells adhered to samples.

	Alginate Coating		Gelatin Coating		No Coating	
	Dynamic	**Static**	**Dynamic**	**Static**	**Dynamic**	**Static**
Cell count, sample 1	12,500	0	12,500	12,500	0	12,500
Cell count, sample 2	0	0	0	0	12,500	12,500
Mean	6,250	0	6,250	6,250	6,250	12,500
Standard Deviation	8,839	0	8,839	8,839	8,839	0

The images taken of each tissue culture plate can be found in Appendix A.10. The cell counts calculated by ImageJ in each 563.2x422.4 μm image are tabulated below in Table 21. The average cell count for both the dynamic and static alginate-containing plates were higher than those of the gelatin-containing plates and uncoated sample-containing plates. The average for the static alginate-containing plate was higher than that of the dynamic plate. The gelatin-containing plate had about the same average cell count for the static and dynamic plates, with the static count slightly higher. The plate containing the uncoated sample also had a higher cell count for the static plate in comparison to the dynamic plate.

Table 21: Cell counts for plates in 563.2x422.4 μm image.

	Alginate Coating		Gelatin Coating		No Coating	
	Dynamic	**Static**	**Dynamic**	**Static**	**Dynamic**	**Static**
Cell count, sample 1	227	310	24	40	11	30
Cell count, sample 2	52	249	26	19	35	45
Mean	139.5	279.5	25	29.5	23	37.5
Standard Deviation	123.7	43.1	1.4	14.8	17.0	10.6

To determine the total number of cells in each plate, the cell count in the above tables is divided by the surface area of the image (0.2379 mm^2) and multiplied by the surface area of the plate (7854 mm^2). The total cell counts are tabulated below, in Table 22. Because these cell count numbers are much higher than 100,000 (the initial amount seeded), they cannot be used to determine the number of cells that adhered to the samples.

Table 22: Total cell counts of plates for motion test.

	Alginate Coating		Gelatin Coating		No Coating	
	Dynamic	**Static**	**Dynamic**	**Static**	**Dynamic**	**Static**
Cell count, sample 1	7.5 million	10 million	790,000	1.3 million	360,000	990,000
Cell count, sample 2	1.7 million	8.2 million	860,000	630,000	1.2 million	1.5 million

Chapter 6. Final Design Validation

We developed these adhesion and cytotoxicity tests to be used for additively manufactured biomedical implants of non-standard shape. While ISO does have standardized cytotoxicity testing, these tests are not suitable for devices made via AM, as they do not account for a complex-shaped device. Therefore, the goal of this project was to develop a test that can account for surface topography and device shape, and to determine how this affects cytotoxicity.

6.1 Impacts

For this project, it is important to consider the ramifications this project may have, both positive and negative. In this section, we discuss the different effects that this project has on the economic sphere, environmental sphere, and other areas.

6.1.1 Economics

As we were allotted $750 for the project, our testing methods are made with a low budget in mind, allowing small research groups to repeat the experiment at a low cost. This will allow for more ease of testing, allowing devices to be tested at higher rates by more research groups. AM is currently found to be less cost effective than traditional manufacturing methods, but the price is decreasing. In the future, additively manufactured devices may be cheaper than traditional devices [24].

6.1.2 Environmental Impact

The environmental impact is likely the same as with other traditional cytotoxicity testing methods. The disposal of biological waste, plastic lab supplies, and printed samples are the biggest environmental concern. Overall, AM is a more energy efficient process than subtractive manufacturing [25]. Polycarbonate can be created in 2 different ways, one of which is more environmentally friendly than the other. One method involves phosgene while the other is phosgene free. The phosgene method involves more steps, leading to more emissions, has the potential for toxic byproducts, and is more costly. The non-phosgene method requires high temperatures and pressures and is slower [26].

6.1.3 Societal Influence

The primary societal impact will be the ability for more implants to be able to be tested at lower costs, allowing implants to reach the market more easily. This will allow more people to receive more customized implants, improving their health and quality of life.

6.1.4 Ethical Concerns

This project does not have ethical concerns, but it has benefits to researchers who can use the tests, as well as patients who can utilize the custom novel implants that can be designed and tested because of this project. Researchers may be able to have new testing methods for devices, allowing more devices to be tested and approved for use if proven safe. The implants that are shown to be safe can then be used by people to help improve their health and lives.

6.1.5 Health and Safety Issues

It is beneficial to do research to mitigate potentially dangerous implants from being used. That factor makes this project a positive one from a healthy and safety standpoint. However, there are some potential health risks to additive manufacturing. For ME, the primary risks include potential inhalation of particle matter and burns are possible if the device is not handled correctly [27]. Cell based tests should be performed in a level 2 biosafety cabinet.

6.1.6 Manufacturability

The samples were manufactured using a ME 3D printer out of polycarbonate and PLA filament. FDM printers are widely available and can be purchased for around $200. Designs can be created in SolidWorks and printed on any ME printer. Polycarbonate filament and PLA filament are readily available and can be found in many places online for cheap prices.

6.1.7 Sustainability

AM processes are more sustainable than traditional manufacturing processes, primarily due to the reduced amount of raw material needed compared to traditional manufacturing processes. It is also less energy intensive, less wasteful, and less polluting than other processes. However, more research is needed to fully understand the sustainability compared to traditional manufacturing [25]. Polymers and metals used in AM are generally not more sustainable than their subtractively manufactured counterparts with some exceptions, including PLA, which is a bioplastic. There is also little potential for recyclability of additively manufactured parts due to a mixture of materials [26].

Biohazardous sharps and waste are disposed of by autoclaving the waste at 121°C and disposing of it in a landfill [28]. Sharps are kept in hard containers, and non-sharp waste is kept in a plastic autoclave/biohazard bag. Liquid biohazard is bleached with 10% bleach and 90% biohazard waste, which is poured down the sanitary sewer after 24 hours.

6.2 Testing Procedures and Project Objectives

Our tests had five main objectives. They were as follows: must be standardized, *in vitro*, accurate, rapid (less than 72 hours), and cost efficient (less than $500). One of our tests, the filter test, was unable to be tested, so we cannot rank it based on the objectives at the time.

The drop test and in motion test were *in vitro*, rapid, and cost efficient. However, we were unable to test it more than once, making them not standardized, and we were unable to test for accuracy at this time.

The manufacturing method had six main objectives: to not leave behind toxic residue, to create complex geometries, to be accessible to the team, to be material efficient, to require minimal to no post processing, and to be time effective. The method chosen fulfilled some of these objectives, while not fulfilling others. Firstly, the material extrusion does not leave behind toxic residue, which was our most important objective, as it is important the devices created are biocompatible and non-toxic. The next objective was to be able to create complex geometries. ME successfully fulfils this objective, as it is capable of printing the device we have chosen, which we classified as complex. ME is also material efficient. The material used is used in the final product without waste. We did not require post processing for the final device, so the method successfully fulfilled that objective. The final objective was time efficiency, which this method did not fulfil. There were extended times where waiting for the objects to print was needed, which we wanted to avoid with our choice of a manufacturing method.

The material had five main objectives. It was intended to be biocompatible, accessible, sterilizable, processable by AM, and clinically approved. The chosen material, polycarbonate, fulfilled all the objectives. It is known to be biocompatible. It is accessible, as it was a polymer our team already had on hand. Polycarbonate is processable by material extrusion, which is the AM process picked for this project. Finally, devices made with polycarbonate have been given clinical approval.

The device used was decided to be representative of a biomedical implant with complex geometries. The implant had curved and flat surfaces and was similarly shaped to knee implants, and it was not testable with current cytotoxicity tests, making it a good candidate for this project. Previous sample considerations included hip cups, flat cylinders, and stents. We decided that hip cups and flat cylinders were not complex enough because they involved fewer surfaces, and we decided not to use stents because we decided to use polymers rather than metals.

Chapter 7. Discussion

In this chapter, we discuss the testing procedures in context of the results.

7.1 Acellular Degradation Test

On day 3, there were issues calibrating the pH meter, so those measurements may be inaccurate. To overcome the issue, pH strips were used to try to approximate the pH. The pH was measured the next day using a different pH meter, and these readings are expected to be more accurate. Because the pH meter was likely uncalibrated when the pH was measured on day 0, we only compared the pH of the sample solutions to pH of the control solution rather than comparing the pH of the same solution on different days.

It was unexpected that the control group had a lower pH than the sample-exposed solution, as it is known that PLA creates acidic byproducts when degrading *in vivo*. This could be because the solution prepared was not a buffer solution and does not contain enzymes that are present in the human body. The plastic of the centrifuge tube that the solutions were stored in may have also interfered.

The weight changes between day 0 and day 4 may have been due to absorption, degradation, or simply user errors. Because the sample size was so small, the t test may not be very accurate to the actual behavior of the material.

7.2 Dimethyl Sulfoxide Experiments

The wells from the first DMSO experiment were seeded at 500,000 cells and showed high confluency. Because the cells were so close together, it was difficult to observe the affected cells. The plate was also stored overnight, allowing for cell multiplication.

For the second DMSO experiment, we seeded cells at much lower numbers, using four different numbers for each concentration of DMSO. This was to identify which seeding number is most appropriate for this experiment. The DMSO concentrations were also changed to include 10% instead of 35%, so that each well increased by 10% concentration relative to the previous one. The plates were incubated for 4-6 hours rather than 16, as 4-6 hours is enough time for DMSO to have a cytotoxic effect.

7.3 Control Coatings

We concluded that the gelatin was better at coating than the poly-l-lysine. While the gelatin did not coat the plate as well as an empty culture plate, but there were adhered cells, while the poly-l-lysine coated plate did not have many adhered cells. We picked gelatin as the coating of choice for the motion test.

7.4 Drop Test: Acellular Feasibility Testing

The only time a drop moved for the first acellular test with the flat-block-like surfaces was when the drop was 50 μL, the surface was the roughest of the three, and the angle was 22.8°. This is because increasing surface roughness increases the hydrophobicity of the surface if it is hydrophobic to begin with [29]. Two of the drops on the knee implant samples only rolled off after the samples moved. This implies that if the samples were fixed in place and unable to move, the drop may not be able to fall off, which is what led us to creating the drop test with the implant embedded in a support structure.

7.5 Qualitative Drop Test

Shortcomings of the qualitative drop test led us to alter it and develop the quantitative drop test procedure. Originally, the positive control was an empty well; however, this cannot be compared to cells rinsed off sample surfaces. Therefore, the positive control needed to be changed. We also realized that it was necessary to test if cells can adhere to a surface when deposited as a 50-μL drop, before testing cytotoxicity. To test adhesion, we changed the positive control to a gelatin-coated sample, which promotes cellular adhesion, and an alginate-coated sample, which cells do not adhere to. In addition to the control groups, the incubation duration was shorted from 16 hours to 4-6 hours. This shorter amount of time does not allow for the population to double but is enough time for the cells to settle and adhere to the surface. Not many cells would multiply in this amount of time, so the number of cells dislodged from the sample can be directly compared to the seeding number.

7.6 Motion Test: Acellular Feasibility Testing

When testing what conditions would cause liquid to spill out of the culture plates, we found that there were no spills for any of the plates. Both a 100mm plate and a 6-well plate are acceptable for the use of this project, as they can hold enough volume for the cells to be able to grow and cover the surface. For the experiment with the 100mm plate, we started with a volume of 11 mL, the recommended minimum volume for cell culture medium in a 100mm plate [28], and stopped at 16 mL, the highest recommended volume of cell medium for this plate [28]. For the 6-well plate, we began with 2 mL. We stopped at 11 mL because adding any more liquid would have caused overflow before the plates were placed on the rocker.

7.7 Motion Test

The cell numbers found by ImageJ were much too high for the number of cells initially seeded. This may be because the cells were not evenly distributed throughout the plate, and a single image was not representative of the entire plate. According to the cell count, very few cells adhered to the samples, which may be because not enough of the sample surface was exposed to the culture medium.

Chapter 8. Conclusions and Future Recommendations

Our team has some recommendations for how to improve this project in the future. These recommendations range from sample fabrication and preparation to changes to the testing procedures.

8.1 Material Samples

Firstly, we would suggest using a different sterilization technique or a different material. Despite carbon-fiber polycarbonate having a high enough glass transition temperature to withstand autoclave sterilization, it still warped in the autoclave, possibly because it may have fillers that lower the temperature it can handle. For future testing with polymer samples, UV or ethylene oxide sterilization should be used instead. Another option would be to experiment with metals, which have higher melting points than polymers. Metals are often used in bone and dental implants and are therefore important to be tested in the context of additively manufactured biomedical implants. Metals are more difficult to print but are available. Another polymer that could be used would be dental resin, given the availability of a vat photopolymerization machine. Vat photopolymerization would have a smoother surface finish than material extrusion would be able to achieve. Dental resin is known to be non-cytotoxic. It is unclear at this time whether it would be able to keep its shape in the autoclave, or if another method would need to be used.

For the motion test, we would suggest using implants small enough to fit into the 6-well plate to fix the problem we had with the samples being too large. The knee implant shape was good for testing, but for all the experiments we would suggest adding other shapes into testing, including flat blocks and individual sections of the knee implant. The test could also be validated by using more types of implants (e.g., hip implants and stents), materials (e.g., PLA and metals), and AM processes (e.g., PBF and vat photopolymerization).

8.2 Control Groups

The alginate coating to be used as a non-adhesive control was very thick, making the entire sample harder to fit in the plate. This could be fixed by soaking the samples in alginate for a shorter duration than 30 minutes, such as 10 minutes. The solution was also difficult to sterilize via vacuum filtration, as it was too thick and would clog the filter. Conversely, we are unsure if the gelatin-coated sample was successfully coated in gelatin, as the procedure was adapted from coating a plate and may not apply to a shape with multiple curves and edges. There may also be a better adhesion coating than gelatin, as results from the well-coating experiment showed that the empty well plate promoted more cellular adhesion than the gelatin coating.

For the filter test, we would suggest using a cytotoxic or a cytotoxic-coated material instead of using a sample soaked in DMSO. This new control group could be a sample coated with latex solution. Further research is needed for other choices of cytotoxic control groups.

8.3 Testing Procedures

For the testing procedures, each experiment would need to be run multiple times to test for reproducibility, which is one of our objectives. After performing the tests multiple times, accurate statistical testing can be done to test for statistical differences among the cell counts. We were unable to test for reproducibility in this project due to time. The filter diffusion test must also be performed in the lab in order to validate it as a testing method.

8.3.1 Acellular Degradation Test

Another suggestion we would make is to improve upon the acellular degradation test. More samples and control groups could be tested to obtain more accurate results. Samples could be tested in glass jars to eliminate the possibility of the solutes interacting with the container. We would also recommend calibrating and using more accurate pH meters every time the experiment is run to get more accurate results.

8.3.2 Drop Test

For the drop test, we would suggest quantitatively testing it with coated samples as positive and negative adhesion controls. The method uses gelatin for the positive adhesion control, alginate for the negative adhesion control, and uncoated samples. The samples have a drop placed on them and are incubated, after which they are rinsed with complete medium. The test uses trypan blue to stain dead cells, and a count is taken from each plate to test for adhesion and cytotoxicity.

8.3.3 Motion Test

Visually inspecting the images from the motion test, it first seemed like there were more cells in the plates containing the alginate-coated samples than the other plates, which means that less cells stuck to the alginate-coated samples than the other samples. However, the statistical tests performed indicate that the cell counts are not statistically different. Future testing should be repeated with a larger number of samples per group to obtain a better sample size for statistical testing.

Due the samples being solid black in color, adhered cells could not be directly observed under the microscope. We attempted to overcome this limitation by Trypsinizing the samples and performing a cell count, but another option would be to perform feasibility testing with transparent samples such as polydimethylsiloxane (PDMS) to ensure that cells have the ability to adhere to sample while the culture plate is in motion. PDMS cannot be fabricated with AM, so it would only be able to be used for adherence feasibility testing. It can also be formed into a complex shape, like a knee implant, if it is cured inside a mold. This mold could be fabricated using AM. Another solution to this would be to image the samples with fluorescence microscopy. This has the possibility of being able to stain the cells and view them rather than performing a cell count with ImageJ.

The cell-counting process for both the cells inside the plates and the cells adhered to the sample can be improved. Instead of counting in ImageJ, the plates can be Trypsinized. For the samples, the entire plate containing the sample could be filled with Trypsin to ensure maximum Trypsin contact. However, implementing both methods would require a large volume of Trypsin.

8.4 Filter Diffusion with Cell Staining in Motion

This test intends to determine whether it is better for a filter test to be in motion while the test is conducted rather than stationary, for the purpose of leachables. A detailed protocol for this experiment can be found in Appendix A.7. Prior to the experiment, cells are cultured and passaged onto a 6-well plate at 250,000 cells for each well. To perform the experiment, cells are first cultured onto 0.45 mm Millipore filters in each well of a 6-well plate and put into the incubator for 4-6 hours to allow the cells to adhere to the filter. In another 6-well plate, an agar layer is prepared at the bottom of each well. Each Millipore filter is transferred to the plate containing the agar layer, with the cell side of the filter facing the agar. Sterilized specimens are placed into their respective wells, with two wells containing gelatin-coated noncytotoxic control, two wells containing DMSO-coated cytotoxic control, and two wells containing uncoated experimental samples. This procedure is repeated for a total of two well plates. One plate is the stationary control, and the other plate on the cell rocker is the dynamic experimental group. Figure 11 shows a schematic representing one of the wells. These plates are incubated for 48-52 hours to allow the cells enough time for 1-2 population doubling periods. Post incubation, the specimens are removed, and the filters are removed as well. They are then stained with trypan blue and microscopically evaluated by performing two cell counts; one for dead cells, and one for alive cells in order to determine viability. The cytotoxicity is measured quantitatively using the reactivity grade table in Appendix A.8.

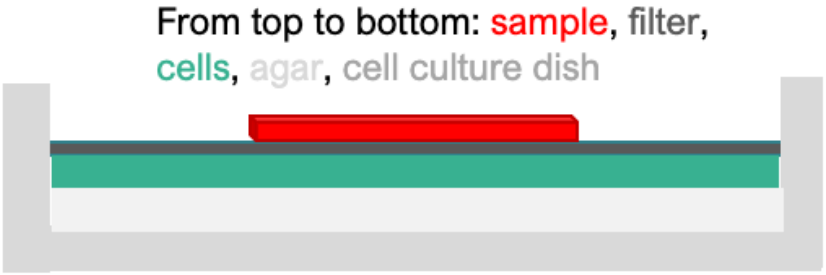


Figure 25: Schematic representation of the sample on top of the filter and agar layer in one of the wells.

8.5 Final Conclusions

The goal of this project was to develop cytotoxicity and adhesion tests for additively manufactured biomedical implants with complex device shapes. To do this, we determined that a

knee implant was a representative sample containing many curves and edges. This knee implant was composed of polycarbonate and fabricated by ME. The three testing proposals we developed and explored were the drop test, motion test, and filter diffusion test. To validate these procedures, more testing must be done to ensure accuracy and reproducibility. The filter diffusion test was not performed due to lack of time and some resources. This procedure did not meet any objectives, and in future work we suggest that this method be performed and evaluated in meeting the objectives.

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Appendices

A.1 Gantt Charts for A-D Terms

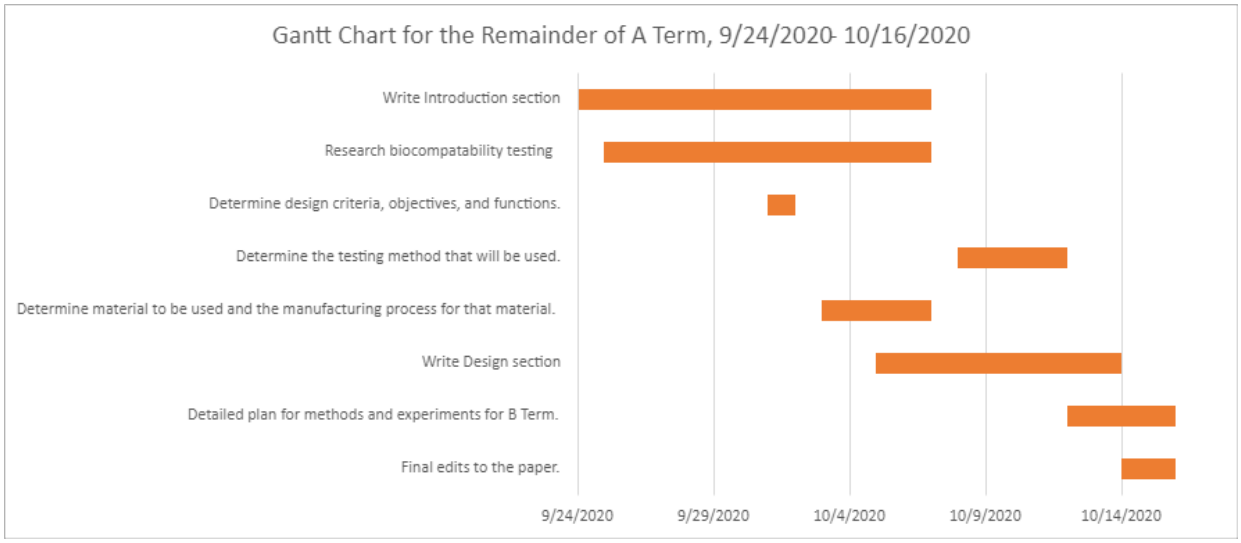


Figure 26: Gantt Chart for A Term.

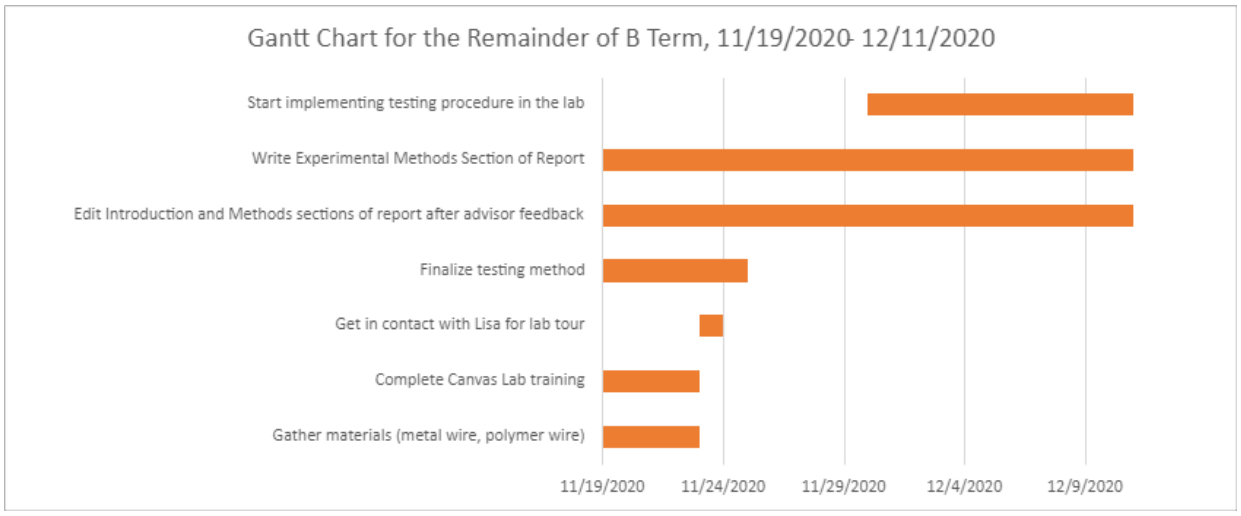


Figure 27: Gantt Chart for B Term.

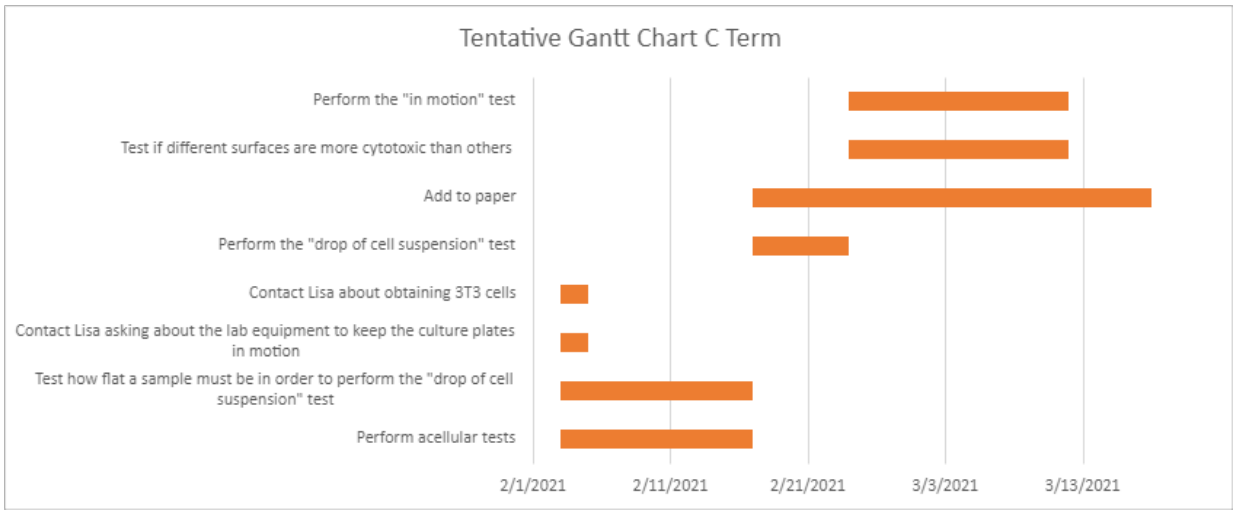


Figure 28: Gantt Chart for C Term.

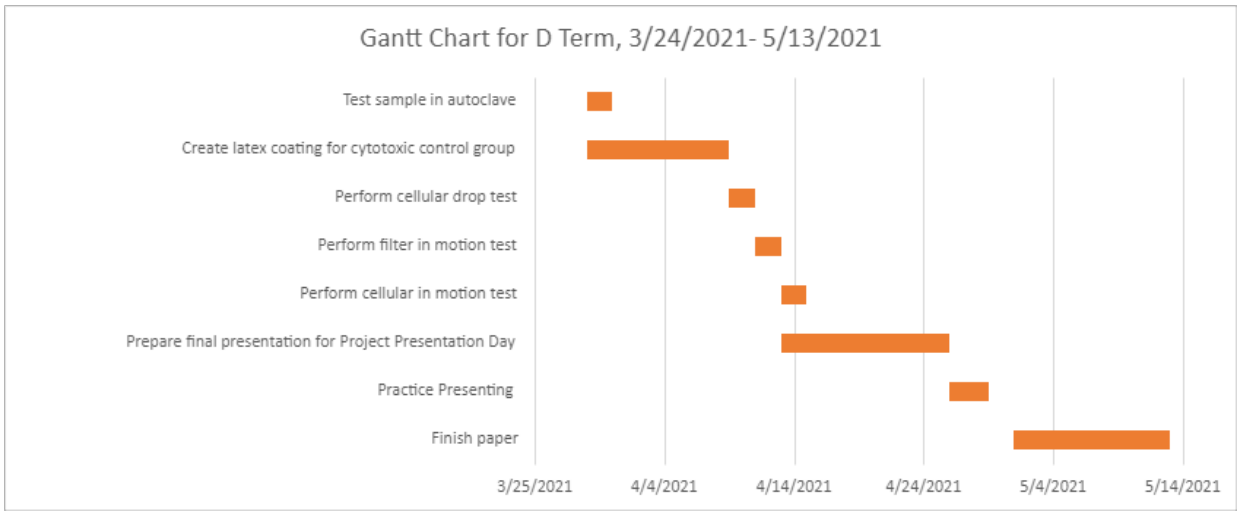


Figure 29: Gantt Chart for D Term.

A.2 Acellular Degradation Test Procedure

This procedure details how we performed the acellular degradation test, including the production of a 100-mL solution of simulated body fluid. The simulated body fluid procedure is adapted from T. Kokubo et. Al. [30].

Materials:

- Two bar-shaped PLA samples (5mmx25mmx2mm)
- Glassware/plasticware
    - Graduated cylinder
    - One 150-mL beaker
    - Stir bar
    - Three 15-mL centrifuge tubes
    - One 250- to 100-mL bottle
    - Pasteur pipets
    - Forceps
- Laboratory equipment
    - Analytical balance
    - Stirrer
    - pH meter
    - 4°C refrigerator
    - Vacuum pump
- Salts
    - NaCl
    - $NaHCO_3$
    - MgCl
    - $CaCl_2$
    - Na_2SO_4
    - Tris
    - KCl
- Solutions and liquids
    - DI water
    - 1.0-M HCl

Part 1: Preparing the samples

1. Using CAD software such as SolidWorks, design a rectangular object with a surface area of 5x25mm and thickness of 2mm.
2. Print two samples of the part for the test.

Part 2: Creating the electrolytic solution

3. Weigh the following amounts of salts:

Table A.1: Salts for simulated body fluid solution.

Salt	Weight (g)
NaCl	0.8035
$NaHCO_3$	0.0355
MgCl	0.0311
$CaCl_2$	0.0292
Na_2SO_4	0.0072
Tris	0.6118
KCl	0.0225

4. Measure 96.1 mL of deionized water in a graduated cylinder.
5. From the graduated cylinder, add about 15 mL of deionized water to a 150-mL beaker.
6. Add the NaCl to the beaker and stir. Once dissolved, add the next salt. Continue until all salts are dissolved.
7. Add the rest of the DI water to the beaker.
8. Add 3.9 mL of 1.0-M HCl solution to the beaker.
9. Measure the pH of the solution using a pH meter and adjust to 7.4 by adding 1.0-M HCl.

Part 3: Addition of samples to solution

10. Weigh each PLA sample and record observations.
11. Put each PLA sample into a centrifuge tube.
12. Transfer about 10 mL of solution to each PLA-containing centrifuge tube, as well as another empty centrifuge tube to act as the control.
13. Transfer remainder of the solution to a bottle for later use. Store at 4°C.
14. Store centrifuge tubes at 4°C.

Part 4: Observation of pH and mass change

15. After 3 days, measure the pH of the solution in each centrifuge tube.
16. For the tubes containing the samples, aspirate the solution so that the sample is as dry as possible.
17. Weigh each sample and record observations.

A.3 Degradation and Leaching with pH and Cell Staining Procedure

This is a step-by-step protocol of how the cellular degradation/leaching test would be performed.

Materials:

- Samples to be tested (sterilized with autoclave)
- Autoclave bag
- pH strips
- Trypan Blue
- Cell culture materials
    - 10% complete medium
    - 3T3 mouse fibroblast cells
    - DPBS (-)
    - Trypsin
- Glassware/plasticware
    - 100 mm tissue culture plates
    - Forceps (sterilized with autoclave)
    - Pasteur pipets
    - Serological pipets
    - Hemocytometer
    - Micropipette
    - Micropipette tips
    - Microfuge tubes
- Laboratory equipment
    - 4°C refrigerator
    - Incubator
    - Vacuum pump
    - Centrifuge
    - Microscope
    - Cell counter clicker

Day 0

1. Prepare 2 samples of each chosen material, each with a surface area of 5mmx25mm and thickness of 1.5mm.
2. Weigh each sample and place each in an autoclave bag.
3. Sterilize materials using an autoclave for 45 minutes. Once sterilized, the following steps should be done in a biosafety cabinet.
4. Add metal/polymer sample to a tissue culture plate, then add 10 mL of cell culture medium.
5. Use a pH strip to identify the pH of the medium.
6. Incubate at 37°C for 3 days.

Day 3

7. Prepare cells for culturing. After adding cell suspension to centrifuge tube, remove a 7-µL sample for a cell count.
8. Take note of the cell count.
9. After centrifuging the cells, aspirate the medium.
10. Remove the culture plate containing the sample from the incubator. Record noticeable observations, such as color change of the medium, cracks in the sample, or dimensional changes of the sample. Record the pH with a pH strip.
11. Add 5 mL of the sample-exposed medium to the centrifuge tube containing the cell pellet. Mix by repeated pipetting. Add 100,000 cells of this cell suspension to the well of a 6-well plate and add sample-exposed medium such that the total volume in the well is 5 mL.
12. Label culture plate with date and cell count. Incubate at 37°C for 3 days.
13. The samples can be disposed of in the regular trash, as they were not exposed to cells.

Day 6

14. Record the pH of the medium with a pH strip.
15. Culture and count cells. To count cells, add a 200-µL sample from the suspension to a 1.5-mL microfuge tube. Add 200 µL of Trypan Blue to the tube and take a 7-µL sample for the cell count.
16. Record the amount of living cells and dead cells.

A.4 Control Coating Procedures

The following protocols detail how the alginate and gelatin coatings were made and applied to the well plates and samples.

A.4.1 Preparation of Alginate Solution

This protocol outlines how to create, sterilize, and store the 1.5% alginate solution that was used to coat the wells and samples.

Materials:

- DI water
- Alginate powder
- Parafilm
- Glassware/plasticware
    - 150-mL beaker
    - Graduated cylinder(s)
    - Stir bar
    - Vacuum filters
    - 50-mL centrifuge tubes
- Laboratory equipment
    - Stirrer
    - Vacuum pump
    - 4°C refrigerator

Procedure:

1. Prepare a 150-mL beaker containing a stir bar and 49.25 mL of DI water, and place it on a stirrer.
2. Measure 0.75 g of alginate powder and slowly add it to the beaker as the stirrer is on.
3. After adding all the powder, let the solution be stirred at medium-high speed for 30-40 minutes.
4. Sterilize the solution using vacuum filtration.
5. Wrap the solution in parafilm and store at 4°C.

A.4.2 Preparation of Gelatin Solution

This protocol outlines how to create, sterilize, and store the 2% gelatin solution that was used to coat the wells and samples.

Materials:

- DI water
- Gelatin powder
- Parafilm
- Glassware/plasticware

    - 150-mL beaker
    - Graduated cylinder(s)
    - Stir bar
    - 100-mL bottle
- Laboratory equipment
    - Stirrer
    - 4°C refrigerator

Procedure:

1. Prepare a 150-mL beaker containing 49 mL of DI water and a stir bar. Place on the stirrer.
2. As the stir bar is stirring the solution, slowly add 1 g of gelatin powder.
3. Let dissolve, and transfer solution to a 100-mL bottle.
4. Store the bottle at 4°C until it can be autoclaved. If not being used immediately after autoclaving, wrap in parafilm and store at 4°C.

A.4.3 Alginate Well Plate Coating

This protocol outlines how we coated the bottoms of a 6-well plate with a 1.5% alginate solution.

Materials:

- DI water
- $CaCl_2$ salt
- Sterilized 1.5% alginate solution
- DPBS (-)
- Parafilm
- Glassware/plasticware
    - 150-mL beaker
    - Graduated cylinder(s)
    - Stir bar
    - 100-mL bottle
    - 6-well plate
    - Serological pipets
    - Pasteur pipets
- Laboratory equipment
    - Balance
    - Stirrer
    - Vacuum pump
    - 4°C refrigerator

Part 1: 5-M $CaCl_2$ solution

1. Weigh 27.745 g of $CaCl_2$ salt to use for the 5-M solution.
2. Prepare a 150-mL beaker containing a stir bar and 50 mL of DI water. Place on stirrer.
3. Turn on stirrer to medium speed. Slowly pour in the salt.

4. Once dissolved, transfer solution to a 100-mL bottle.
5. Autoclave the solution.
6. Add 5 mL of the sterilized $CaCl_2$ solution to two wells of a 6-well plate. Let sit overnight in the biosafety cabinet.

Part 2: Coating the well

7. Aspirate $CaCl_2$ solution.
8. Add 1.5 mL of sterilized 1.5% alginate solution to each well that had the $CaCl_2$ solution.
9. Let dry for 40 minutes.
10. Rinse each well containing the coating with 5 mL of DPBS (-) or other buffer (not containing $CaCl_2$).
11. If the plate is not being used right away, wrap in parafilm and store at 4°C.

A.4.4 Gelatin Well Plate Coating

This protocol outlines how we coated the bottoms of a 6-well plate with a 2% gelatin solution.

Materials:

- Sterilized 2% gelatin solution
- Parafilm
- Glassware/plasticware
    - 6-well plate
    - Micropipette
    - Micropipette tips
- Laboratory equipment
    - 4°C refrigerator
    - Incubator

Procedure:

1. Incubate bottle of sterilized 2% gelatin solution for 10 minutes to allow it to liquify.
2. To coat the entire well in a thin layer of gelatin, first add 95 µL of the solution. Add another 95 µL, and then finally 50 µL. This is a total of 240 µL.
3. Let coating dry for two hours. Store at 4°C in parafilm if not being used immediately.

A.4.5 Alginate Sample Coating

This protocol outlines how we coated the knee implant samples with 1.5% alginate solution.

Materials:

- Four knee implant samples (sterilized with autoclave)
- DI water
- $CaCl_2$ salt

- Sterilized 1.5% alginate solution
- DPBS (-)
- Parafilm
- Glassware/plasticware
    - 150-mL beaker
    - Graduated cylinder(s)
    - Stir bar
    - 100-mL bottle
    - 50-mL centrifuge tubes
    - Serological pipets
    - Pasteur pipets
    - Forceps (sterilized with autoclave)
    - 6-well plates
- Laboratory equipment
    - Balance
    - Stirrer
    - Vacuum pump
    - 4°C refrigerator

Procedure:

1. Make and sterilize a 5-M $CaCl_2$ solution as described in Appendix A.4.3 Part 1.
2. Add the four knee implant samples to a 50-mL centrifuge tube.
3. Fill the tube with the sterile 5-M $CaCl_2$ solution such that the tube can be closed with minimal air bubbles.
4. Leave overnight in the biosafety cabinet.
5. The next day, aspirate the $CaCl_2$ solution from the tube.
6. Pour the samples into a new 50-mL centrifuge tube.
7. Fill the tube with sterilized 1.5% alginate solution such that there are no air bubbles, as done in step 2.
8. Let soak for 30 minutes.
9. Remove samples one at a time using forceps.
10. Rinse each sample with DPBS (-) or another buffer that does not contain $CaCl_2$.
11. Let each sample dry in a 6-well plate for 30-60 minutes.
12. To dispose of the solution that the samples were soaking in, add sodium polyacrylate powder and dispose in the trash.
13. If not being used immediately, store the samples in a 6-well plate, wrap with parafilm, and store at 4°C.

A.4.6 Gelatin Sample Coating

This protocol outlines how we coated the knee implant samples with 2% gelatin solution.

Materials:

- Four knee implant samples (sterilized with autoclave)

- Sterilized 2% gelatin solution
- Parafilm
- Glassware/plasticware
    - Sterilized forceps
    - 50-mL centrifuge tube
    - Serological pipets
    - Forceps (sterilized with autoclave)
    - 6-well plate
- Laboratory equipment
    - 4°C refrigerator
    - Incubator

Procedure:

1. Incubate bottle of sterilized 2% gelatin solution at 37°C for 10 minutes.
2. Using forceps, place the four knee implant samples in a 50-mL centrifuge tube and fill with gelatin solution so that there are no air bubbles. Let soak for 30 minutes.
3. Remove samples from tube and place in a 6-well plate to dry for two hours.
4. To dispose of the solution that the samples were soaking in, add sodium polyacrylate powder and dispose in the trash.
5. If not being used immediately, store the samples in a 6-well plate, wrap with parafilm, and store at 4°C.

A.5 Qualitative Drop Test Procedure

This protocol outlines how we performed the qualitative drop test.

Materials:

- Four modified knee implant samples (sterilized with autoclave)
- Dimethyl sulfoxide
- Cell culture materials
    - 10% complete medium
    - 3T3 mouse fibroblast cells
    - DPBS (-)
    - Trypsin
- Glassware/plasticware
    - Forceps (sterilized with autoclave)
    - 6-well plate
    - Serological pipets
    - Pasteur pipets
    - 15-mL centrifuge tubes
    - Hemocytometer
    - Micropipette
    - Micropipette tips
- Laboratory equipment
    - Vacuum pump
    - Microscope
    - Cell counter clicker
    - Incubator

Day 1:

1. Sterilize all samples and forceps prior to experiment with an autoclave.
2. Using the forceps, place each sample inside well of a 6-well plate and label plate; accordingly, two samples are the experimental group and two are the cytotoxic control.
3. Culture cells and reseed at 500,000 cells in a 15-mL centrifuge tube.
4. Place 17.5 µL of DMSO on the surface of the two cytotoxic control samples.
5. Place a 50-µL drop of cell suspension (this contains 5,000 cells) on top of the DMSO drop on the cytotoxic controls. Place two more 50-µL drops on the experimental samples and two more in the remaining empty wells.
6. Observe positive control (empty well) under microscope to ensure that cells are present and image cells.
7. Incubate for 16 hours.

Day 2:

8. Turn samples on their side using sterile forceps.
9. Gently rinse surface containing the drop with 1 mL of complete medium. Remove samples.
10. Observe cells under microscope and image.

A.6 Motion Test Procedure

This protocol outlines how we performed the motion test.

Materials:

- Four experimental knee implant samples (sterilized with autoclave)
- Four alginate-coated knee implant samples – negative control (sterilized with autoclave)
- Four gelatin-coated knee implant samples – positive control (sterilized with autoclave)
- Lab tape
- Cell culture materials
    - 10% complete medium
    - 3T3 mouse fibroblast cells
    - DPBS (-)
    - Trypsin
- Glassware/plasticware
    - Serological pipets
    - Pasteur pipets
    - 15-mL centrifuge tubes
    - Hemocytometer
    - Micropipette
    - Micropipette tips
    - 100 mm tissue culture plates
    - Forceps (sterilized with autoclave)
    - 6-well plates
- Laboratory equipment
    - Microscope
    - Cell counter clicker
    - Incubator
    - Rocker

Procedure:

1. Prior to the experiment, sterilize (via autoclave) and coat samples as necessary. Forceps are also sterilized using the autoclave.
2. Subculture and count cells.
3. In twelve 100 mm tissue culture plates, seed 100,000 cells per plate with 10 mL of medium.
4. Label the culture plates according to coating, sample number, and whether it will be stored stationary or in motion.
5. Using the forceps, place each sample inside each appropriate culture plate. Tape is necessary to keep the covers on.
6. Place the plates labeled as the "motion" plates on the rocker inside the incubator. Place the plates labeled "stationary" in the incubator on the shelf, not moving.
7. Turn on the rocker and set it to full speed.
8. Incubate for 4-6 hours.
9. Turn off rocker and remove plates from incubator.

10. Add 3 mL of Trypsin to each well of two 6-well plates.
11. Remove all twelve samples with sterile forceps and transfer them to the two 6-well plates, ensuring that the part of the sample exposed to the medium is soaked in Trypsin. Incubate for 10 minutes.
12. Add 2 mL of culture medium and transfer each suspension to a 15-mL centrifuge tube.
13. Remove a 7-μL sample for a cell count of the cells adhered to the samples.
14. For the remaining empty 100 mm plates, image each plate under the microscope to perform a cell count in ImageJ.

A.7 Filter Diffusion with Cell Staining Procedure

Materials:

- Four experimental samples (sterilized with autoclave)
- Four alginate-coated samples – noncytotoxic control (sterilized with autoclave)
- Four DMSO-coated samples – cytotoxic control (sterilized with autoclave prior to the coating with sterilized DMSO)
- Twelve 0.45 mm Millipore filters
- Agarose
- Trypan blue
- Cell culture materials
    - 10% complete medium
    - 3T3 mouse fibroblast cells
    - DPBS (-)
    - Trypsin
- Glassware/plasticware
    - Serological pipets
    - Pasteur pipets
    - 15-mL centrifuge tubes
    - Hemocytometer
    - Micropipette
    - Micropipette tips
    - 6-well plates
    - Forceps (sterilized with autoclave)
- Laboratory equipment
    - Microscope
    - Cell counter clicker
    - Incubator
    - Cell rocker

Procedure:

1. Prior to the experiment, sterilize (via autoclave) and coat samples as necessary. Forceps are also sterilized using the autoclave.
2. Establish a monolayer of 10^6 cells/mL of fibroblast cells onto a sterilized 0.45 μm Millipore filter and store 4-6 hours in the incubator at 37°C, 5% CO_2. Repeat this for a total of twelve Millipore filters.
3. Prepare two 6-well plates with a 3-mm agar layer on the bottom of the wells.
4. Place the incubated filters onto each agar layer, cell side down, using sterilized forceps.
5. Place sterilized test specimen(s) (recommended dimensions: 5 mm x 25 mm x 1.5 mm) onto the filters. Label the plate accordingly.
6. Place one plate onto the shelf in the incubator (stationary) and the other on the cell rocker (in motion) in the incubator.
7. Turn on the rocker and set to full speed.
8. Incubate for 48-52 hours.

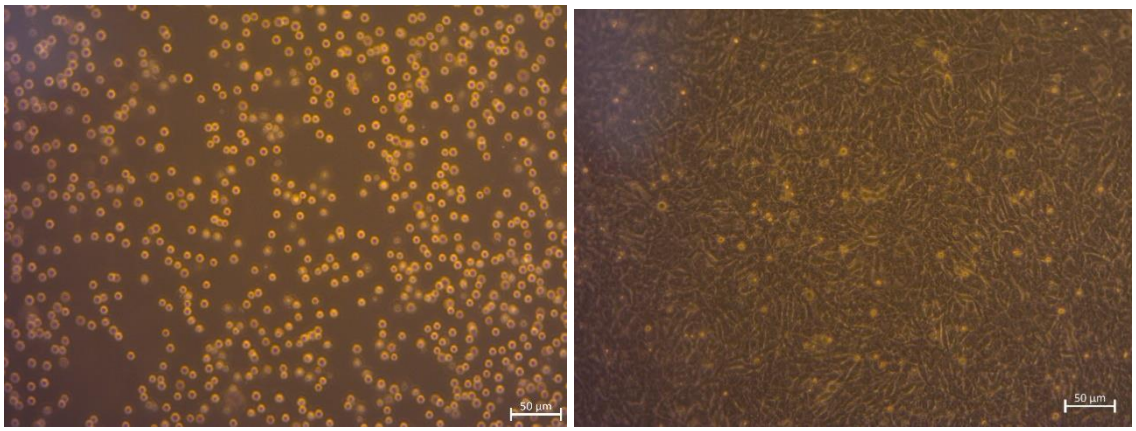
9. Turn the rocker off and remove the plates from the incubator.
10. Remove specimens and stain the filters with 1mL of trypan blue.
11. Perform two cell counts for the stained (dead) cells and the unstained (alive) cells.
12. Determine viability by dividing the total cell (alive + dead) by the alive cells. Multiply by 100 to get a percentage.
13. Measure the cytotoxicity using the reactivity grade table from Appendix A.8.

A.8 Reactivity Grade Table

Table A.8: Reactivity grade table.

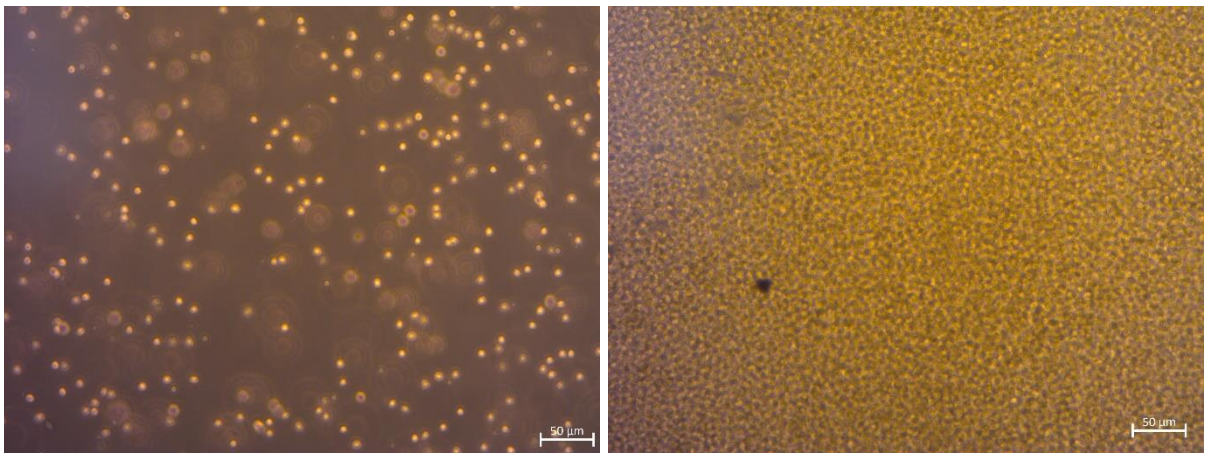
Grade	Reactivity	Conditions
0	None	No malformed cells, 100% viability
1	Slight	Few malformed cells under specimen, 90% viability
2	Mild	Malformed cells limited to the zone under the specimen, 80% viability
3	Moderate	Malformed cells extend the zone up to 1 cm, 70% viability
4	Severe	Zone of malformed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

A.9 Images from DMSO Experiments



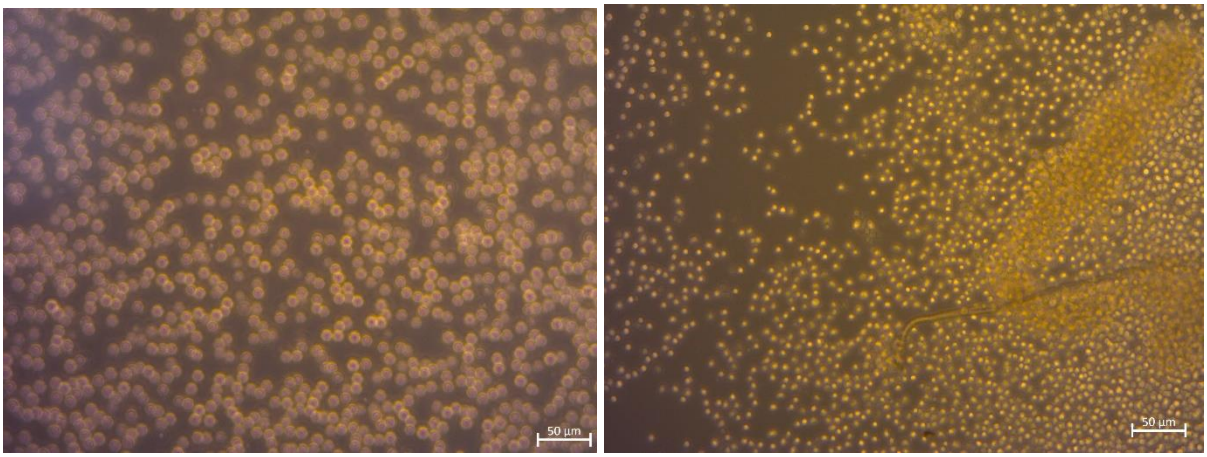
a. Before incubation *b. After incubation*

Figure 30: 500,000 cells at 0% DMSO concentration before (a) and after (b) incubation.



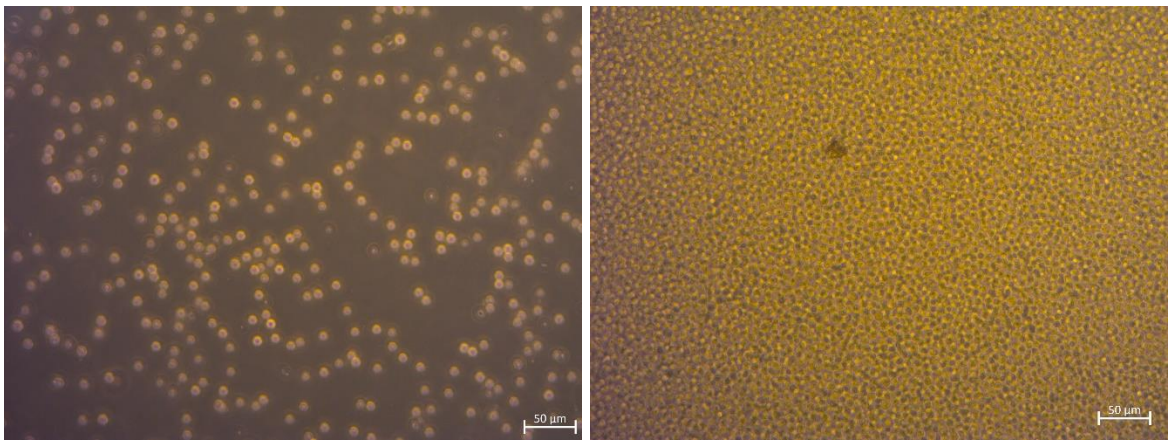
a. Before incubation *b. After incubation*

Figure 31: 500,000 cells at 20% DMSO concentration before (a) and after (b) incubation.



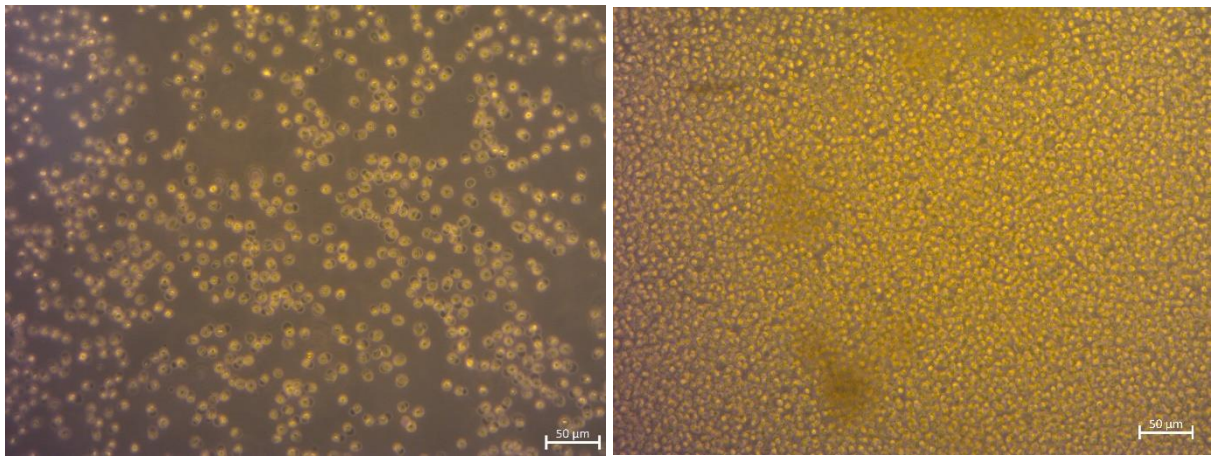
a. Before incubation *b. After incubation*

Figure 32: 500,000 cells at 30% DMSO concentration before (a) and after (b) incubation.



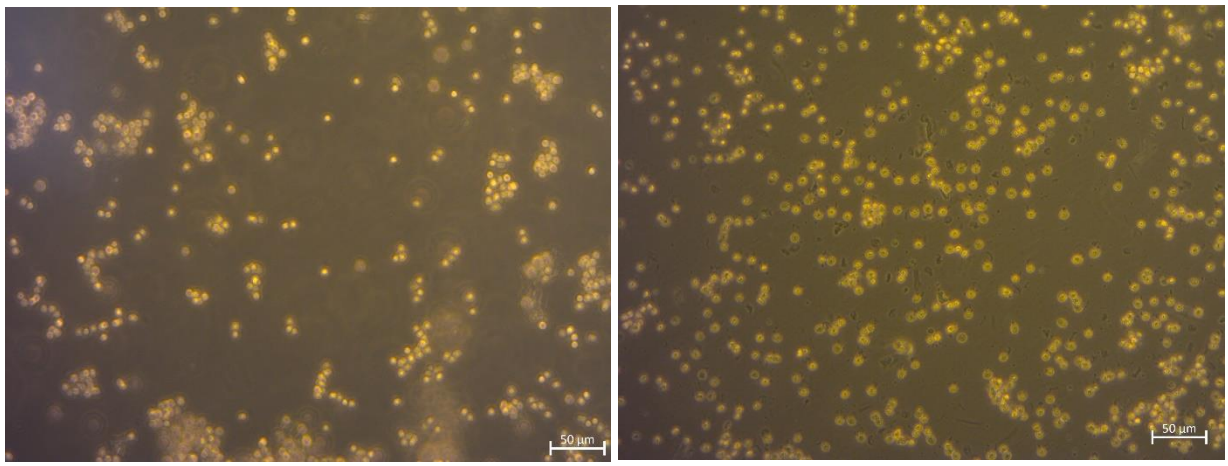
a. Before incubation *b. After incubation*

Figure 33: 500,000 cells at 35% DMSO concentration before (a) and after (b) incubation.



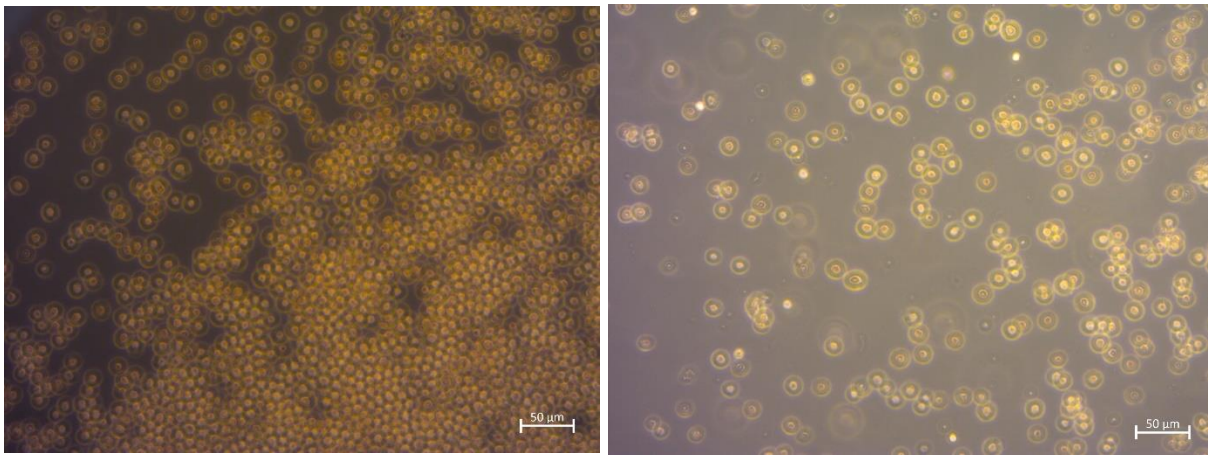
a. Before incubation *b. After incubation*

Figure 34: 500,000 cells at 40% DMSO concentration before (a) and after (b) incubation.



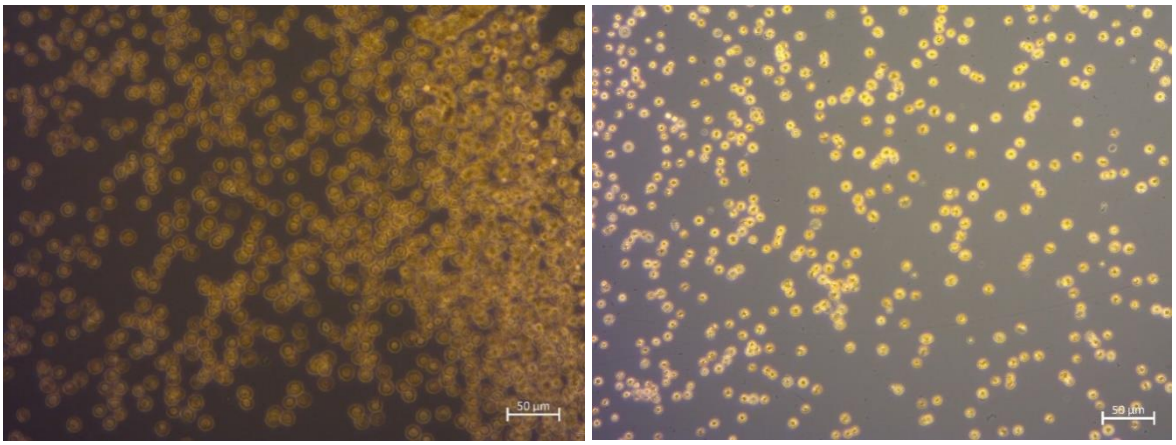
a. Before incubation *b. After incubation*

Figure 35: 500,000 cells at 50% DMSO concentration before (a) and after (b) incubation.



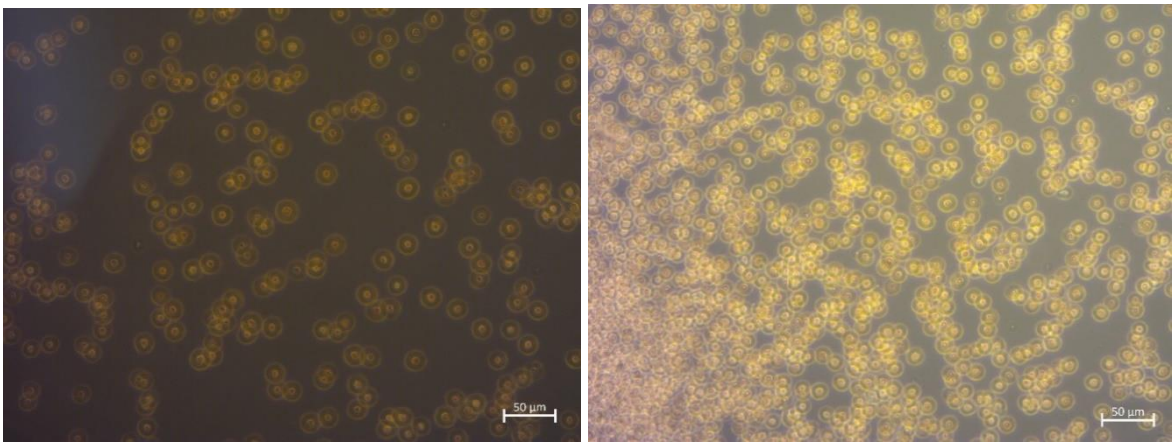
a. Before incubation *b. After incubation*

Figure 36: 25,000 cells at 20% DMSO concentration before (a) and after (b) incubation.



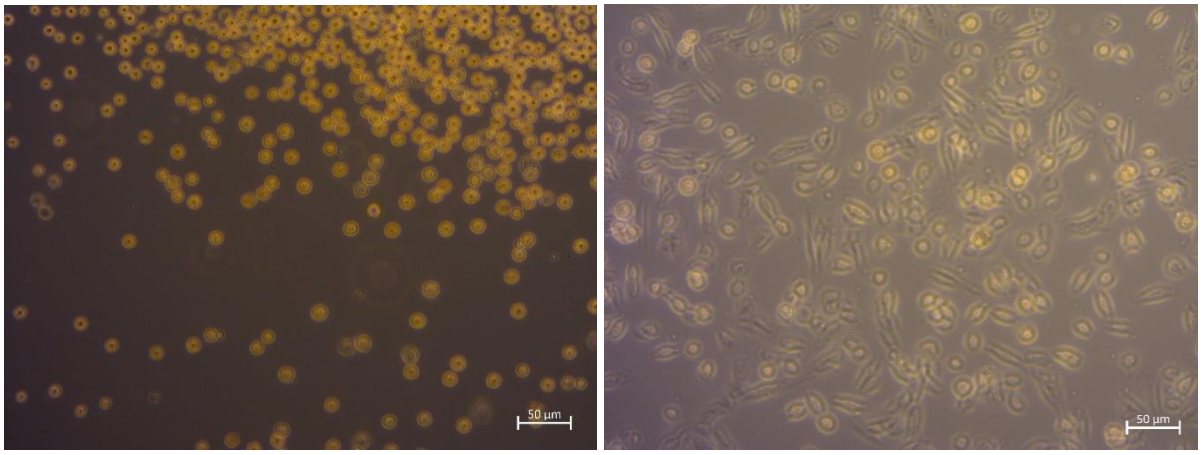
a. Before incubation *b. After incubation*

Figure 37: 25,000 cells at 30% DMSO concentration before (a) and after (b) incubation.



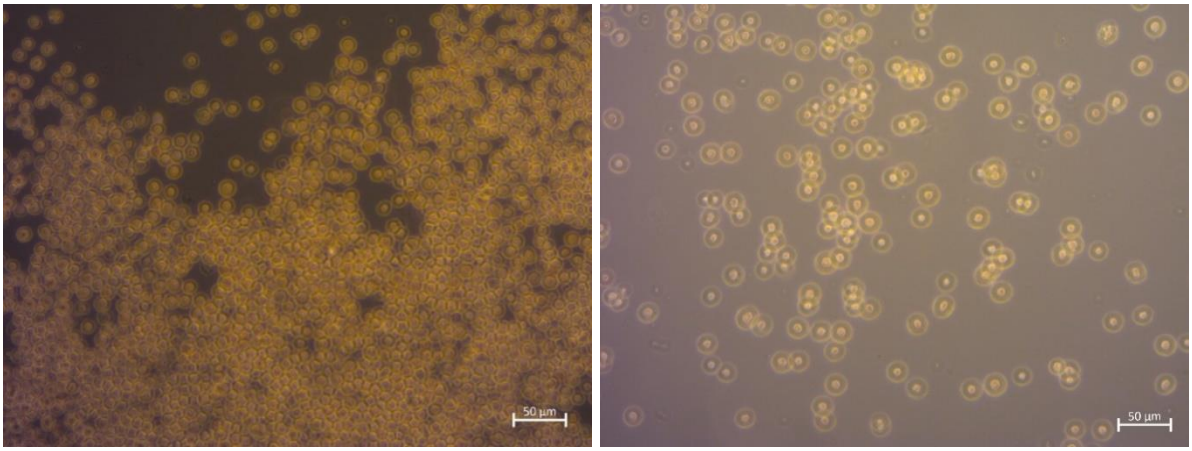
a. Before incubation *b. After incubation*

Figure 38: 25,000 cells at 40% DMSO concentration before (a) and after (b) incubation.



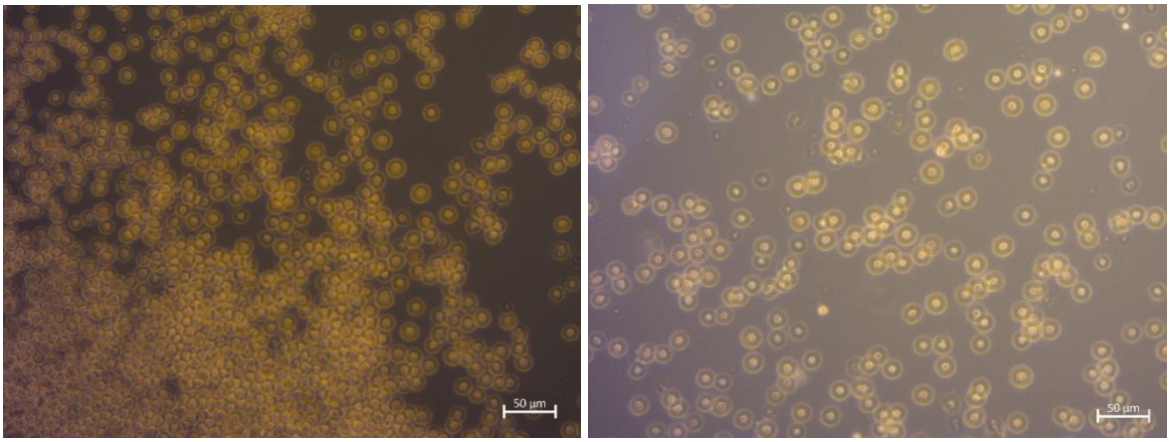
a. Before incubation *b. After incubation*

Figure 39: 50,000 cells at 0% DMSO concentration before (a) and after (b) incubation.



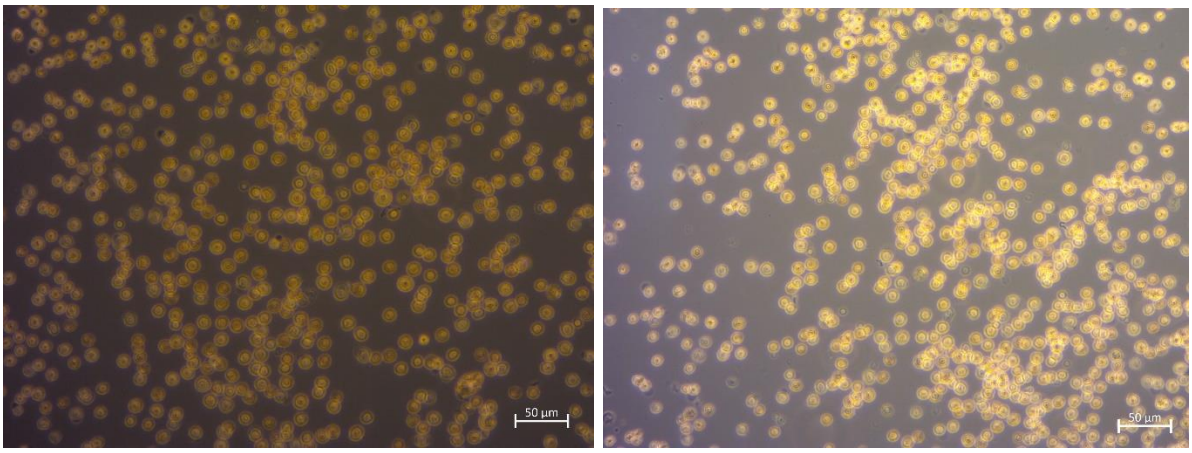
a. Before incubation *b. After incubation*

Figure 40: 50,000 cells at 10% DMSO concentration before (a) and after (b) incubation.



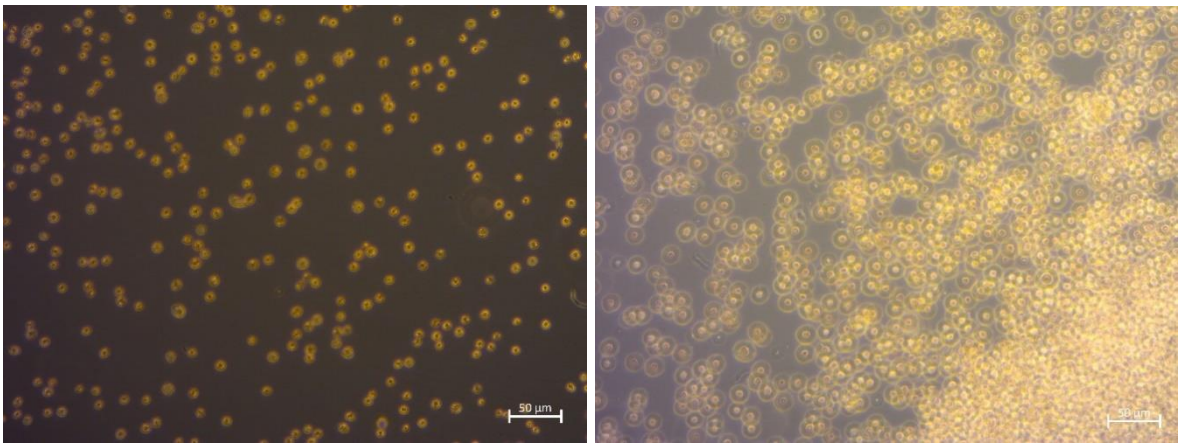
a. Before incubation *b. After incubation*

Figure 41: 50,000 cells at 20% DMSO concentration before (a) and after (b) incubation.



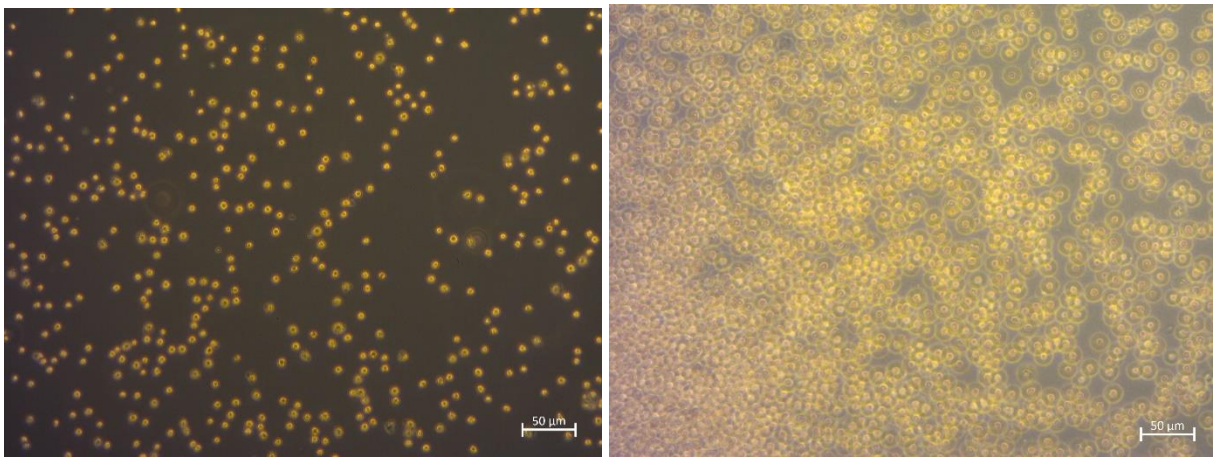
a. Before incubation *b. After incubation*

Figure 42: 50,000 cells at 30% DMSO concentration before (a) and after (b) incubation.



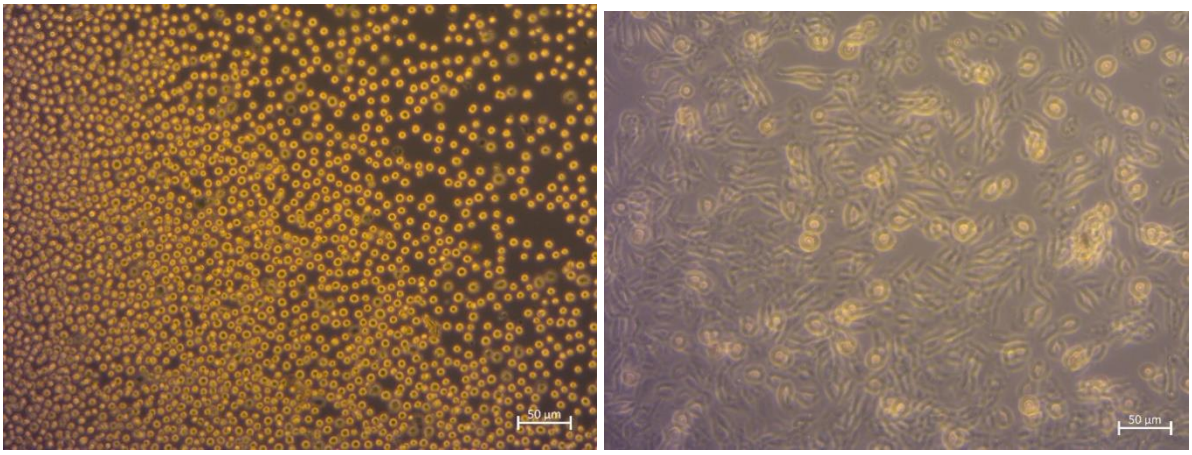
a. Before incubation *b. After incubation*

Figure 43: 50,000 cells at 40% DMSO concentration before (a) and after (b) incubation.



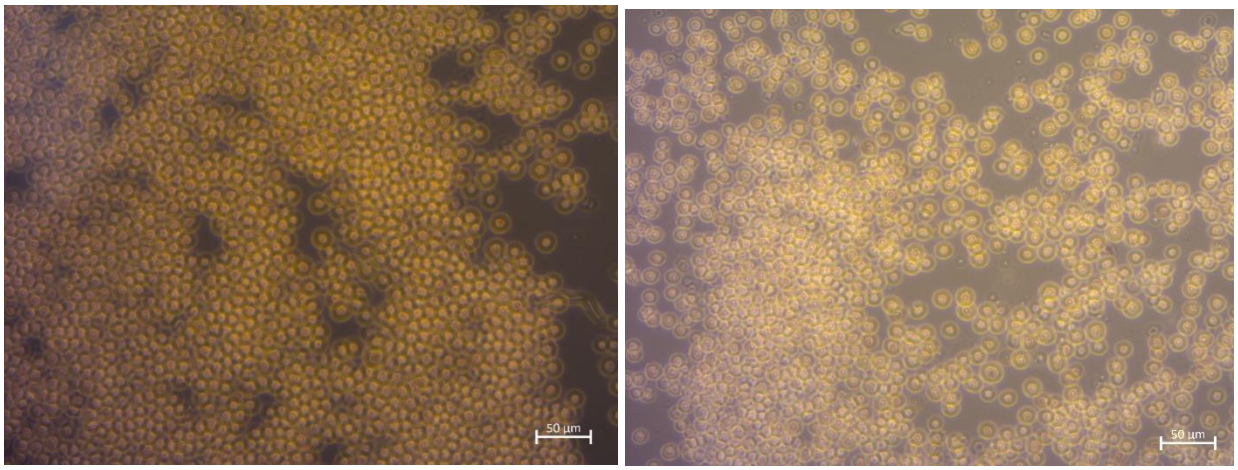
a. Before incubation *b. After incubation*

Figure 44: 50,000 cells at 50% DMSO concentration before (a) and after (b) incubation.



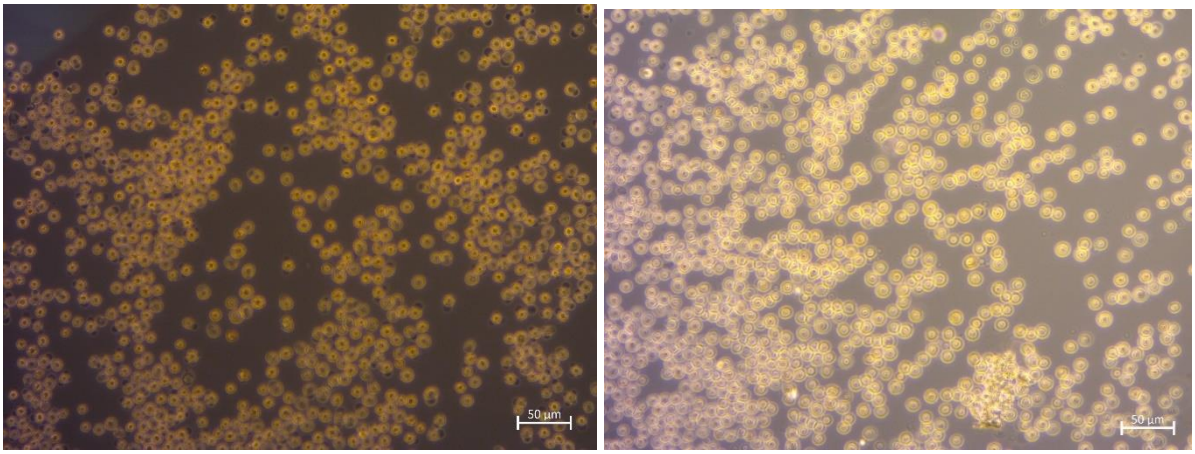
a. Before incubation *b. After incubation*

Figure 45: 75,000 cells at 0% DMSO concentration before (a) and after (b) incubation.



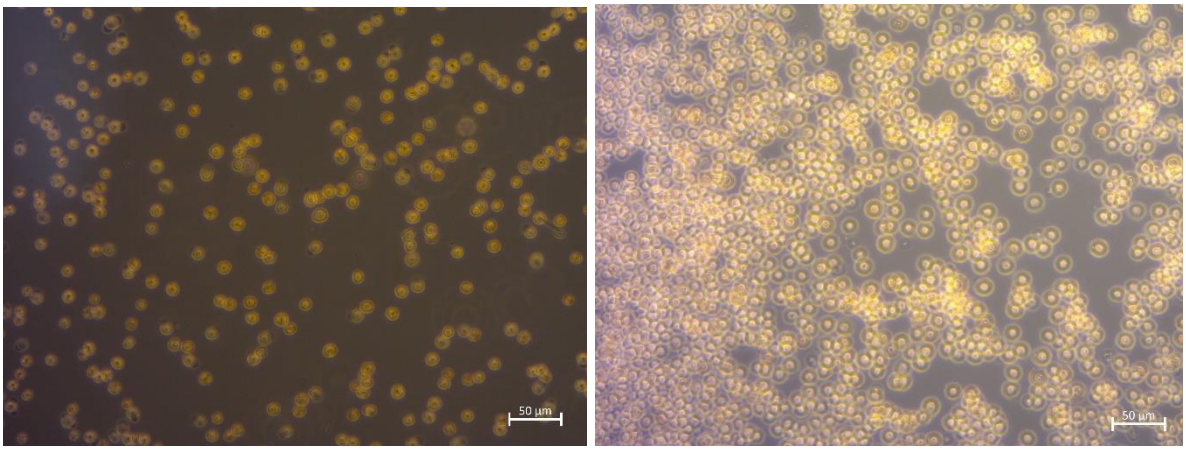
a. Before incubation *b. After incubation*

Figure 46: 75,000 cells at 10% DMSO concentration before (a) and after (b) incubation.



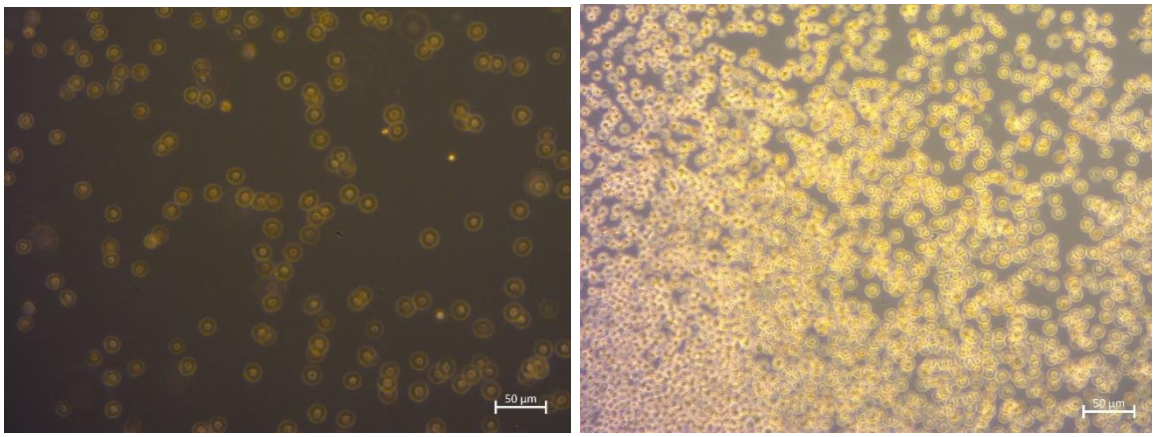
a. Before incubation *b. After incubation*

Figure 47: 75,000 cells at 20% DMSO concentration before (a) and after (b) incubation.



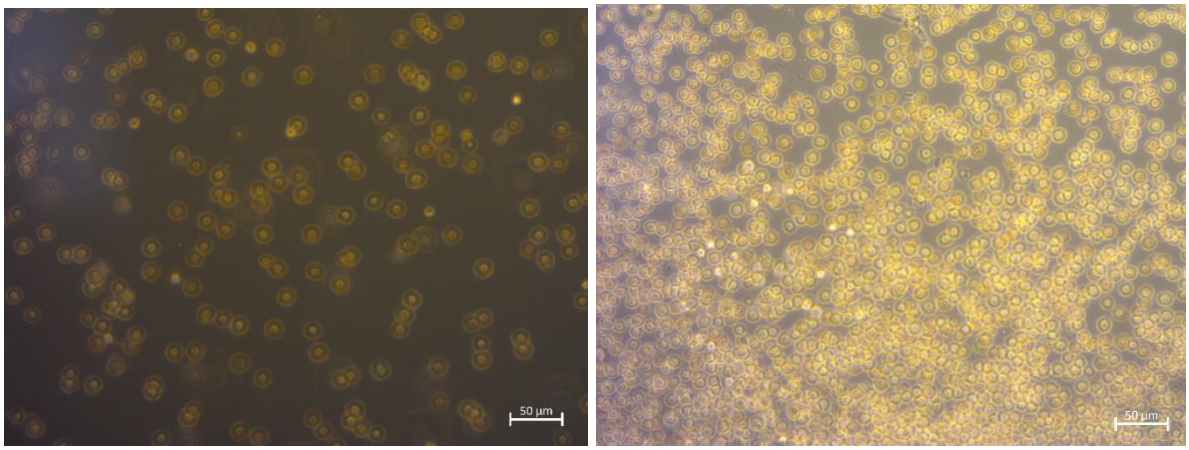
a. Before incubation *b. After incubation*

Figure 48: 75,000 cells at 30% DMSO concentration before (a) and after (b) incubation.



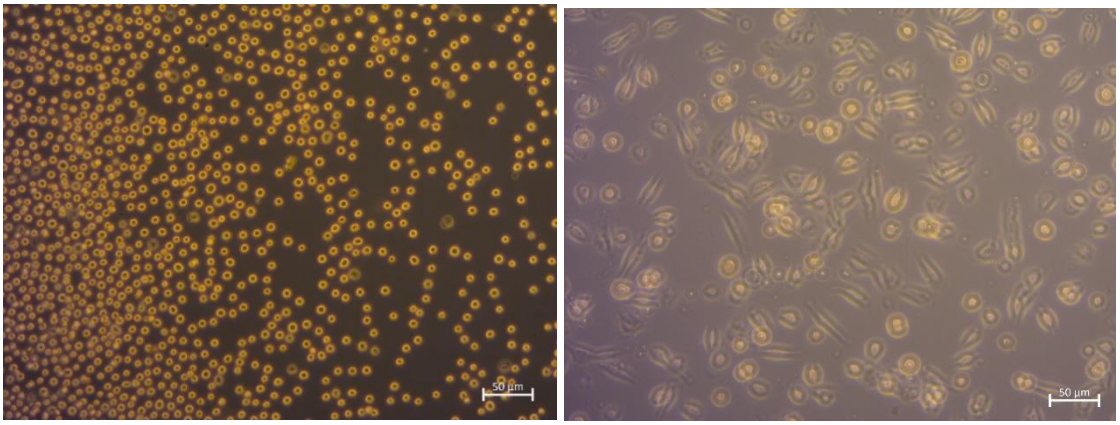
a. Before incubation *b. After incubation*

Figure 49: 75,000 cells at 40% DMSO concentration before (a) and after (b) incubation.



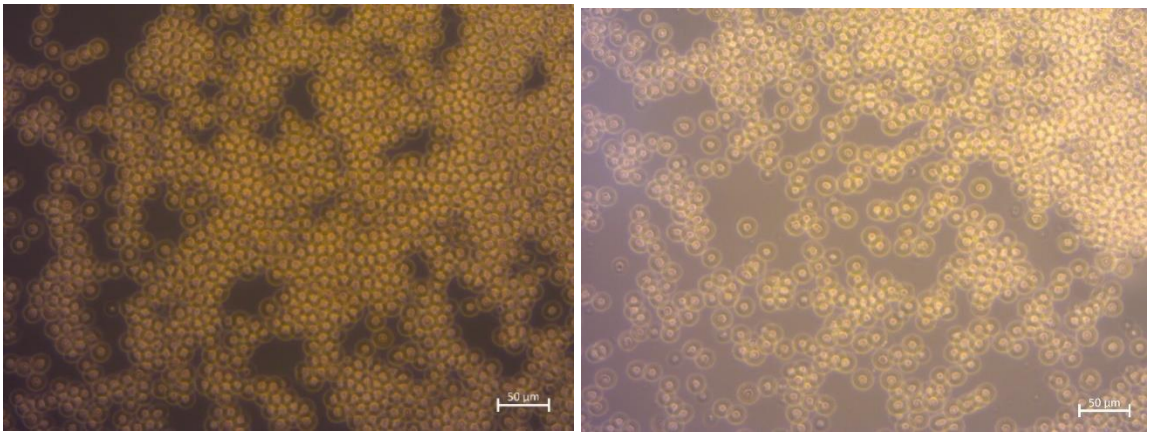
a. Before incubation *b. After incubation*

Figure 50: 75,000 cells at 50% DMSO concentration before (a) and after (b) incubation.



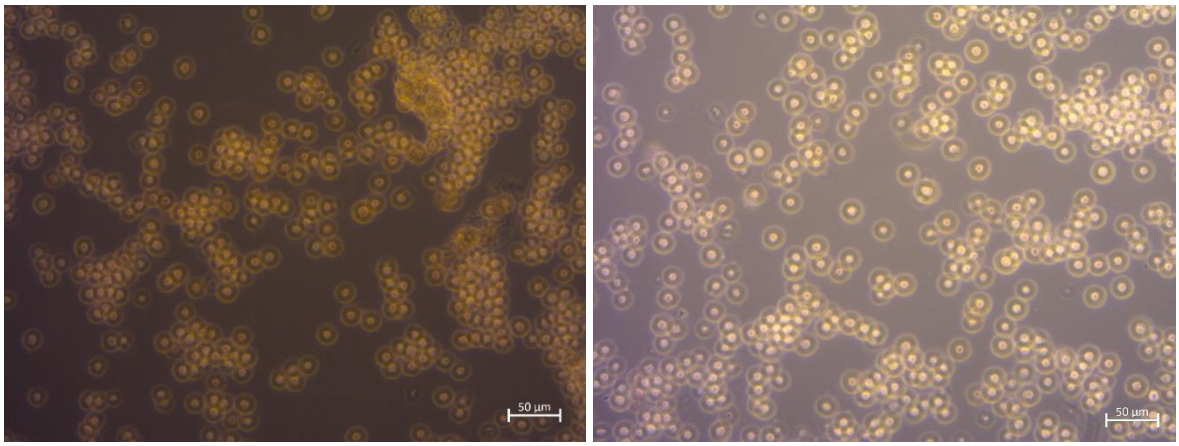
a. Before incubation *b. After incubation*

Figure 51: 100,000 cells at 0% DMSO concentration before (a) and after (b) incubation.



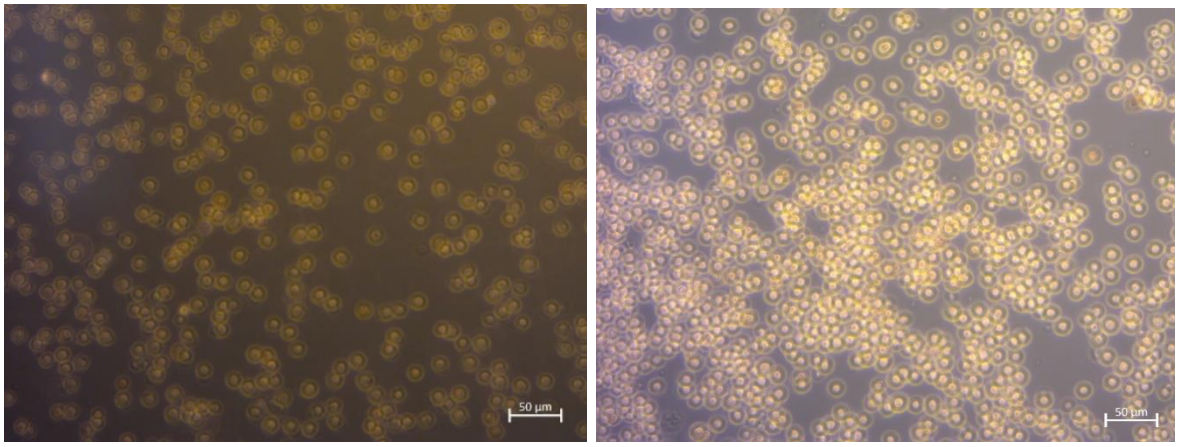
a. Before incubation *b. After incubation*

Figure 52: 100,000 cells at 10% DMSO concentration before (a) and after (b) incubation.



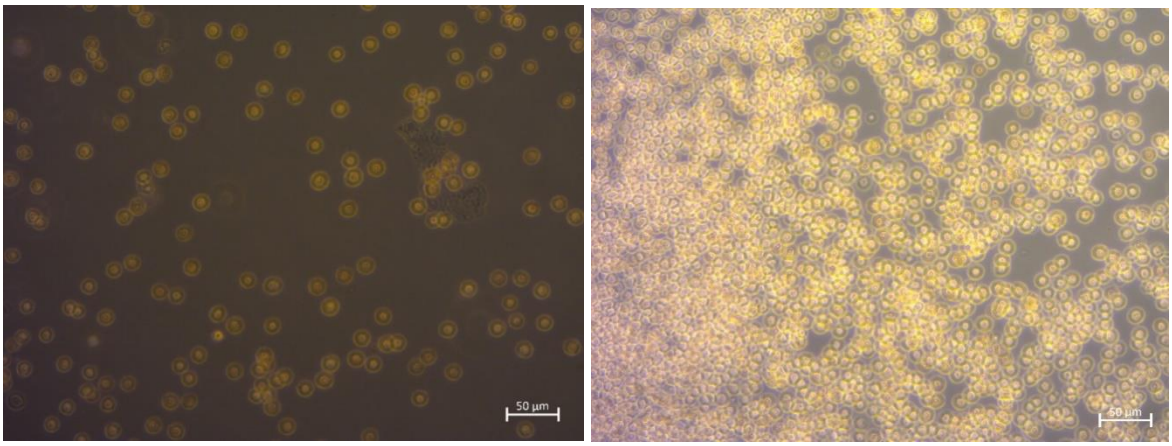
a. Before incubation *b. After incubation*

Figure 53: 100,000 cells at 20% DMSO concentration before (a) and after (b) incubation.



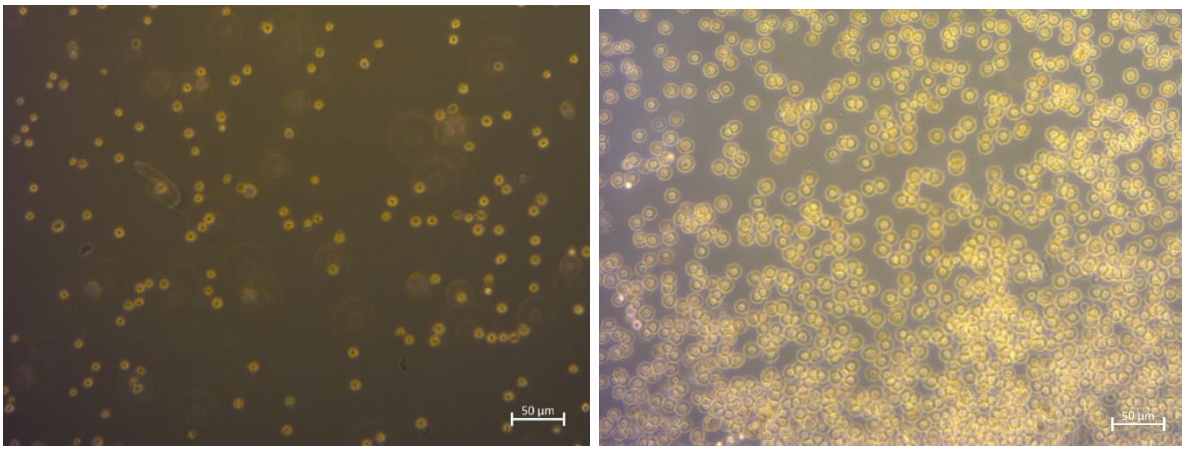
a. Before incubation *b. After incubation*

Figure 54: 100,000 cells at 30% DMSO concentration before (a) and after (b) incubation.



a. Before incubation *b. After incubation*

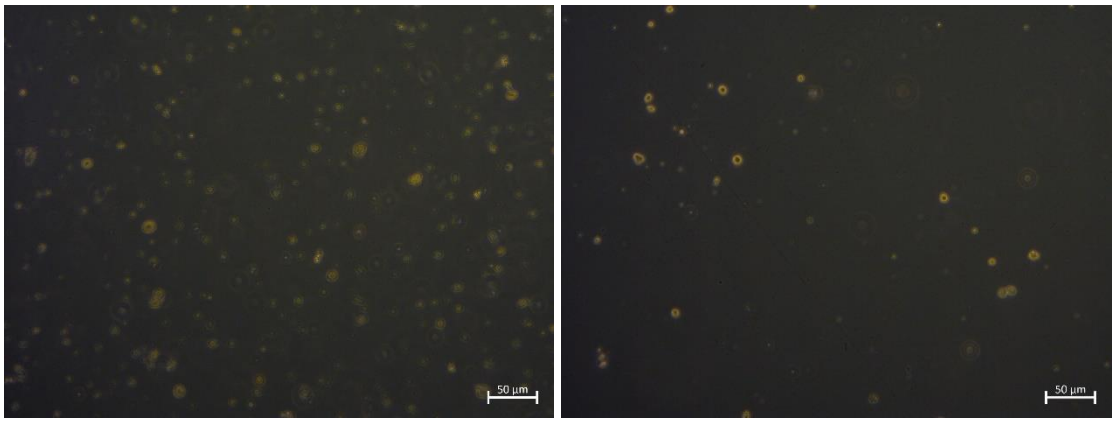
Figure 55: 100,000 cells at 40% DMSO concentration before (a) and after (b) incubation.



a. Before incubation *b. After incubation*

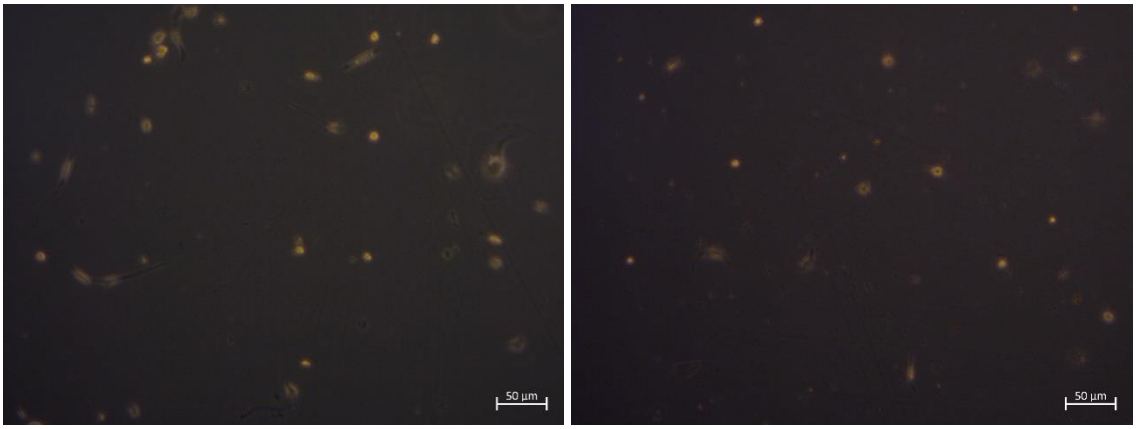
Figure 56: 100,000 cells at 50% DMSO concentration before (a) and after (b) incubation.

A.10 Motion Test Cell Images



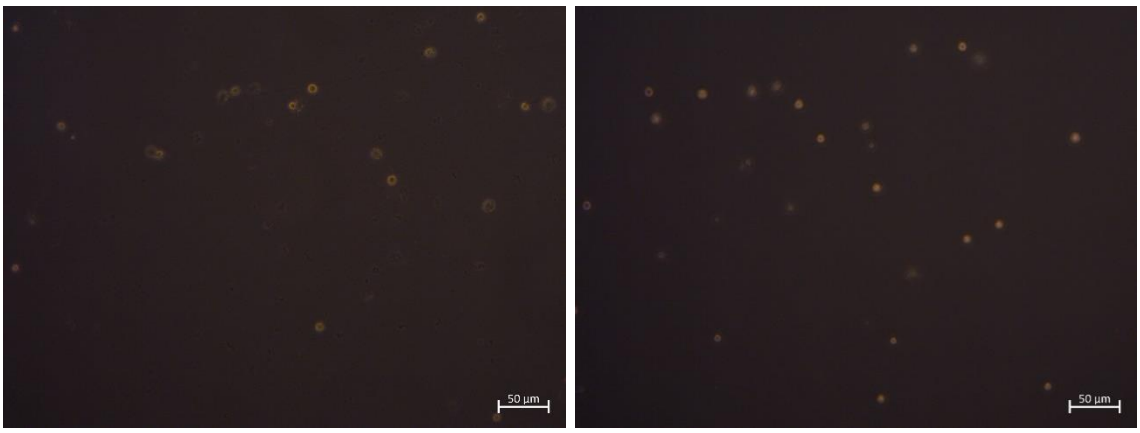
a. Sample 1 *b. Sample 2*

Figure 57: Well that contained dynamic alginate-coated sample.



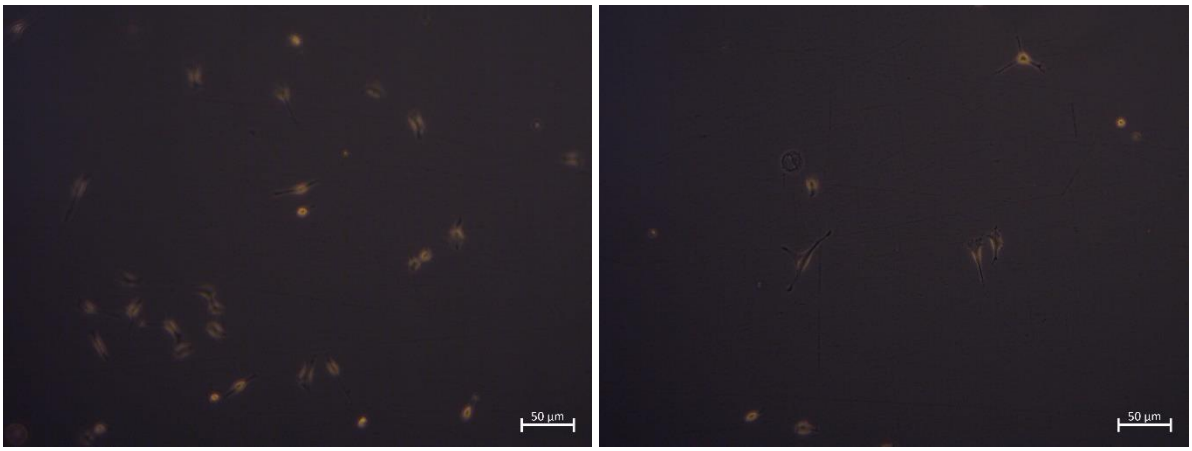
a. Sample 1 *b. Sample 2*

Figure 58: Well that contained static alginate-coated sample.



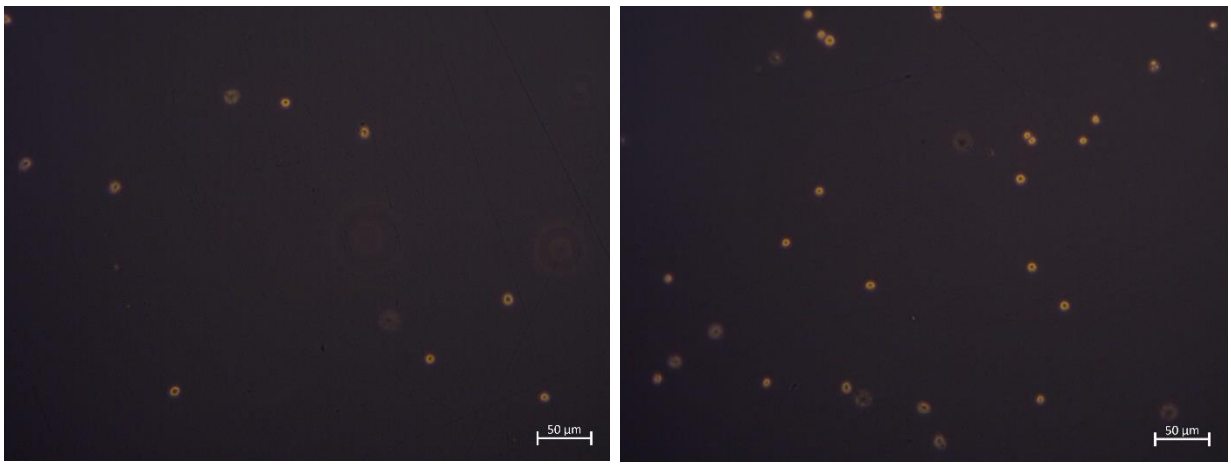
a. Sample 1 *b. Sample 2*

Figure 59: Well that contained dynamic gelatin-coated sample.



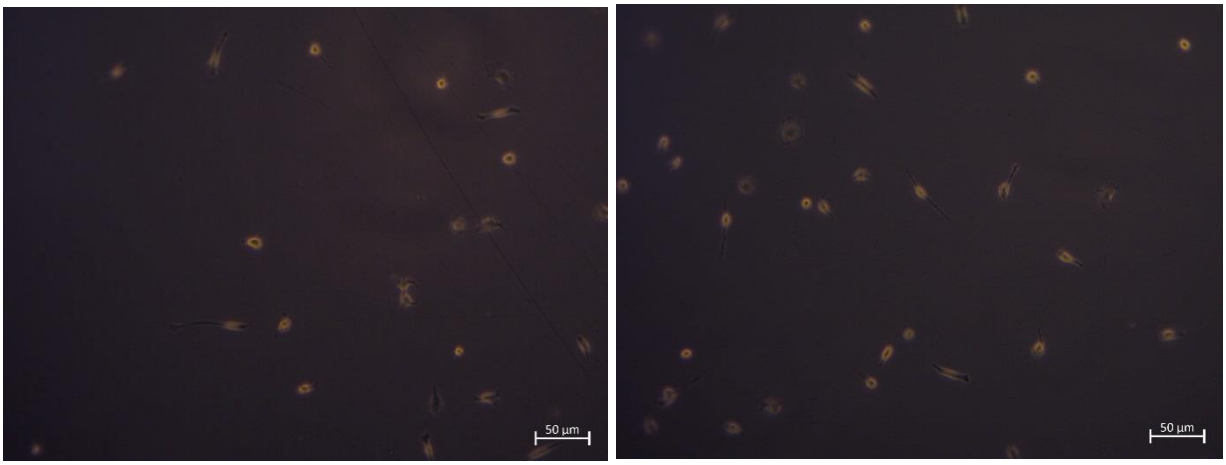
a. Sample 1 *b. Sample 2*

Figure 60: Well that contained static gelatin-coated sample.



a. Sample 1 *b. Sample 2*

Figure 61: Well that contained dynamic uncoated sample.



a. Sample 1 *b. Sample 2*

Figure 62: Well that contained static uncoated sample.