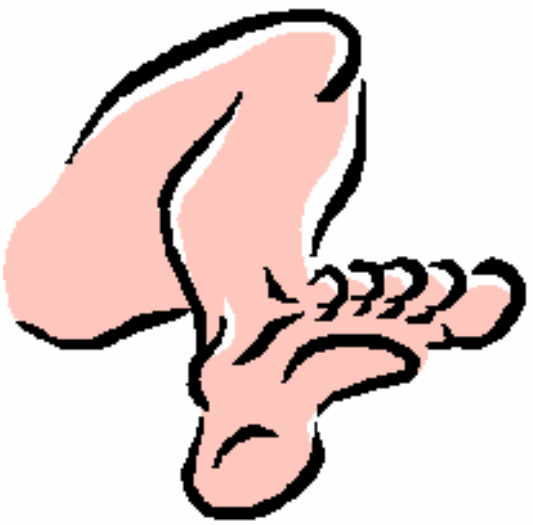
N.K.O.B.®

(New Kim on the Block)



Injectable Polymer Construct for Tissue Engineering Articular Cartilage

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1. Executive Summary

N.K.O.B.® Corporation's express goal is to develop a more viable solution to the increasing need for articular cartilage repair. There are thousands each year that receive treatment for articular cartilage trauma or degeneration, and due to an aging population this number is expected to grow. Total success is not realizable with current treatments. Thus, N.K.O.B.® will develop and market an injectable polymer scaffold, with encapsulated autologous cells and growth factors which may be injected into an articular cartilage defect, resulting in regeneration of tissue. This treatment will be wholly less invasive than any treatment on the market as of today, and will decrease the need for follow-ups while increasing the success rate of articular cartilage regeneration.

Through an evaluation of the current market and the FDA approval process, N.K.O.B.® has estimated that it will take an expected value of $108,000,000 to pass the FDA approval process. The process may take approximately 16 years to complete on average, however, this amount of time may fluctuate due to any successes or failures throughout the process. The most financially burdensome portion of the process involves the clinical human trials, which are estimated to cost about $100,000,000. Through a risk simulation, N.K.O.B.® has chosen to utilize 1 PhD and 10 Laboratory Technicians for the pre-FDA testing phase. This reduces the risk that N.K.O.B.® will take due to a better chance of approval in the FDA approval process.

The Fixed Capital Investment required by N.K.O.B.® is estimated to be about $3,000,000. This cost includes construction of a facility and purchase of all required equipment. This brings the total investment for the project to be $111,000,000, which includes the fixed capital investment, FDA approval costs, and clinical costs.

N.K.O.B.® has chose to determine the price of the product through existing demand and competitor's prices. Based on this, N.K.O.B.® will charge a fee of about $11,000 for the cost of culturing, preparation of the polymer, and shipping. This will allow N.K.O.B.® to break even in three years time. After 10 years the expected cash flow will total $112,000,000 for that year.

2. Introduction

2.1 Objective

Every year millions of Americans suffer from trauma, disease, or malformation of cartilage tissue, especially in the articular cartilage of the knee.[1] Articular cartilage is a thin layer of tissue that covers the ends of diarthroidal joints in the knee and provides mainly mechanical support, distributes forces during loading of the joint, and acts as a lubricating surface to prevent physical wear of the joint. Patients who suffer from even minor lesions or lacerations to their articular cartilage can suffer great pain and discomfort, and this can lead to accelerated degeneration of the joint. The reason for this degeneration is due to the fact that cartilage tissue lacks an inherent means of fully regenerating itself.

There are many current procedures used today, such as microfracture, drilling, allo- and auto-grafts, and more recently, autologous chondrocyte implantation (ACI). However, these methods, though effective in restoring some regeneration of cartilage, have not had a success that is required in this particular region of the musculoskeletal system. Therefore, it is N.K.O.B.®'s goal to develop and implement a solution that will involve the complete regeneration of the missing or malformed cartilaginous region through the use of an injectable implant that is biodegradable and biocompatible that contains autologous cells and growth factors in a porous network.

As stated previously, the amount of musculoskeletal deformations, injuries, failure totals in the millions for Americans every year, and with a progressively aging population this number is sure to climb to even greater heights and the demand for procedures that have long-lasting effects without the need for constant renewal or

replacement will increase. If cartilage had the inherent ability to repair itself, there would not be such a great need for procedures to restore injured cartilage.

2.2 Cartilage Anatomy Background

Cartilage is primarily composed of three components: chondrocytes, collagen, and proteoglycans, as well as being comprised of about 80% water. Chondrocytes are the cells that function primarily to maintain the cartilage structure and function, through the maintenance of the extra-cellular matrix of the tissue. These cells occupy only about 10% of the total volume of the cartilage tissue, as seen in Figure 1. Since cartilage has no nerve supply, as well as no vascular supply, these cells cannot receive neural impulses to signal repair, and immune response cannot penetrate due mainly to steric hindrance. Collagen is a macromolecule that has a characteristic triple helical structure, which comes in many different types. The type of collagen prevalent in articular cartilage is collagen type II. Collagen gives cartilage its phenomenal shear and tensile properties, and also functions to maintain the proteoglycans in the extra-cellular matrix. Proteoglycans are mainly complex macromolecules that consist primarily of a protein core with covalently bound glycosaminoglycan chains. The structure of these proteoglycans is mostly an aggregate, which consistes of a long protein core with hundreds of bound glycosaminoglycans, and the distribution of these are not homogeneous throughout the cartilage. The main function of these proteoglycans is to promote proteoglycan-collagen and proteoglycan-proteoglycan interactions that ultimately hold the tissue together and provide it with its mechanical properties.[2]

The aforementioned components of cartilage are not uniformly distributed throughout the cartilage depth. Figure 1 illustrates the arrangement of the chondrocytes throughout cartilage.

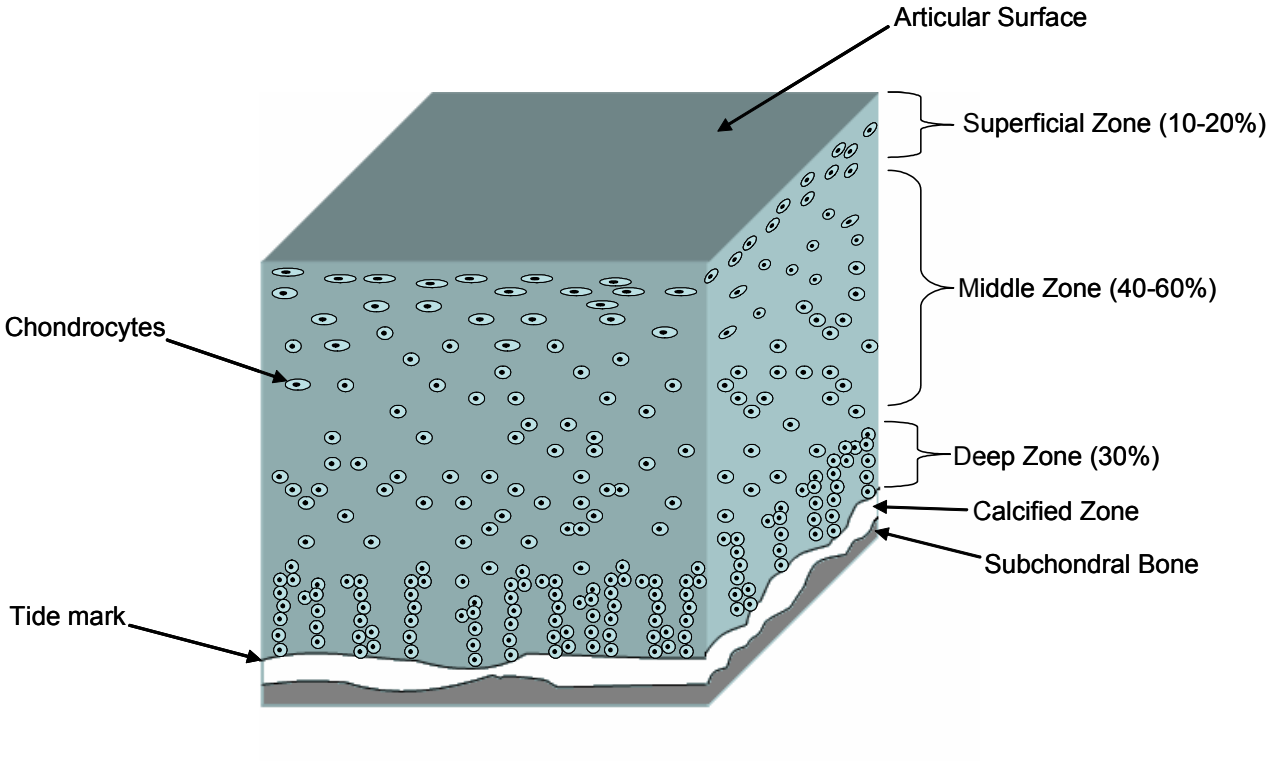

Figure 1. Illustration of varying arrangement of cells with depth in articular cartilage

Figure 1 shows four clear zones in the cartilage, superficial, middle, deep, and calcified cartilage zones. In the superficial zone the collagen fibrils are arranged parallel to the surface while the chondrocytes are elongated and the proteglycan content is at its lowest and the water content is at its highest. In the middle zone the chondrocytes are more rounded and the collagen fibrils are more random in arrangement. In the deep zone the collagen fibrils are much thicker and the proteglycan content is at its highest, with the water content at its lowest. The chondrocytes are arranged in a columnar fashion. The calcified cartilage region acts

to separate the hyaline cartilage from the underlying subchondral bone.[2] On the top of the cartilage seen in Figure 1 would be another section of cartilage, but flipped upside down on the otherside of the joint.

There are two types of cartilage present in the body, fibrocartilage and hyaline cartilage. Fibrocartilage is the type that makes up many non-load bearing cartilaginous regions of the body, such as the ear or the nose. This is made up of mainly collagen type I, and has much less resilient mechanical properties as hyaline cartilage. Hyaline cartilage, which is what articular cartilage is composed of, is prevalent in all diarthroidal joints and has good mechanical properties. The typical compressive modulus ranges from 0.4 to 1.5 MPa whereas equilibrium shear moduli have been found to range from 0.05 to 0.33 MPa. The tensile modulus can range from 5 MPa even up to 50 MPa. Due to these intense mechanical stimulations of the knee joint a material must be selected that can withstand such arduous conditions.[2]

2.3 Articular Cartilage Damage

There are many different types of trauma that may result in articular cartilage defects in the knee. The main type of defect in the knee is a cartilage lesion. Depending on the severity of the lesion, the underlying bone is often exposed and results in damage to the bone and can throw off the balance of the joint and lead to resulting damage of the surface of surrounding cartilage. These lesions, if left untreated can become larger and lead to degradation of the cartilage and surrounding tissue.[3]

Another common cause of cartilage defects is osteoarthritis. Osteoarthritis is characteristic of degeneration of the articular cartilage in the knee and other joints in

the musculoskeletal system. In many cases, once the cartilage degenerates, the underlying subchondral bone can be worn away as well, and this can lead to further complications in the defect site.[3]

2.4 Current Therapies

Due to the prevalence of articular cartilage trauma and degeneration many different procedures have been implemented to alleviate, recede, or even repair an articular cartilage defect in a patient. There are two main types of therapies that may be used: reparative and restorative.

Reparative surgeries include arthroscopic debridement, abrasion arthroplasty, and microfracturing. Arthroscopic debridement involves an arthroscopic procedure, one in which the surgeon uses a tiny camera inserted into the knee to view the joint, to clean the joint of rough edges and wash it out. Abrasion arthroplasty is performed when a cartilage lesion has become hard, after which the surgeon scrapes off the hard tissue which instigates a healing response and fills the defect with fibrocartilage. Microfracturing involves the surgeon using an awl to drill holes into the underlying subchondral bone in order to induce bleeding which will also produce a healing response. However, each of these methods is not only temporary, but produces the wrong type of cartilage for the joint. Fibrocartilage lacks the mechanical properties and integrity for the loading that the joint experiences.[3]

Restorative therapies include osteochondral autografts, osteochondral allografts, and autologous chondrocyte implantation. Osteochondral autografts involve obtaining a section of bone and cartilage from non-loadbearing regions of the patient's own knee and placing them in the defect site. Allografts are the same as

autografts except that the cartilage is taken from a donor individual and not harvested from the patient. Autologous chondrocyte implantation (ACI) is a recently implemented procedure which involves harvesting small sections of articular cartilage, degrading the cartilage to isolate the chondrocytes, proliferating the chondrocytes *in vitro*, injecting the chondrocytes into the defect site, and suturing a periosteal flap on top to secure the chondrocytes in place. However, this procedure has not been met with very high success, often because the chondrocytes tend to migrate from the defect site since the periosteal flap can not sustain the mechanical loading of the knee.[3]

2.5 N.K.O.B.® Corporation's Plan

Since therapies that are currently implemented to repair or restore articular cartilage are not sufficient to meet the demands of the consumer, N.K.O.B.® has developed a new procedure to fully restore the function of the joint in a noninvasive manner. This procedure will involve harvesting of autologous cells from the patient in a first surgery, proliferation of these cells *in vitro* to obtain a sufficient amount, microencapsulation of these cells for delivery, preparation of microspheres containing growth factors released in controlled amounts over time, injected into the defect in a biodegradeable, biocompatible polymer that will have sufficient mechanical properties until the autologous cells begin to form extra-cellular matrix to fully regenerate the tissue. Figure 2 illustrates this plan.

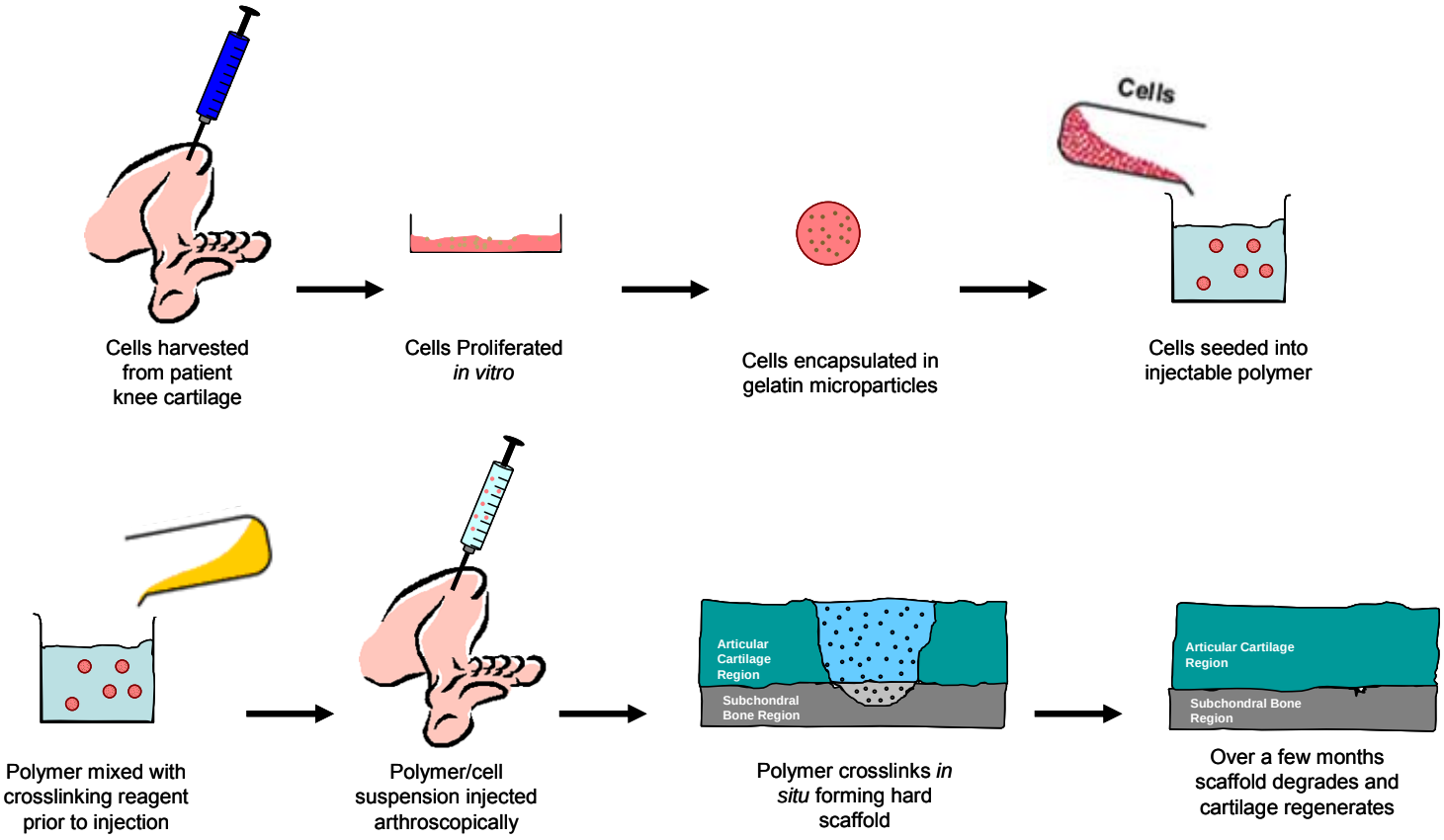

Figure 2. Overview of N.K.O.B.® Corporations Procedure

In formulating a procedure to regenerate cartilage in the knee many considerations were taken into account to develop the proper and appropriate procedure to obtain the desired result.

2.5.1 Minimally Invasive

The first and foremost consideration that N.K.O.B.® needed to address was to develop a procedure that would involve a short surgery time period and be as minimally invasive to the patient as possible. Therefore, since arthroscopic surgeries of the knee, such as those procedures used in ACI, have been established and are minimally invasive[16], an injectable polymer was chosen to serve as the delivery vehicle for the cells and growth factors. The polymer

chosen, poly(propylene fumarate), has been studied extensively and its biocompatibility and biodegradability are well established.[12] Also, PPF has the ability to be chemically crosslinked *in situ* in a matter of 15 minutes, thus reducing surgery time greatly reducing surgery costs associated with hospital fees and doctors fees.[6]

2.5.2 Mechanical Properties

Since the knee articular cartilage region is constantly under considerable stress, from tensile, shear, and compressive stimuli, the polymer chosen must have mechincal properties on the same order of magnitude as articular cartilage. PPF, when combined either with particles of β-tricalcium phosphate or co-polymerized with poly(ethylene glycol), has been shown to posses mechanical moduli of a similar scale as articular cartilage.[9,10]

2.5.3 Mimicking Nature

Since, as stated previously, most cartilage lesions or defects of osteoarthritis in the knee affect not only the articular cartilage, but also the underlying subchondral bone, N.K.O.B.® has chosen to implement a procedure that involves using a bilayer implant into the defect region. This injection will be comprised of two layers that will be injected one at a time. The lower layer will be comprised of PPF mixed with β-TCP particles to give it sufficient mechanical properties required that will contain microparticles with both mesenchymal stem cells inside and also microspheres with growth factor Bone Morphogenetic Protein-2 (BMP-2) contained within. BMP-2 will elicit the responding tissue to respond in a healing fashion to repair the damaged tissue.[2] For the cartilaginous

portion, or upper portion, of the construct PPF will be co-polymerized with poly(ethylene glycol) (PEG) in order to again give the polymer mechanical characteristics required. Autologous chondrocytes, harvested from the patient's articular cartilage will be encapsulated as well as Transforming Growth Factor Beta (TGF-β), which has been shown to stimulate cartilage formation.[2] Figure 3 below illustrates what the injected composite will look like after injection into the defect site.

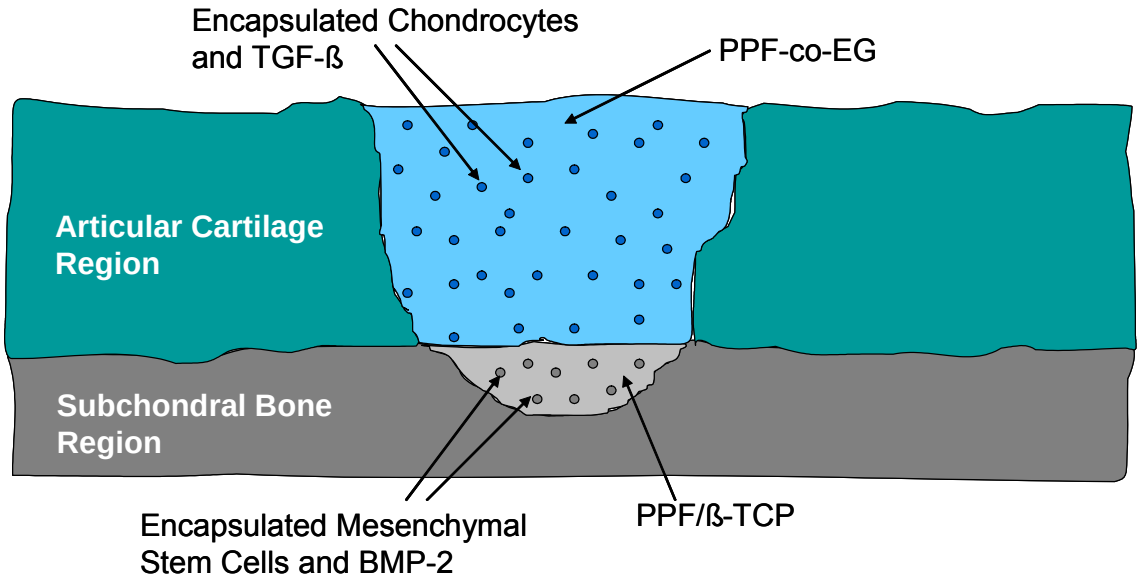

Figure 3. Side view of Construct after injection into a subchondral cartilage defect

Consequently, in utilizing a bilayered construct, the lower bone region of the construct should serve to better anchor the total construct into place. This will address one of the main issues of keeping the cells in the defect site, and will help the construct to better integrate with the surrounding tissue, which is one of the main problems with current applications of cartilage tissue engineering.[17]

3. Production Plan

3.1 Overview of Process

The following section describes N.K.O.B.® process of cartilage regeneration from harvesting of cells to injection arthroscopically. Briefly, chondrocytes or mesenchymal stem cells are harvested from the patient in an initial surgery. These cells are then proliferated *in vitro* and encapsulated in gelatin microparticles and seeded into an injectable polymer along with growth factors, which is ultimately injected into the defect site in a second surgery. The reason that these cells need to be encapsulated is two fold. The *in situ* crosslinking reaction will produce a local increase in temperature that would be detrimental to the cells contain within the composite. Also, once the cell encapsulations disperses, a porous network is produced because the volume occupied by the cell encapsulations provides pores throughout the hardened polymer.

3.2 Harvesting, Proliferation, and Preparation of Cells

Two types of cells are needed for N.K.O.B.®, chondrocytes and mesenchymal stem cells. Chondrocytes are harvested from the patients own articular hyaline cartilage, while mesenchymal stem cells are harvested from the patients bone marrow. The chondrocytes are needed for the cartilaginous region of the construct and mesenchymal stem cells are needed for the bone region.

3.2.1 Chondrocytes

For the cartilaginous region of the implant autologous chondrocytes are first obtained from the patient from a low-load bearing section in an uninvasive

manner and proliferated *in vitro* to obtain a sufficient amount and readied for encapsulation by the following procedure:

1. Patient is first immobilized in a supine position
2. A section of cartilage from the patient's knee is then removed from a non-weight bearing area of the knee articular cartilage
3. This biopsy is then degraded with specific enzymes, mentioned below, to isolate the chondrocytes as described previously[4], all steps performed in a sterile laminar flow hood:
    a. Biopsy dissected into pieces <2mm in each dimension using sterile disposable scalpels and their weights recorded. They are cut small to increase the surface area that the enzymes can reach.
    b. Cartilage pieces then placed in 75 cm^2 tissue culture flasks, because this is the standard size for cell culturing.
    c. Then 10 mL culture medium is added to each flask containing 0.8mg/mL collagenase, and 1.0mg/mL DNase
    d. Flasks then placed in an incubator at 5% (v/v) CO_2 in air for 24 hours at 37ºC.
    e. Then each tissue digest is passed through 70 μm mesh cell strainers and then cells are recovered from the elutate by centrifugation at 2000 rpm for 10 minutes.
    f. Cell pellet then suspended in 10 mL DMEM containing ascorbic acid (50μg/ml), Gentamicin (50μg/mL), and Amphotericin (2.5μg/mL). Then they are centrifuged again at 2000 rpm.

g. The cells are then resuspended in 1 mL of media and are counted using a hemacytometer.

h. The chondrocytes are then seeded at a density of 10,000 cells/cm^2 in a 75 cm^2 flask in media with antibiotics and ascorbic acid as stated preciously and also containing fetal bovine serum at a concentration of 20% (v/v).

i. These flasks are then placed in an incubator for approximately 4 weeks to provide a sufficient number of cells. Media in the flasks is changed by decanting the old media and replacing it with 10 mL of fresh media every 2 days.

4. Once a sufficient number of cells have been obtained the culture media is removed and the flasks are washed with 5 mL of phosphate buffered saline (PBS). The PBS is then removed and 1mL of Trypsin-EDTA is added to the flask.

5. The flask is then incubated for 5 minutes at 37ºC to allow the cells to detach, after which 9mL of DMEM medium is added to the flask and the solution of cells and media is centrifuged for 5 min.

6. The resulting cell pellet is washed with 10mL of DMEM medium and centrifuged again at 2000 rpm for 5 minutes

7. Cells are now ready to be encapsulated in gelatin microparticles

3.2.2 Mesenchymal Stem Cells

For the bone region of the injectable implant mesenchymal stem cells must be harvested from the patient at the same time that the patient has

chondrocytes harvested in an uninvasive biopsy, proliferated and readied for encapsulation by the following procedure, described by Mendes et al[5].

1. Bone marrow aspirates are first obtained from the patient in a volume of 20 mL usually from the hip bone.
2. This aspirate is then mixed with essential medium (α-MEM) with 10% FBS, antibiotics such as gentamicin (50 μg/mL).
3. Cells then resuspended with a 20-gauge needle at a density of $5x10^5$ cells/cm^2 and cultured in α-MEM with 10% FBS, antibiotics, ascorbic acid (50ug/mL) and basic fibroblast growth factor (bFGF, 1ng/mL) at 37ºC in an incubator with 5% CO_2. Culture medium refreshed twice a week.
4. Cells are then cultured for 4 weeks on 75 cm^2 tissue culture flasks.
5. After 4 weeks the cells are lifted by first washing with PBS and then adding 1mL of 0.25% trypsin-EDTA, incubated for 5 minutes, 9mL medium added, then centrifuged, then washed with 10mL medium, then centrifuged again.
6. Cells now ready to be encapsulated in the gelatin microparticles.

3.3 Gelatin Microencapsulation of Cells

Since the polymer that will deliver the cells has an undesirable increase in temperature during its crosslinking reaction, the cells must be encapsulated in microparticles to protect them for a sufficient time during crosslinking. This cell delivery method must have the four following criteria established by Payne et al[6]: (1) carrier must maintain and preserve the phenotypic expression of the encapsulated cells during crosslinking reaction in vivo, (2) carrier must maintain its mechanical

strength and composition during injection and crosslinking reaction, (3) must allow cells to attach and function in their new environment after the crosslinking reaction, (4) carrier must only encapsulate cells for a very short time after injection.

3.3.1 Preparation of Gelatin Solution for microcapsules

The following is the procedure used by Payne et al[6] and will be used to prepare the gelatin solution for the microparticles:

1. First a 30% solution of gelatin is prepared as follows:
    a. A 30% (w/v) porcine gelatin is prepared by heating 70 mL ddH_2O to 95ºC on a hotplate with a magnetic stirrer.
    b. 30 g of porcine gelatin is then added in 0.25 g portions allowing each portion to dissolve until another is added.
    c. Volume then increased to 100mL after all gelatin dissolved with ddH_2O.
    d. The resulting solution is then allowed to solidify at room temperature and then stored at 4ºC until later use.
2. Next an 11% gelatin solution is made from the 30% gelatin solution previously prepared as follows:
    a. DMEM and the 30% gelatin solution are warmed to 50ºC
    b. 33.33g of the 30% gelatin solution and 56.67 mL of DMEM are combined in a beaker.
    c. pH is adjusted to 7.2 by either addition of either NaOH or HCl
    d. This resulting 11% solution is then warmed to 50ºC

e. In a sterile laminar flow hood the solution is then sterilized via vacuum filtration into a sterile media bottle.

f. This sterile 11% solution is then stored at 4ºC until later use.

3.3.2 Encapsulation of Cells in gelatin microparticles

In order to encapsulate these cells, chondrocytes and mesenchymal stem cells, the following procedure is used as described by Payne et al[6]:

1. First, 45 mL of the 11% gelatin solution at 37ºC is diluted with a 5 mL sterile suspension of either chondrocytes or marrow stromal cells in DMEM and mixed thoroughly. The resulting solution should contain approximately 4.0×10^5 cells/mL.
2. This solution is equilibrated at 37ºC
3. In a sterile laminar flow hood the solution is placed in 30mL syringes with 22G needles.
4. These are allowed to cool to 34ºC.
5. The solution is then dripped into 400mL of sterile mineral oil at 10ºC agitated by a magnetic stirrer (this mineral oil solution contains 5mM of Dithiobis(succinimidylpropionate) (DSP) to initiate a crosslinking reaction on the particle surface). This causes the suspension to form droplets because of hydrophobic interactions and causes the droplets to harden from the temperature change.
6. The microparticles are then collected by filtering on a cold 80 μm nylon mesh.

7. Then the microparticles are transferred to a beaker containing 400mL of sterile PBS at 15ºC with a magnetic stirrer.
8. After 2 minutes the agitation is stopped and the remaining mineral oil was decanted.
9. The microparticles are then again filtered on the nylon mesh and then this mesh is placed on sterile absorbent towels on an aluminum tray in an ice bath to eliminate residual PBS and to ensure that the microparticles stay cool.

3.3.3 Gelatin Microparticle Chemistry

In order to protect the autologous chondrocytes or mesenchymal stem cells contained within the actively crosslinking PPF polymer network from thermal shock and eventual cell death. The cells will be encapsulated in gelatin microparticles that have their surface crosslinked with a homo-bifunctional cleavable crosslinker, Dithiobis(succinimidylpropionate) (DSP). Presently most researchers wish to microencapsulate for an extended period of time, but in this case the cells only need to be encapsulated for a short period of time. Payne et al[6] have proven that using the aforementioned procedure to temporarily encapsulate cells successfully protects the cells from thermal shock associated to the polymer crosslinking reaction.

Gelatin is essentially a denatured form of collagen[6] and will participate in protein chemistry reactions, such as reaction of primary amines in the gelatin with local NHS-esters. The crosslinking reaction can be seen in Figure 4.

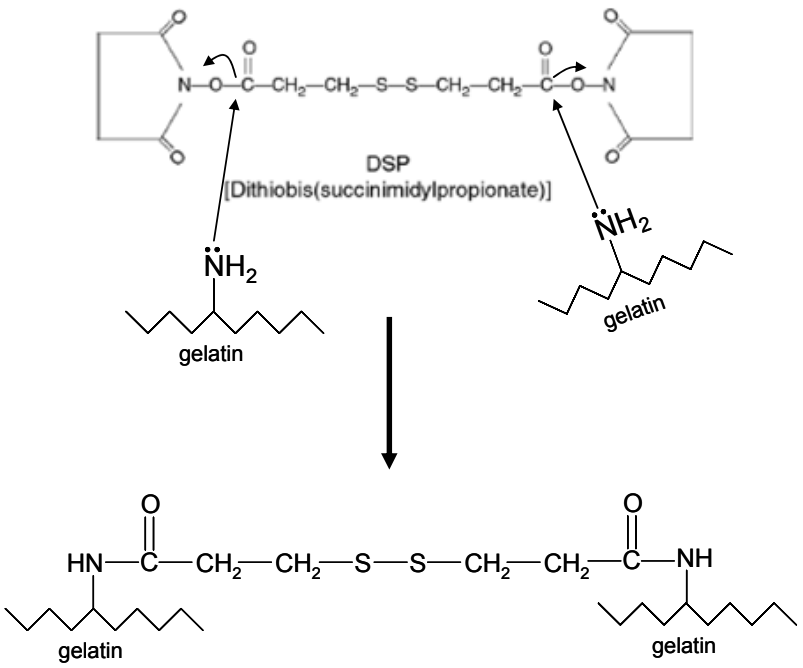

Figure 4. DSP Crosslinking Reaction

The crosslinking of the surface of the gelatin will serve to give the gelatin microparticle better mechanical integrity and serve to temporarily prevent the reverse thermal gelation of the particle. Since DSP is water insoluble it should not penetrate far into the surface of the water soluble gelatin microparticles and should crosslink the gelatin mainly on the surface.[6] Payne et al have shown that it is possible to crosslink the surface of gelatin microparticles, and that this crosslinking gives these particles improved mechanical properties when compared to uncrosslinked gelatin microparticles.[6] It was found that the DSP crosslinked the surface, essentially forming a shell protecting the microparticle as seen in Figure 5. This membrane formed should also prevent enzymatic digestion of the gelatin for a short amount of time, mainly due to steric hindrance.[15]

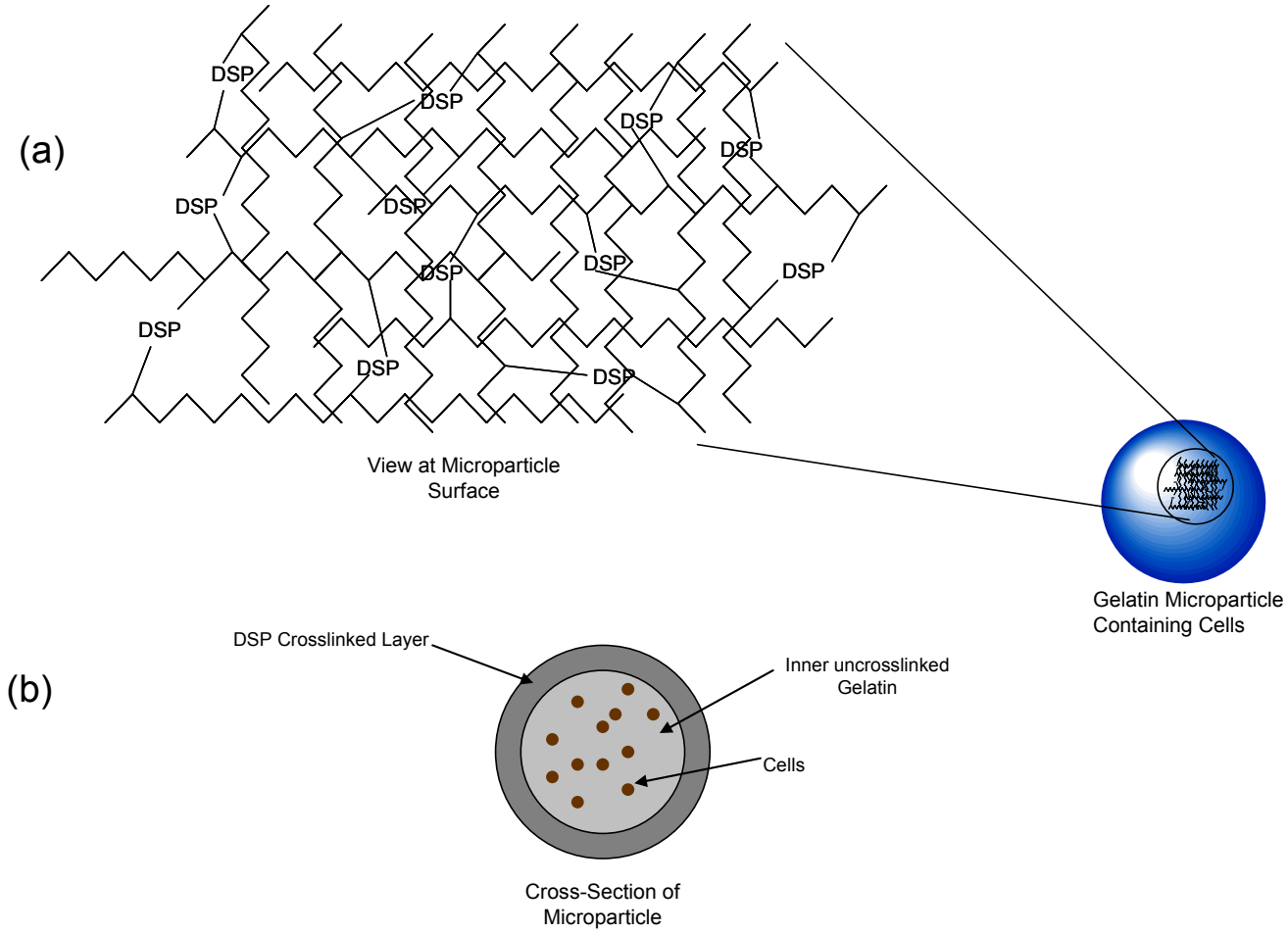

Figure 5. (a) View of Crosslinked Surface of Microparticles and (b) Crossection of Microparticle Surface

3.4 Growth Factor Microsphere Production

In order to recruit the surrounding healthy tissue to assist in the regeneration of the lost tissue and also to stimulate the autologous cells injected to produce the appropriate tissue growth factors must be applied to the construct. However, it is critical that these growth factors be released in a controllable fashion over time to facilitate the regenerative potential. Therefore, N.K.O.B.® has chosen to apply growth factor TGF-β for the cartilaginous region of the construct and BMP-2 for the

bone region of the construct. The following procedure, established by King et al.[7], outlines this procedural step:

1. Microspheres are prepared using a solid-encapsulation/single emulsion/ solvent extraction technique as described by King et al[7]
2. 500 mg of 50/50 PLGA and 5 mg of polyethylene glycol (PEG) is dissolved in 2 mL of methylene chloride creating a 25% (w/v) solution. PEG addition increases the degradation rate of the microspheres.
3. 100 mg of bovine serum albumin (BSA) and 50 μg of either TGF-β or BMP-2 (in a mass ratio of 1:2000) depending on the region of intended use are added to the polymer solution and vortexed vigorously at a medium-high setting for 30 seconds.
4. Immediately after, 10 mL of 0.3% (w/v) polyvinyl alcohol (PVA) is added and vortexed vigorously for another 30 seconds.
5. This solution is then added to a beaker containing 90 mL of the PVA and 100 mL of 2% isopropyl alcohol and this is continuously stirred for 90 minutes at room temperature. This allows the organic solvent to be extracted from the solution.
6. The microspheres resulting are then centrifuged and washed in distilled water 5 times.
7. The microparticles are then frozen to -80ºC and lyophilized until use in injectable implant.

3.5 Production of Injectable Polymer

The polymer that N.K.O.B.® has selected is poly(propylene fumarate). In the bone region this will be combined with particles of β-tricalcium phosphate in order to give it sufficient mechanical strength for a bone implant. For the cartilaginous region of the construct the polymer will be co-polymerized with poly(ethylene glycol) in order to also give it sufficient mechanical strength, as well as to make it more receptive to cells.

3.5.1 Preparation of PPF/β-TCP composite

The following procedure outlines the production of PPF and also its combination with β-TCP:

1. poly(propylene fumarate) (PPF) is prepared in a two step reaction process as described by Payne et al.[8]
    a. diethyl fumarate (DEF) and propylene glycol (PG) are combined in a 1:3 molar ratio in a 11 round bottom 3-neck reaction vessel.
    b. Zinc chloride ($ZnCl_2$) is added at a molar ratio of 0.01:1 $ZnCl_2$:DEF and hydroquinone (Hq) is added at a molar ratio 0f 0.002:1 Hq:DEF. $ZnCl_2$ is the catalyst and Hq prevents undesired reactions
    c. Attached to the vessel is a cold water condenser with a flask to collect condensate and the system is slowly purged with nitrogen to make it a nitrogen atmosphere.
    d. The vessel is first submerged in an oil bath and raised to 100ºC. Then the temperature is raised to 150ºC over 5 hours. When condensate

ceases to form the first step is complete, forming the diester bis(2-hydroxypropylfumarate) (BHPF)

e. Then the reaction mixture is allowed to cool to room temperature

2. The second step of PPF synthesis is as follows:

a. The products of step 1 (BHPF and PG) are then combined in a 31, 3-neck reaction flask of the same set-up

b. The system is then purged with nitrogen gas

c. The temperature is then raised to 100 ºC over 45 minutes and the pressure is reduced to 0.1 Torr. The unreacted PG is distilled and condensed.

d. Next the temperature is raised to 140 ºC over 2 hours. PG produced during the transesterification is removed and condensed resulting in formation of PPF.

e. The temperature is then maintained at 140 ºC for 2 hours and then raised to 150 ºC for 1 hour. The system is then purged with nitrogen and allowed to cool to room temperature.

f. The PPF is then dissolved in an equal volume of methylene chloride.

g. This solution is then shaken with and organic phase removed form solutions as follows: 5% HCl in ddH_2O, ddH_2O twice, ddH_2O saturated with sodium chloride twice, and ethyl ether.

h. The PPF/methylene chloride solution is then added dropwise to cold ethyl ether to remove the Hq.

i. The remaining solvent is then removed by rotovaporation followed by vacuum drying. Product can be stored at -20 ºC for later use.

3. Then β-TCP is combined with the PPF to improve mechanical properties according to Peter et al.[9]

   a. 0.33 g β-TCP is used for every 1 g of PPF.

   b. The β-TCP is added to the PPF/N-VP mixture in the crosslinking reaction described below.

3.5.2 Preparation of PPF-co-EG composite

In the following procedure PPF is synthesized as above and this described the co-polymerization with PEG:

1. PPF is synthesized exactly as described above, except in order to make the copolymer with poly ethylene glycol, PEG is added to the transesterification reaction mixture, second step in reaction process as described by Suggs et al[10] in a 50/50 molar ratio of BHPF to PEG .
2. This resulting copolymer is then purified by solution-precipitation with chloroform and petroleum ether, followed by rotoevaporation to eliminate excess solvents.

3.6 Injection of Construct

The following described the mixing of the constituents for the injection and the injection into the defect site.

3.6.1 Microparticle seeding and PPF Crosslinking

The following procedure outlines the mixing of the constituents of the composite which must be done directly before injection:

1. PPF (with TCP or EG), N-vinylpyrrolidinone (N-VP) and benzoyl peroxide are first sterilized by placing them in glass vials and exposing them to UV light in a sterile laminar flow hood.
2. The N-VP and BP are sterilized for 2 hours, while the PPF is sterilized overnight.
3. Also, dimethyl p-toluidine (DMT) is obtained and sterilized.
4. The amounts of each component in the PPF composite per gram of PPF are as follows: 1 g PPF/b-TCP or PPF-co-EG, 0.1 g N-VP, 0.0015 g BP, and 2.5uL DMT.
5. First, these components are prepared by mixing the respective PPF, DMT and half of the N-VP, and also mixing together the BP and the other half of the N-VP.
6. The previously prepared microparticles are then obtained as well as the microspheres with growth factors.
7. Then, the PPF, DMT, N-VP solution is then added to the BP, N-VP solution and briefly mixed, after which the microparticles containing either chondrocytes or marrow stromal cells are added to the solution, as well as the microcapsules containing the respective growth factors.
8. This composite is then loaded into a 10 cm^3 sterile syringe and is ready for injection.

3.6.2 Injection of composite into defect site

The following procedure will be performed arthroscopically:

1. Once the composites for both regions are prepared the knee is prepared for injection.
2. The surgery will be performed arthroscopically, therefore there is no need for large incisions into the knee. The patient is immobilized in a supine position and anesthesia is applied.
3. The defect area is first cleaned out (give details of this procedure!!)
4. Then the bone region PPF/β-TCP composite is injected in the proper volume for the defect size. Since the composite retains its volume once crosslinked there is no need to over compensate for any volume loss.
5. 20 minutes must be allowed for the composite to crosslink and harden.
6. Then the cartilage region PPF-co-EG composite is injected on top of the bone region composite in the appropriate volume for the defect.
7. Another 20 minutes are allowed for the crosslinking reaction the incisions are sutured and the surgery is finished
8. After approximately 60 minutes the cell encapsulations will degrade and the PPF composite scaffolds will contain pores, while the microspheres containing growth factors release their growth factors slowly over time.

3.7 Polymer Chemistry

PPF production

Diethyl fumarate ($C_8H_{12}O_4$) first reacts with propylene glycol ($C_3H_8O_2$) to form the diester bis(2-hydroxypropyl fumarate) (BHPF) ($C_{10}H_{16}O_6$) with a side product of ethyl alcohol (C_6H_6O) using zinc chloride ($ZnCl_2$) as a catalyst

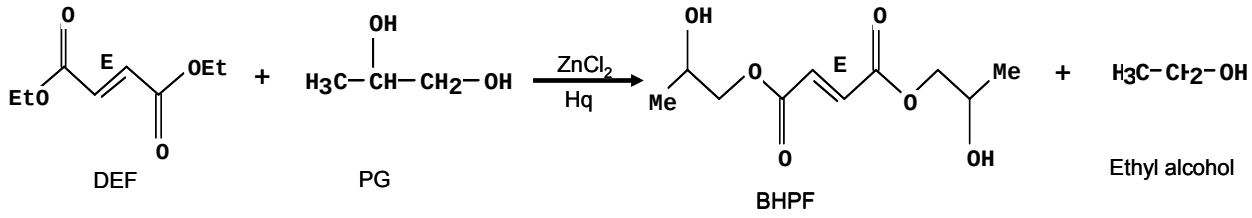

Figure 6. Reaction to form diester BHPF

In the second step of the reaction scheme BHPF and the excess PG left from the first step are transferred to a larger flask and are purged with nitrogen and the pressure is reduced to 0.1 Torr. This causes the PG to distill and produces the PPF through a transesterification of the BHPF wish also produces more PG which is removed as well. The reaction is shown in Figure 7.

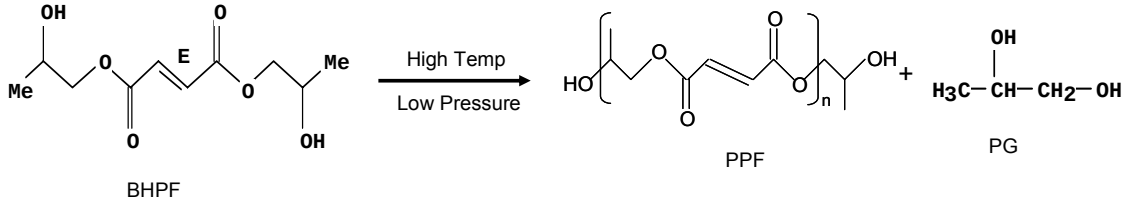

Figure 7. Transesterification to form PPF

In order to form PPF-co-EG, PEG is mixed into the reaction mixture for step 2, the transesterification, in a 50/50 molar ration in order to form the co-polymer.

PPF Crosslinking

The PPF crosslinking reaction is initiated directly prior to injection by the addition of N-VP, BP, and DMT in the following amounts relative to PPF illustrated in Table 1.

Table 1. Relative Amount of each Component in Crosslinking Reaction

Component	Amount
PPF/β-TCP or PPF-co-EG	1.0 g
N-VP	0.1 g
Benzoyl Peroxide	0.0015 g
DMT	2.5 µL

The reaction is shown below in Figure 4. In this reaction the double bonds on the polymer are crosslinked via a vinyl monomer, N-VP, with an initiator benzoyl peroxide and through the use of an accelerator, DMT. The reason for such a high mass ratio of PPF to N-VP is because this high ratio reduces the possibility of N-VP to form any undesireable formation of monomer vinyl pyrrolidinone, as investigated and described by Greeser[11].

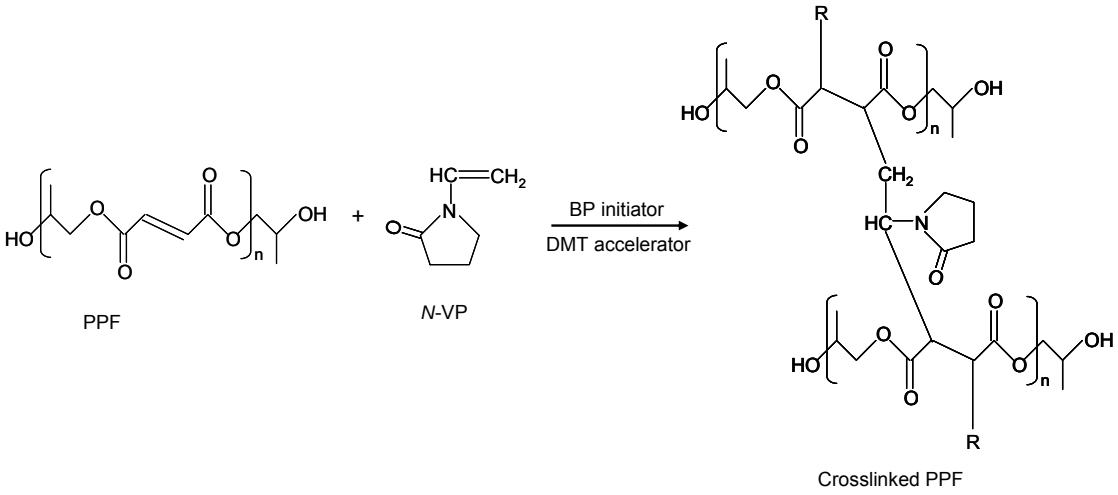

Figure 8. Crosslinking of PPF *in situ*

PPF degradation

This polymer will degrade into the following products *in vivo*: fumaraic acid, propylene glycol, poly(acrylic acid-*co*-fumaric acid), and fumaric acid, as seen in

Figure 9.[12,13] The main pathway of degradation of this polymer *in vitro* is through hydrolytic degradation.[12]

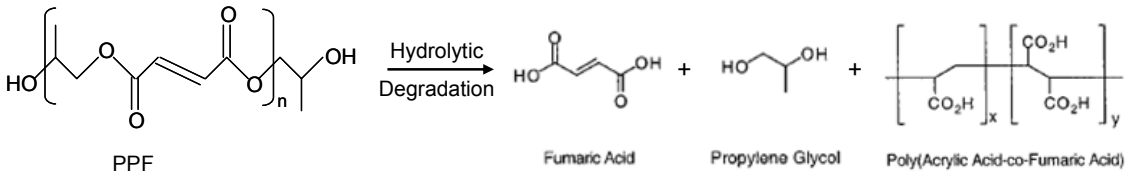

Figure 9. Degradation of PPF

This degradation scheme occurs mainly by the hydrolytic cleavage of the polymer into the aforementioned constituents. Peter et al[14] performed studies on the *in vivo* degradation of the polymer in male Lewis rats, to not only determine the degradation behavior but also to analyze the toxicity of the polymer. PPF/β-TCP cylinders were place in the hind quarters of the rats and specimens were analyzed at 3, 6, 9, and 12 weeks post implantation. They found that the compressive strength and modulus decreased to posses no mechanical strength by 6 weeks after implantation, which is accelerated more than in *in vitro* studies. They attribute this to the decrease in local pH *in vivo*.

Suggs et al[10] conducted similar studies using the PPF-co-EG formulation. They also evaluated the *in vivo* degradation of a 50/50 formulation of PPF-co-EG in rats for 12 weeks. Their results were similar as Peter et al[14], in that the samples decreased in mechanical integrity within the first 3 weeks, with a total loss of mechanical integrity after 6 weeks. After 1 week the loss in mass steadied to a constant rate, resulting in about a 60% loss in mass after 12 weeks *in vivo*. The samples did swell some, but only to about 0.5% increase in volume. The bulk degradation of these polymers is

evidenced by the decrease in mechanical properties without a noticeable change in dimension.[10]

3.8 Temperature Profile

Due to the undesired release of heat during the cross-linking reaction, it is necessary to model the temperature profile in order to accurately determine the behavior of the cells and microcapsules during this reaction. Unfortunately, at increased temperatures, the gelatin inside the microcapsules undergoes reverse gelation and the cell survival rate decreases. The DSP cross-linking surface on the microcapsules is used to provide a form of protection during this increase in temperatures which was previously described in more detail. This DSP will help the gelatin to maintain its mechanical integrity as well as protect the cells from thermal shock.

Payne et al. have experimentally measured the temperature profile of a composite of the PPF polymer without the gelatin microcapsules. The following graph represents the temperature rise during the cross-linking reaction as a function of time. These experimental results reveal that the maximum temperature during the reaction is 45.7 ^{o}C and the temperature is above the critical temperature of 37 ^{o}C for approximately five minutes. This representation is only an estimation to the behavior, as it does not take into consideration the microcapsules or the external temperature of the body. A more complex temperature profile will entail all boundary conditions of the injectable polymer as a function of the heat generated and the external boundary conditions.

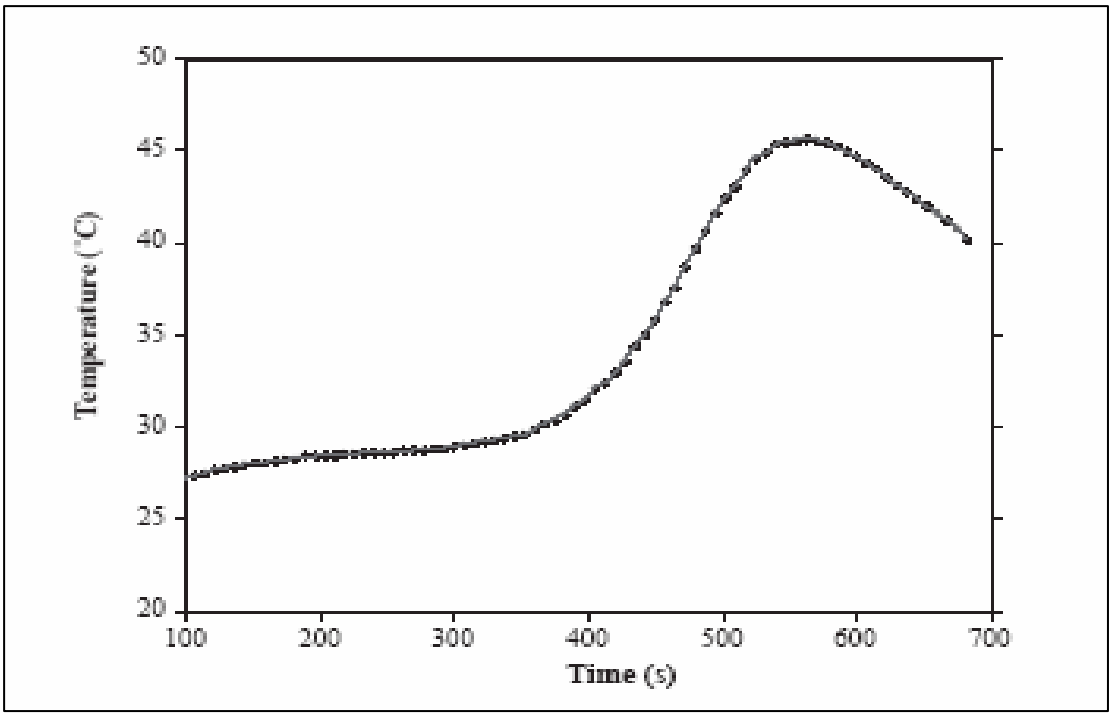

Figure 10. Experimental temperature profile of cross-linking reaction versus time.

It is necessary to measure the amount of heat transfer as a result of the heat generation in order to accurately assess the amount of cell survival. The following graph relates the percentage of cell survival to temperature. From the graph, at increased temperatures (42 ^{o}C), the cell survival decreases substantially with time. At temperatures greater than this, the percent cell survival is similar, as the cell death increases steadily in the first 5-10 minutes. At the internal body temperature of 37 ^{0}C, this is ideal for cell survival; therefore, at this temperature the rate of cell death is assumed to not be dependent on the temperature. The other temperatures are assumed to be closely related to function at 42 ^{o}C, and are approximated with the according curves in the figure. The calculation of the temperature profile will reveal the extent of the temperature increase, and from this, it will be possible to analyze the amount of cell survival.

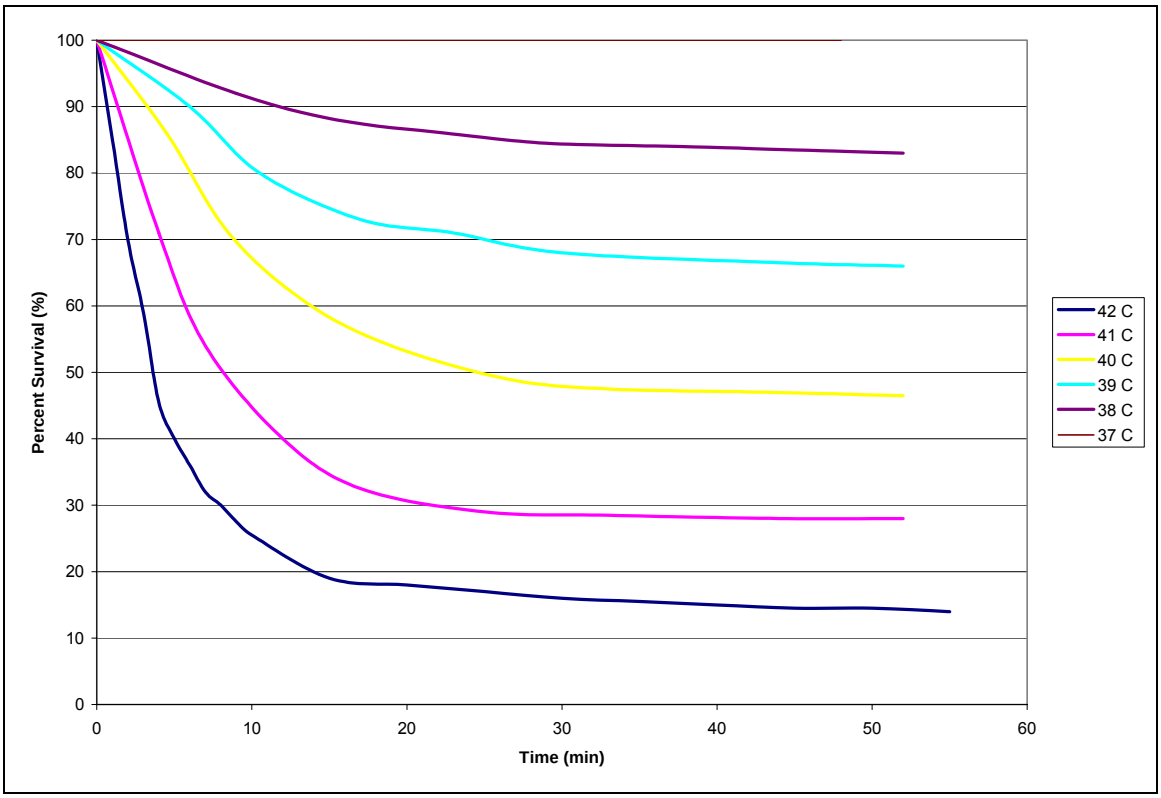

Figure 11. Cell survival versus time for various temperature[26]

In order to accurately model the behavior of the temperature in the knee after injection, it is necessary to mathematically represent temperature profile during the cross-linking of the polymer. The defect is assumed to be rectangular in size, with approximate dimensions of 2 cm x 2 cm x 7 cm. Figure 12 shows a mathematical model to be used in calculations.

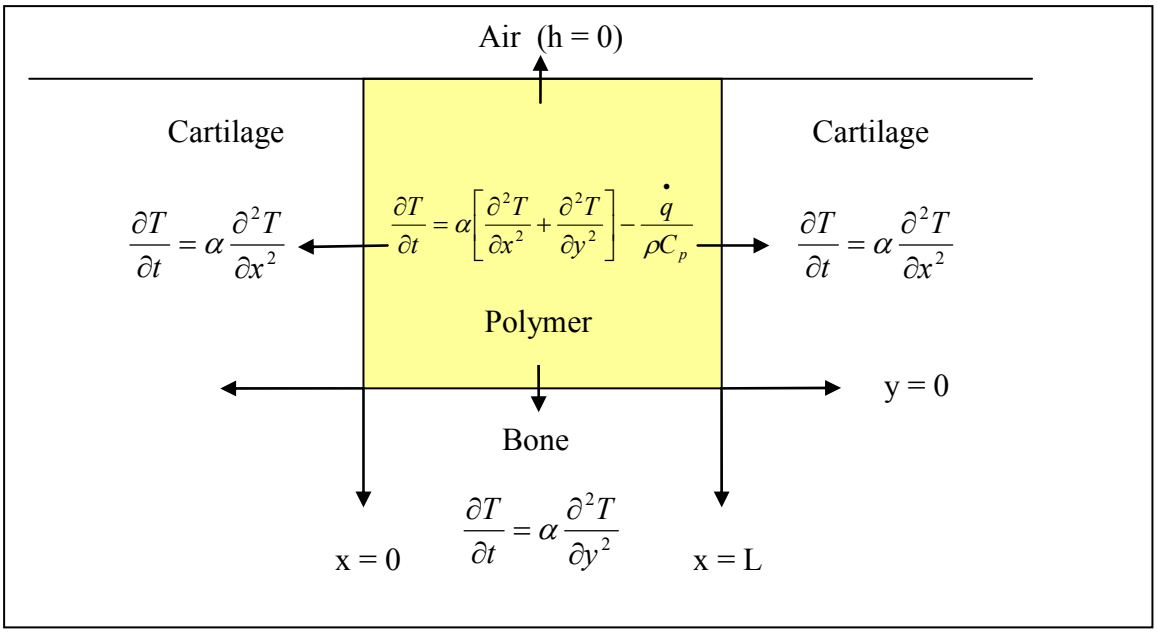

Figure 12. Mathematical Representation of Temperature Flux within Defect

In order to simplify the problem, first the problem is solved in one dimension. The inside term is represented by the following equation:

$$\alpha \frac{\partial T}{\partial t} = \frac{\partial^2 T}{\partial x^2} + q(t)$$

The heat term is only a function of time, since it is assumed that the heat is not a function of x in order to simplify the problem. The first boundary condition is as follows:

$$\alpha_u \left. \frac{\partial T}{\partial x} \right|_{x=L,t} = \alpha_T \left. \frac{\partial T}{\partial z} \right|_{z=0,t}$$

where the inside flux as a function of *x* at the surface (x=L) is equal to the outside flux as a function of *z* at the surface (z=0).

Inside Term:

From separation of variables, the function of temperature with respect to *x* and *t* is described as follows:

$$T(x,t) = C(t) + Dx + \sum A_n(t)\sin\lambda_n x + \sum B_n(t)\cos\lambda_n x$$

$T_x(L,t) = -T_x(-L,t)$ from symmetry and $\lambda_n = \frac{n\Pi}{L}$ from the boundary terms

From the original differential equation the following equation can be formed:

$$\alpha\frac{\partial T}{\partial t} - \frac{\partial^2 T}{\partial x^2} - q(t) = \alpha C'(t) + \alpha\sum B_n{}'(t)\cos\lambda_n x + \alpha\sum A_n{}'(t)\sin\lambda_n x -$$
$$\sum \lambda_n{}^2 B_n(t)\cos\lambda_n x - \sum \lambda_n^2 A_n(t)\sin\lambda_n x - k_1 e^{-k_2 t} = 0$$

The following relationships allow for further simplification:

$$\alpha C'(t) = k_1 e^{-k_2 t} \quad \text{or} \quad C(t) = K - \frac{k_1 e^{-k_2 t}}{\alpha k_2}$$

$$\alpha B_n{}'(t) + \lambda_n^2 B_n(t) = 0 \quad \text{or} \quad B_n(t) = b_n e^{\left(\frac{-\lambda_n^2}{\alpha}\right)t}$$

$$\alpha A_n{}'(t) + \lambda_n^2 A_n(t) = 0 \quad \text{or} \quad A_n(t) = a_n e^{\left(\frac{-\lambda_n^2}{\alpha}\right)t}$$

Simplifying:

$$T(x,t) = \left(K - \frac{k_1}{\alpha k_2}e^{-k_2 t}\right) + Dx + \sum a_n e^{\left(-\lambda^2/\alpha\right)t}\sin\lambda_n x + \sum b_n e^{\left(-\lambda^2/\alpha\right)t}\cos\lambda_n x$$

Another boundary condition: $T(x,0) = T_o$ or the initial temperature is applied to this equation:

$$\left(T_o - K + \frac{k_1}{k_2\alpha}\right) = Dx + \sum b_n\cos\lambda_n x + \sum a_n\sin\lambda_n x$$

Multiplying by $\cos\lambda_n x$:

$$0 = \int_{-L}^{L} Dx\cos\lambda_n x dx + \sum a_m\int_{-L}^{L}\sin\lambda_m x\cos\lambda_n x dx + \sum b_m\int_{-L}^{L}\cos\lambda_m x\cos\lambda_n x dx$$

Using the rules of orthogonality and integrating, the equation is solved for a constant

$$b_n = \frac{-2D}{\lambda_n^2 L}$$

where $\int_{-L}^{L} \cos^2 \lambda_n x dx = L$ and $\sin n\Pi = 0$.

Using the same concept of orthogonality and multiplying by $\sin \lambda_n x$, the equation is further simplified:

$$a_n = 2\left[\frac{T_o - K + \left(k_1 \diagup k_2\right)\alpha}{L\lambda_n} - \frac{DL}{\lambda_n}\right]$$

where $\int_{-L}^{L} \sin^2 \lambda_n x dx = L$.

The new simplified equation becomes:

$$T(x,t) = \left(K - \frac{k_1}{\alpha k_2} e^{-k_2 t}\right) + Dx + \sum 2\left[\frac{T_o - K + \left(k_1 \diagup k_2\right)\alpha}{L\lambda_n} - \frac{DL}{\lambda_n}\right] e^{\left(-\lambda^2/\alpha\right)t} \sin \lambda_n x +$$

$$\sum \frac{2D}{\lambda_n^2 L} e^{\left(-\lambda^2/\alpha\right)t} \cos \lambda_n x$$

From the symmetric boundary condition that $\left.\frac{\partial T}{\partial x}\right|_{x=L} = \left.\frac{\partial T}{\partial x}\right|_{x=-L}$, further evaluation determines that the constant *D* must equal zero. The following equation represents the temperature profile as a function of *x, t* with one constant, *K*, remaining. In order to solve for *K,* the outside equation must now be solved.

$$T(x,t) = \left(K - \frac{k_1}{\alpha k_2} e^{-k_2 t}\right) + \sum 2\left[\frac{T_o - K + \left(k_1 \diagup k_2\right)\alpha}{L\lambda_n}\right] e^{\left(-\lambda^2/\alpha\right)t} \sin \lambda_n x$$

Outside Term:

The outside differential equation is determined from the conduction equation in the cartilage region in one dimension:

$$\frac{\partial T}{\partial t} = \alpha \frac{\partial^2 T}{\partial z^2}$$

Using the Laplace transformation, the partial differential simplifies to:

$$s\hat{T} - T_o = \alpha \frac{\partial^2 \hat{T}}{\partial z^2} \quad \text{or} \qquad \alpha \hat{T}'' - s\hat{T} + T_o = 0$$

where $\hat{T}$ is the Laplace transform of T. This simple ordinary differential equation is solved for $\hat{T}$:

$$\hat{T} = Ae^{\sqrt{\left(\frac{s}{\alpha}\right)}z} + Be^{-\sqrt{\frac{s}{\alpha}}z}$$

From evaluation of the boundary conditions, A is 0 since T is bounded as z goes to 0.

The Laplace transform of $\left.\frac{\partial \hat{T}}{\partial z}\right|_{z=0,s} = F(s)$ where $F(s)$ is the Laplace transform of *f(t)*.

$$\frac{\partial \hat{T}}{\partial z} = \frac{-B\sqrt{s}}{\sqrt{\alpha}} e^{-\sqrt{\frac{s}{\alpha}}z}$$

$$\hat{T}(z,s) = Be^{-\sqrt{\frac{s}{\alpha}}z}$$

The internal solution can now be used with the boundary condition:

$$T_{out}(0,s) = T_{in}(L,s)$$

$$\hat{T}(0,s) = B = T(L,s)$$

$$\hat{T}(z,s) = T(L,s)e^{-\sqrt{\frac{s}{\alpha}}z}$$

$$T(L,t) = \left(K - \frac{k_1}{\alpha k_2} e^{-k_2 t} \right)$$

The resulting Laplace transform is:

$$\hat{T}(z,s) = F(s)\cdot G(s) \text{ where } F(s) = T(L,s) \text{ and } G(s) = e^{-\sqrt{\frac{s}{\alpha}}z}$$

Taking the inverse Laplace transformation:

$$T(z,t) = f(t) * g(t)$$

Laplace transform of *G(s)*.

$$g(t) = \frac{ze^{-\frac{z^2}{4\alpha t}}}{2\alpha\sqrt{\Pi}t^{3/2}}$$

From the theory of convolution:

$$T(z,t) = f(t) * g(t) = \int_0^t f(t-\xi)g(\xi)d\xi$$

$$T(z,t) = \int_0^t \left(K - \frac{k_1}{\alpha k_2} e^{-k_2(t-\xi)} \right) \frac{ze^{-\frac{z^2}{4\alpha\xi}}}{2\alpha\sqrt{\Pi}\xi^{3/2}} d\xi$$

By separating the integral:

$$T(z,t) = K\int_0^t \frac{ze^{-\frac{z^2}{4\alpha\xi}}}{2\alpha\sqrt{\Pi}\xi^{3/2}} d\xi - \frac{k_1}{\alpha k_2} e^{-k_2 t} \int_o^t \frac{ze^{-\frac{z^2}{4\alpha\xi}} e^{k_2\xi}}{2\alpha\sqrt{\Pi}\xi^{3/2}} d\xi$$

The first integral can be solved as follows:

$$\frac{K}{\sqrt{\Pi}} \int (-2) e^{-\frac{z^2}{4\alpha\xi}} d\left(\frac{z}{2\alpha\xi}\right) = -2\frac{K}{\sqrt{\Pi}} \int e^{-u^2} du$$

$$K\int_0^t \frac{ze^{-\frac{z^2}{4\alpha\xi}}}{2\alpha\sqrt{\Pi}\xi^{3/2}} d\xi = -2\frac{K}{\sqrt{\Pi}} \int_\infty^{\frac{z}{2\alpha t}} e^{-u^2} du = -2\frac{K}{\sqrt{\Pi}} erfc\left(\frac{z}{2\alpha t}\right)$$

The second integral is more complex and is simplified to the following form:

$$2\int_0^{\frac{2\alpha t}{z}} e^{-\frac{1}{r^2}} e^{pr}\,dr \quad \text{where } r=\frac{1}{u},\ u=\frac{z}{2\alpha\xi},\ \text{and } p=\frac{zk_2}{2\alpha}$$

From this integral, as $t\to\infty$, $e^{-\frac{1}{r^2}}\to 1$; so the major contributor to the function of r is e^{pr}. This integral is approximated by taking the average of an estimation of the upper bound and lower bound of the function, e^{pr}. Using four sections of the function between 0 and $\frac{2\alpha t}{z}$, the area underneath the curve is approximated to be:

$$\left(\frac{\alpha t}{2z}\right)\left(1+2e^{\frac{k_2 t}{4}}+2e^{\frac{k_2 t}{2}}+2e^{\frac{3k_2 t}{4}}+e^{k_2 t}\right)$$

Therefore, the entire solution to the integral for the outside term is estimated to be:

$$T(z,t)=K\int_0^t \frac{ze^{-\frac{z^2}{4\alpha\xi}}}{2\alpha\sqrt{\Pi}\xi^{3/2}}d\xi-\frac{k_1}{\alpha k_2}e^{-k_2 t}\int_o^t \frac{ze^{-\frac{z^2}{4\alpha\xi}}e^{k_2\xi}}{2\alpha\sqrt{\Pi}\xi^{3/2}}d\xi \quad =$$

$$\left[-2\frac{K}{\sqrt{\Pi}}erfc\left(\frac{z}{2\alpha t}\right)\right]-\left[\frac{k_1}{\alpha k_2\sqrt{\Pi}}e^{-k_2 t}\left(\frac{\alpha t}{2z}\right)\left(1+2e^{\frac{k_2 t}{4}}+2e^{\frac{k_2 t}{2}}+2e^{\frac{3k_2 t}{4}}+e^{k_2 t}\right)\right]$$

The constant *K* can be solved for by using the boundary condition $T_{out}(0,t)=T_{in}(L,t)$.

$$T_{out}(0,t)=-2\frac{K}{\sqrt{\Pi}}$$

$$T_{in}(L,t)=K-\frac{k_1}{\alpha k_2}e^{-k_2 t}$$

By equating the two solutions together, the constant, *K*, is determined to be:

$$K=\frac{\frac{k_1}{\alpha k_2}e^{-k_2 t}}{\left(1+\frac{2}{\sqrt{\Pi}}\right)}$$

Therefore, the temperature flux in the implant is estimated to be:

$$\frac{\partial T}{\partial x} = \frac{2\left(T_o - \frac{k_1}{\left(1+\frac{2}{\sqrt{\Pi}}\right)\alpha k_2} e^{-k_2 t} + \left(k_1 / k_2\right)\alpha \right)}{L} \sum e^{\left(\lambda_n^2/\alpha\right)t} \cos\lambda_n x$$

The other constants used in the equation are defined as followed: L is half of the length of the implant into the defect site, assumed to be 3.5 cm, or 0.035 m. The constant α is the thermal diffusivity of the cartilage and the polymer. Both are assumed to be the same, and are assumed to be the thermal diffusivity of water, since cartilage is 80% water, and the polymer is assumed to have physical characteristics close to cartilage. The thermal diffusivity, α, of water is 0.00145 cm^2/s, or 0.145 x 10^{-6} m^2/s . The initial temperature, T_o, is the temperature at which the polymer is injected, assumed to be the same as the internal temperature of the body, or 310 K. The constants k_1 and k_2 come from the heat generation term that is a result of the cross-linking reaction of the polymer. These constants are determined through the next series of equations and assumptions.

Heat Generated Term:

Assuming the reaction follows first order kinetics:

$$q(t) = k_1 e^{-k_2 t}$$

It is also assumed that the reaction is dependent on the amount of N-vinylpyrrolidinone (N-VP) used prior to the injection.

$$r = \frac{dM}{dt} = k_2 M$$

$$\ln\left(\frac{M_t}{M_o}\right) = -k_2 t$$

$$M_t = M_o e^{-k_2 t}$$

It was previously determined that 0.1 g of N-VP is required for the reaction, so M_o = 0.1 g, and the time it takes for the reaction was determined to be approximately 15 minutes. So if all the N-PV is assumed to react with the polymer, $k_2 = 214.75$ min^{-1}.

From the temperature profile plot in Figure 10, the heat of reaction is approximated to be 50 J/g. For 1 g, the heat of reaction is 50 J.

$$50 = k_1 \sum_{t=0}^{15} e^{-214.75t}$$

From this summation, the constant k_1 is calculated to be 50 J. Therefore the heat generation term is:

$$q(t) = 50e^{-214.75t}$$

Due to the complexity of the equation, the actual graphical representation of the temperature profile is unavailable for this report. However, Figure 13 represents the expected temperature profile of the injectable polymer. It is expected that as time increases, initially the temperature will rise according to the cross-linking reaction, and as time increases towards infinity, the temperature will return to the original internal body temperature of 37 ^{o}C. Future work will completely analyze the temperature flux of the defect site and its effect on embedded microcapsules.

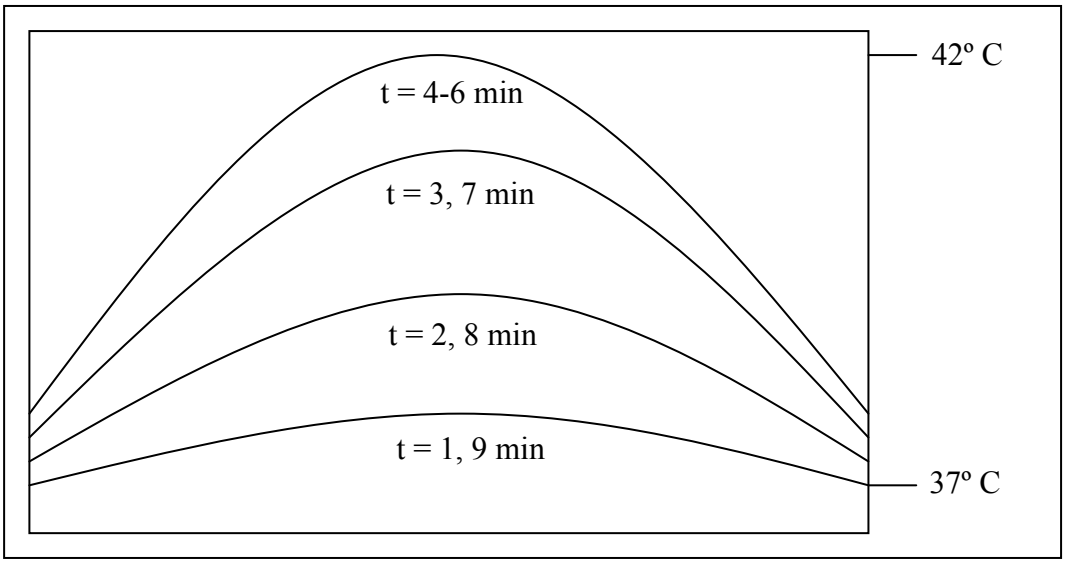

Figure 13. Expected behavior of the internal temperature during polymerization

From the results of the temperature profile, the behavior within the defect site can be analyzed as a function of the position and time. Further work will incorporate the amount of heat transfer within the microcapsules in order to determine the actual temperature increase though the DSP shield into the gelatin spheres. This will accurately determine the amount of cell survival as a function of temperature and will determine the necessary number of injected cells to maintain a desired level of cell survival following the cross-linking reaction of the polymer. From this information, an optimal microcapsule diameter can also be chosen to maximize cell survival. This diameter may include the necessary amount of protection provided by the thickness of the DSP on the surface.

Also the injection temperature may also be evaluated in order to assess the amount of cell survival. At lower injection temperatures, the maximum temperature during the reaction will be lower. However, this lower temperature will allow for a

slower reaction time, which may ultimately lead to greater cell death. This analysis will be dependent on the kinetics of the reaction and will be calculated in future work.

In order to simplify this complex solution, cylindrical coordinates may be used in the future in order to eliminate the necessity for two dimensions within the defect. Also the solution will be complete with the graphical representation in order to fully model the behavior of the temperature profile. Following these solutions, the optimal diameter of the microcapsules and injection temperature can be determined in order to maximize the cell survival for cartilage regeneration.

3.9 Porosity

After injecting the composite material (polymer, cells, growth factor, and scaffolds) into the defect site, it is necessary to consider the mechanical integrity of the material. Due to the intensity of stress asserted onto the knee, the material would need to uphold the same mechanical properties of healthy cartilage. The microspheres form the pores within the polymer. After crosslinking and forming the network of pores, the composite will provide a certain amount of mechanical strength. Increasing the porosity will increase the amount of cells that can be injected, which will increase the chances of a successful implant. However, increasing the cells will also increase the porosity, which will decrease the mechanical strength. Finding the maximum acceptable porosity is the key to having a successful implant.

To choose the porosity of the composite, the relationship between the compressive strength and porosity is evaluated. In order to determine the acceptable porosity, the compressive modulus versus porosity is assessed. In the following

study, porous rocks were tested with varying porosity. From the figure below, Figure 14, it is seen that the relationship is exponential, and given through the equation $\sigma_c = ae^{-bn}$, where a and b are empirical coefficients.[29]

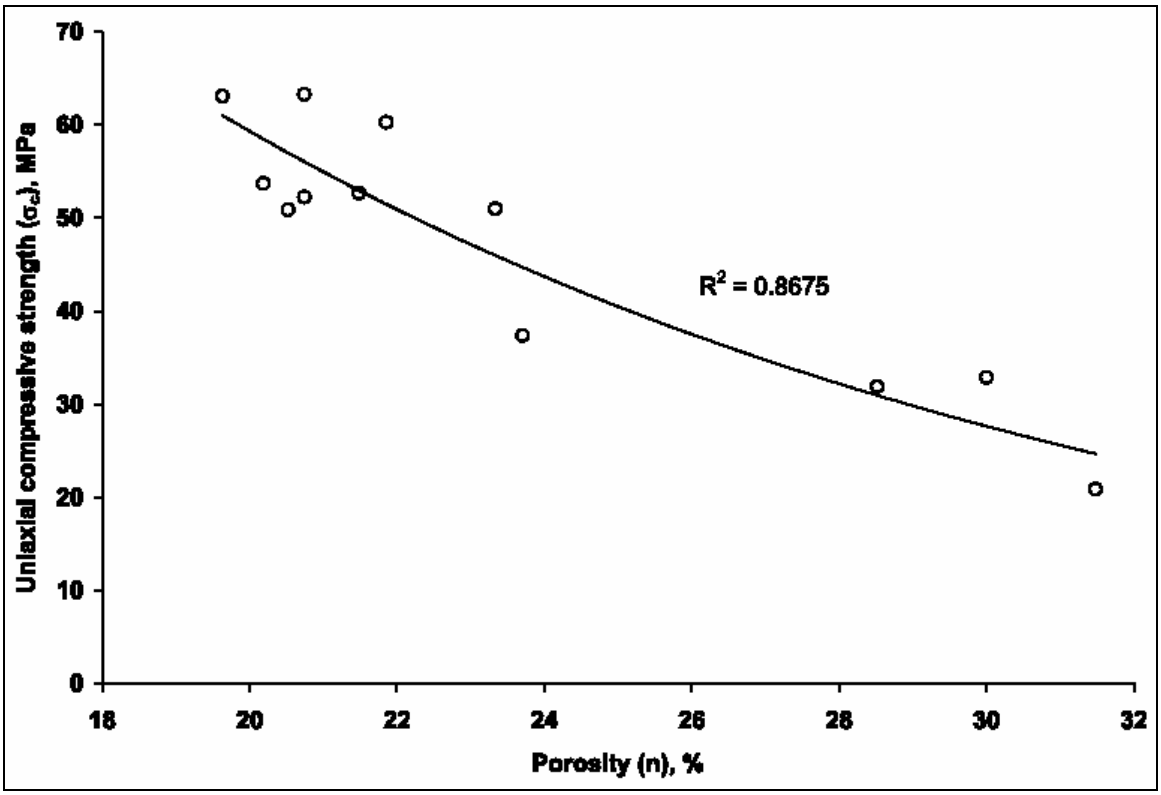

Figure 14. Relationship between compressive strength and porosity[29]

For the composite used for PPF, there is also a similar relationship. The figure below, Figure 15, depicts the analogous correlation.

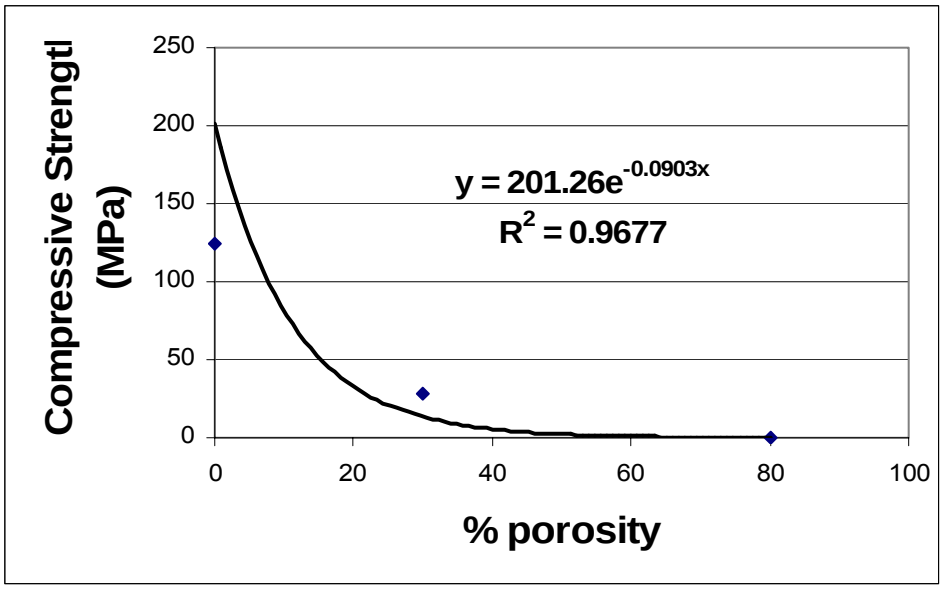

Figure 15. Relationship of compressive strength and porosity for PPF

Using the equation given on Figure 15, the acceptable porosity is obtained.[9,28] Healthy cartilage has a compressive modulus between 0.4 and 1.5 MPa.[2] Using this range with the above correlation, the acceptable porosity is found to be between 54 and 69%. With this range, the highest porosity of 69% will be chosen for the implant. It is also understood that the material will degrade over time. With the degradation, the mechanical integrity is also expected to decrease. According to Hile et al, following three weeks of *in vitro* degradation, 50% of the initial mechanical strength of the composite will be lost. However, it is also noted that 50% of the mechanical integrity of the defect region is recovered after three weeks. Therefore, the mechanical stability will be upheld for the initial few weeks. Thereafter, the mechanical properties will be upheld by the new cartilage and bone. The defect site should be balanced without noticeable compromise.[28]

Furthermore, an additional concern is the homogenous mixture of microspheres and polymer. As the microspheres and polymer are mixed together and injected into the defect site, the microspheres are expected to be evenly distributed within the composite. However, it is necessary to form the network of pores within the new structure. After PPF crosslinks, the cells are released from the capsules. In order to promote growth, the pores need to create continuous voids within the material. Thus, the microspheres need to be adjacent to each other before crosslinking.

For our use, creating 69% porosity will be achieved through injecting enough microspheres to achieve close to a body centered cubic (BCC) alignment. With BCC alignment, a single sphere touches four neighboring spheres. The microspheres will form the void spaces and the polymer will take up the remaining area. Thus, the ratio of void space divided by total space will equal the porosity of the material. A perfect BCC structure will have 68% porosity. At this percentage, the spheres will be packed close enough to create the network of pores. Injecting additional smaller spheres in the place of an equivalent volume of larger spheres can accomplish a higher porosity to obtain the 69%, while keeping the desired volume. However, it is also understood that it is not necessary to create different sized spheres. The packing will most likely not be a uniform BCC structure; however, it will not greatly affect the overall porosity of the material. A less organized placement of the spheres, such as a simple cubic structure will not be possible since this type of formation will not allow porosity greater than 52%. A higher organization, such as the face centered cubic structure will allow a higher packing efficiency of the spheres, and will lead to a maximum of 72% porosity. Creating a composite with 69 volume percent

microspheres will force the spheres to be evenly distributed. An even distribution will most closely resemble the BCC structure. It is understood that the spheres will be forced to touch neighboring spheres; therefore they will be in close enough proximity to promote the porous structure. Since the material will most closely resemble a BCC structure, the average sphere will be touching four surrounding spheres. In addition, the porosity is not largely dependent on the diameter of the spheres. As long as the spheres are the same size, the void space per total space will remain conserved.

4. FDA Approval Process

The FDA approval process will be the most critical step of N.K.O.B.®. This process will be the most expensive and time consuming step and will ultimately determine the fate of the development of the venture. Since this new treatment involves implantation in the body and lacks information on the effectiveness and safety, the approval process will follow the most stringent evaluation.

The Center for Device and Radiological Health is the branch of the FDA that is responsible for the regulation of the evaluation of medical devices. The N.K.O.B.® implant will be defined as a medical device since it is "an implant [that is] … intended to affect the structure or any function of the body[25]." Medical devices are classified under three different classes. Class III is the most risky type of medical device that lacks sufficient proof of safety and effectiveness and could result in unnecessary harm or injury to the body. Since our device is to be implanted into the body, and could result in adverse side effects, the implant will be under this classification.

Class III devices require Pre-Market Approval (PMA) because General Controls may not be sufficient enough to evaluate the new device. General Controls include Establishment Registration, Medical Device Listing, and Labeling[25]. A PMA includes the necessary data collected through non-clinical and clinical studies that back up the safety and effectiveness of the device.

There are two methods of filing a PMA, either through a modular or traditional PMA. N.K.O.B.® will follow a modular PMA which allows for the application to be submitted in separate modules. In comparison, a traditional PMA application is

submitted after all non-clinical and clinical studies have been completed and includes all necessary data needed for approval. The modular PMA application allows for feedback for initial submissions that will help determine appropriate paths to take. After request for a Module PMA, a review board will assign a PMA shell that will describe the individual steps taken and the required information for each step. The shell will also provide a timeline for which each module should be submitted.

N.K.O.B.® will follow three main modules in the PMA application. Module 1 will include laboratory testing on biocompatibility on the scaffold, cell/scaffold interactions, and basic chemistry involved with the production of the polymers and microspheres. Module 2 will consist of non-clinical studies involved animal experiments and studies evaluating the effectiveness of the implants and success of the regeneration of the cartilage. Module 3 will consist of clinical studies on humans.

4.1 FDA Modeling and Risk Analysis

In order to determine how risky N.K.O.B.® will be a model was developed in order to determine how much money could potentially be lost or made over the course of the project, how long the project will last and what are the likely costs.

It was determined that the main factors that determined success or failure of the project were the initial research and development process. During this process N.K.O.B.® chose to vary in its model the number of lab technicians and the number of experiments that were performed. The way these variables affect the probability, cost, and time of the process is as follows: The more lab workers that are employed the more it costs to perform the experiments, but the experiments can be performed in a timelier manner. However, the number of workers does not have an effect on the

probability of the project because the number of experiments performed for a certain scenario would still be the same. The number of experiments affects the probability, cost and time of the project. If more experiments are performed initially, the chances of the project receiving PMA approval and passing all the modules of the approval process increases, whereas if fewer experiments are performed the chances consequently decrease. However, more experiments entail more money and more time spent.

4.1.1 Pre-FDA Testing

In order to develop a model that would aid in quantifying the amount of risk associated with the FDA approval process, a two-stage stochastic modeling procedure was utilized. Two-stage stochastic programming involves quantifying the risk associated with certain planning decisions by attaching a probability to certain events that may happen in the future. In two-stage stochastic modeling, first stage decision variables are set, such as amount of employees or number or experiments. These first stage decision variables must be determined before a process can be undertaken, and are called "here an now" decisions.[27] After the first stage decision variables, the course of the project will be determined by chance, but the first stage decision variables in this case will aid in either decreasing or increasing the chance of success or failure. Usually the value determined to compare the riskiness of the project is profit or expected net present value. A second stage decision variable occurs after an outcome, which in this case is whether or not to continue with the FDA approval process after a failure has occurred.

It is impossible to determine exactly how these variables affect the project merely by observation because of the associated uncertainties; therefore a decision tree-type simulation was developed to quantify the expected values and the risk. The number of workers was set to 10, 5, or 2 workers and 1 PhD. Also, the number of experiments performed was set to 70, 60, or 45 experiments. This created essentially nine different decisions that could be made. The goal was to determine which of these decisions would render the highest Net Present Value, with the least amount of risk involved in the venture, i.e. how much profit must be given up in order to reduce the risk. Figure 16 shows a decision tree for these first stage decision variables which must be made prior to the start of the project. These first stage decision variables are ‘’here and now” decisions which must be made prior to beginning a project venture.

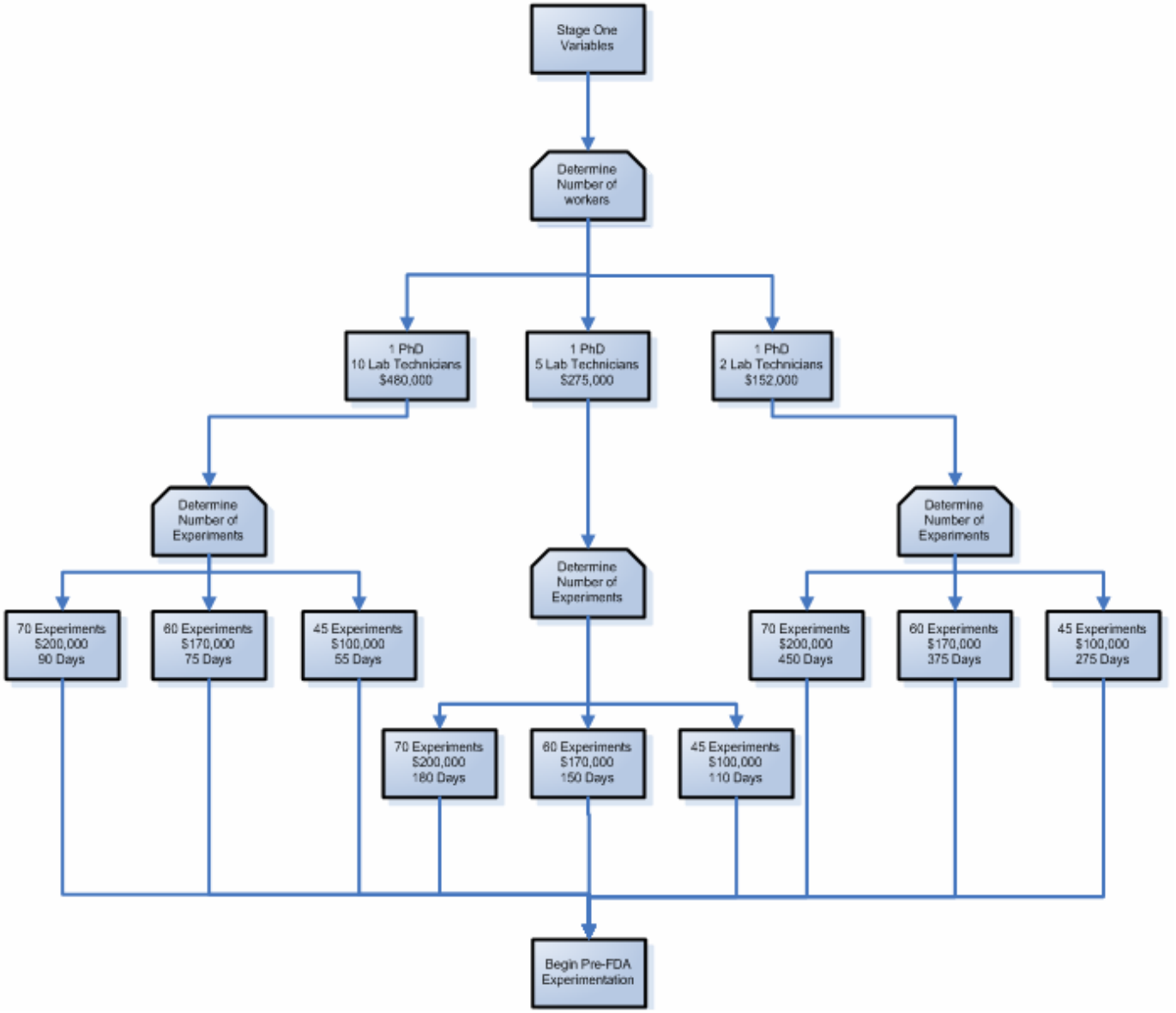

Figure 16. Pre-FDA testing decisions

The experiments to be performed prior to FDA approval will be mainly on the synthesis and laboratory bench scale level. The goal of these initial experiments is to prove to the FDA that the procedure could be a potentially marketable product and that it will be successful in passing the FDA approval process. Therefore, the more experiments performed the more chances that the procedure will pass the approval process in a more timely manner, and with much

less of a monetary burden. For each of the first stage decision variables the number, type, and depth of the initial pre-testing experiments are different, such that the 70 experiment decision will contain more experiments than the 45 experiment decision.

For the 45 experiment decision variable the following five types of experiments will be performed, with each being run nine times (three times in triplicate for verification of results obtained): synthesis of poly(propylene fumarate), gelatin microencapsulation assay, evaluation of cell growth on polymer, growth factor encapsulation and effect, and basic biocompatibility tests.

4.1.1.1 Synthesis of Poly(propylene fumarate)

This experiment will deal with the synthesis of the polymer as described below:

1. Poly(propylene fumarate) (PPF) is prepared in a two step reaction process as described by Payne et al.[8]
    a. diethyl fumarate (DEF) and propylene glycol (PG) are combined in a 1:3 molar ratio in a 11 round bottom 3-neck reaction vessel.
    b. Zinc chloride ($ZnCl_2$) is added at a molar ratio of 0.01:1 $ZnCl_2$:DEF and hydroquinone (Hq) is added at a molar ratio 0f 0.002:1 Hq:DEF. $ZnCl_2$ is the catalyst and Hq prevents undesired reactions
    c. Attached to the vessel is a cold water condenser with a flask to collect condensate and the system is slowly purged with nitrogen to make it a nitrogen atmosphere.

d. The vessel is first submerged in an oil bath and raised to 100ºC. Then the temperature is raised to 150ºC over 5 hours. When condensate ceases to form the first step is complete, forming the diester bis(2-hydroxypropylfumarate) (BHPF)

e. Then the reaction mixture is allowed to cool to room temperature

2. The second step of PPF synthesis is as follows:

a. The products of step 1 (BHPF and PG) are then combined in a 31, 3-neck reaction flask of the same set-up

b. The system is then purged with nitrogen gas

c. The temperature is then raised to 100 ºC over 45 minutes and the pressure is reduced to 0.1 Torr. The unreacted PG is distilled and condensed.

d. Next the temperature is raised to 140 ºC over 2 hours. PG produced during the transesterification is removed and condensed resulting in formation of PPF.

e. The temperature is then maintained at 140 ºC for 2 hours and then raised to 150 ºC for 1 hour. The system is then purged with nitrogen and allowed to cool to room temperature.

f. The PPF is then dissolved in an equal volume of methylene chloride.

g. This solution is then shaken with and organic phase removed form solutions as follows: 5% HCl in ddH_2O, ddH_2O twice, ddH_2O saturated with sodium chloride twice, and ethyl ether.

h. The PPF/methylene chloride solution is then added dropwise to cold ethyl ether to remove the Hq.
i. The remaining solvent is then removed by rotovaporation followed by vacuum drying.

Once the polymer has been synthesized, the number average and weight average molecular weights will be determined through chromatography. This will determine the success of the synthesis and will provide data on the proper synthesis procedure, as any failures will be fixed in order to determine the proper procedure required. This experiment is a minimally essential experiment for the success of the project.

4.1.1.2 Gelatin Microcapsule Assay

This experiment, also minimally essential experiment, will evaluate the success of the microencapsulation of the different cell types, chondrocytes and mesenchymal stem cells. First the gelatin solution will be prepared as follows:

1. First a 30% solution of gelatin is prepared as follows:
    a. A 30% (w/v) porcine gelatin is prepared by heating 70 mL ddH_2O to 95ºC on a hotplate with a magnetic stirrer.
    b. 30 g of porcine gelatin is then added in 0.25 g portions allowing each portion to dissolve until another is added.
    c. Volume then increased to 100mL after all gelatin dissolved with ddH_2O.
    d. The resulting solution is then allowed to solidify at room temperature and then stored at 4ºC until later use.

2. Next an 11% gelatin solution is made from the 30% gelatin solution previously prepared as follows:
    a. DMEM and the 30% gelatin solution are warmed to 50ºC
    b. 33.33g of the 30% gelatin solution and 56.67 mL of DMEM are combined in a beaker.
    c. pH is adjusted to 7.2 by either addition of either NaOH or HCl
    d. This resulting 11% solution is then warmed to 50ºC
    e. In a sterile laminar flow hood the solution is then sterilized via vacuum filtration into a sterile media bottle.
    f. This sterile 11% solution is then stored at 4ºC until later use.

Then, in order to encapsulate the cells, the following procedure will be followed:

1. First, 45 mL of the 11% gelatin solution at 37ºC is diluted with a 5 mL sterile suspension of either chondrocytes or marrow stromal cells in DMEM and mixed thoroughly. The resulting solution should contain approximately 4.0×10^5 cells/mL.
2. This solution is equilibrated at 37ºC
3. In a sterile laminar flow hood the solution is placed in 30mL syringes with 22G needles.
4. These are allowed to cool to 34ºC.
5. The solution is then dripped into 400mL of sterile mineral oil at 10ºC agitated by a magnetic stirrer (this mineral oil solution contains 5mM of Dithiobis(succinimidylpropionate) (DSP) to initiate a crosslinking reaction

on the particle surface). This causes the suspension to form droplets because of hydrophobic interactions and causes the droplets to harden from the temperature change.

6. The microparticles are then collected by filtering on a cold 80 μm nylon mesh.
7. Then the microparticles are transferred to a beaker containing 400mL of sterile PBS at 15ºC with a magnetic stirrer.
8. After 2 minutes the agitation is stopped and the remaining mineral oil was decanted.
9. The microparticles are then again filtered on the nylon mesh.

After the microparticles have been seeded with the different cell types various assays will be performed on them in order to determine the success rate of encapsulation. A DNA assay will be performed in order to determine the number of cells that were successfully encapsulated. This number will be compared with the number seeded initially to determine the success rate of encapsulation. This is an essential experiment to be performed since if microencapsulation is not achieved, the procedure would not be viable for use in humans.

4.1.1.3 Evaluation of Cell Growth on Polymer

This experiment will evaluate the cell growth success of both encapsulated and non-encapsulated cells on both PPF/β-TCP and PPG-co-EG polymer sections. These polymer sections will be both actively crosslinking and already crosslinked.

The polymer will be prepared as previously described. Also, the cells will be encapsulated as described in the previous experiment. The polymer will then be crosslinked using *N*-VP and seeded with both encapsulated and non-encapsulated cells by the following procedure:

1. PPF (with TCP or EG), N-vinylpyrrolidinone (N-VP) and benzoyl peroxide are first sterilized by placing them in glass vials and exposing them to UV light in a sterile laminar flow hood.
2. The N-VP and BP are sterilized for 2 hours, while the PPF is sterilized overnight.
3. Also, dimethyl p-toluidine (DMT) is obtained and sterilized.
4. The amounts of each component in the PPF composite per gram of PPF are as follows: 1 g PPF/b-TCP or PPF-co-EG, 0.1 g N-VP, 0.0015 g BP, and 2.5uL DMT.
5. First, these components are prepared by mixing the respective PPF, DMT and half of the N-VP, and also mixing together the BP and the other half of the N-VP.
6. The previously prepared encapsulated or non-encapsulated cells are then added in the appropriate seeding density.
7. Then, the PPF, DMT, N-VP solution is then added to the BP, N-VP solution and briefly mixed, after which the cells are added to the solution, as well as the microcapsules containing the respective growth factors.
8. This composite will then be set in a large Petri dish and incubated in a sterile environment for a specified time (1, 4, 10, or 30 days).

Once the composite has been prepared histological analysis will be performed at certain time points. These histological analysis will test for cell growth and proliferation. Alkaline phosphotase and osteocalcin assays will be performed in order to determine the production of cell extra-cellular matrix formation. Also, DNA assays will be performed in order to determine the proliferation of the cell numbers on the composites. These numbers will be contrasted between the encapsulated and non-encapsulated cells in order to be certain that microencapsulation does increase the cell survival rate in the crosslinking polymer.

4.1.1.4 Growth Factor Encapsulation and Effect

This experiment will deal with encapsulation of the growth factors and their observed effect on the success of cell proliferation and growth. The growth factors will be encapsulated by the following procedure:

1. Microspheres are prepared using a solid-encapsulation/single emulsion/ solvent extraction technique as described by King et al[7]
2. 500 mg of 50/50 PLGA and 5 mg of polyethylene glycol (PEG) is dissolved in 2 mL of methylene chloride creating a 25% (w/v) solution. PEG addition increases the degradation rate of the microspheres.
3. 100 mg of bovine serum albumin (BSA) and 50 μg of either TGF-β or BMP-2 (in a mass ratio of 1:2000) depending on the region of intended use are added to the polymer solution and vortexed vigorously at a medium-high setting for 30 seconds.

4. Immediately after, 10 mL of 0.3% (w/v) polyvinyl alcohol (PVA) is added and vortexed vigorously for another 30 seconds.
5. This solution is then added to a beaker containing 90 mL of the PVA and 100 mL of 2% isopropyl alcohol and this is continuously stirred for 90 minutes at room temperature. This allows the organic solvent to be extracted from the solution.
6. The microspheres resulting are then centrifuged and washed in distilled water 5 times.

Once these microspheres are produced they will be applied to respective cell cultures of chondrocytes and mesenchymal stem cells. These cell cultures will be incubated for various time periods and the resulting effect of the growth factors will be compared to cultures not containing growth factors. Various assays such as alkaline phosphotase, osteocalcin, and DNA will be conducted in order to compare the effect of growth factors on cell growth and extra cellular matrix production.

4.1.1.5 Basic Biocompatibility Tests

In order to evaluate basic biocompatibility issues resulting from the injection of the construct into the defect site, this experiment will deal with implantation of a test construct subcutaneously in male Wistar rats.

The experiment will begin with the preparation of the construct as described previously. These constructs will be prepared in sections that are 2mm thick and 8mm in diameter. Once the constructs are prepared, they will be surgically implanted under the skin of the male Wistar rats and left for 2

months. After 2 months various organs of the rats will be examined, such as the kidneys, liver, spleen, and pancreas, and compared to rats without implantation. Other various test will be run in order to determine any toxic effects to these organs. Also, surrounding tissue of the implant will be examined for any local necrosis or morbidity. This experiment will provide minimally essential data for the biocompatibility of the procedure.

These experiments for the 45 experiment set are estimated to cost approximately $100,000 (see Figure 13). The amount of time required for these experiments will depend on the number of laboratory technicians working, which will be 55 days for 10 lab technicians and 275 days for 2 lab technicians (see Figure 13).

For the 60 experiment first stage decision variable the aforementioned experiments will be performed in addition to three more experiment types. These additional experiments include: evaluation of polymer mechanical properties pre- and post-implantation, evaluation of the optimal cell seeding density of the gelatin microparticles, and evaluation of the optimal growth factor and cell density ratio within the construct.

4.1.1.6 Evaluation of Polymer Mechanical Properties

The main purpose of this additional experiment is to determine the mechnical properties of the polymer and if needed reformulate the synthesis of the polymer. If the mechanical properties are not sufficient enough for use in

the articular knee joint, then the polymer must be altered in some way to make them adequate.

Specimens of the polymer will first be prepared by the procedure described previously. The mechanical properties (tensile, shear, and compressive) will be evaluated before implantation. The specimens will then be implanted into wistar rats and taken out at different time points (1, 3, 6, 10, and 30 days). The extracted specimens will then be tested for their mechanical properties, as well as being tested for their percent mass lost and determine the swelling ratio.

4.1.1.7 Optimal Cell Seeding Density

The purpose of this experiment will be to evaluate the optimal seeding density of the gelatin microparticles containing the encapsulated cells. There would obviously be an optimal number of cells to seed, since too many cells may crowd the space and prevent cell growth and too little cells may prevent cell networks being formed and thus prevent cell growth. This optimum will need to be determined by the success of growth both *in vitro* and *in vivo*.

Various amounts of cells will be seeded into the gelatin microparticles and these microparticles will be seeded into the polymer constructs and various volume ratios. All of the samples will be incubated *in* vitro for various time periods and the success of cell survival and tissue growth will be evaluated through various histological assays.

Also, to evaluate the optimum *in vivo*, the constructs will be implanted into the articular joints of male wistar rats and samples will consequently be extracted at various time points for histological analysis.

4.1.1.8 Optimal Growth Factor Density

Because the previous experiment dealing with growth factors was merely a basic test to determine the effect of growth factor stimulation, a further experiment to determine the optimal density of microparticles containing growth factors can be implemented.

In this experiment, which is similar to the previous experiment, various concentrations of growth factor microspheres will be evaluated both *in vitro* and *in vivo*. Histological assays will then be performed to again determine the optimal growth factor concentration.

These experiments for the 60 experiment set are estimated to cost approximately $170,000 (see Figure 13). The amount of time required for these experiments will depend on the number of laboratory technicians working, which will be 75 days for 10 lab technicians and 375 days for 2 lab technicians (see Figure 13).

For the 70 experiment first stage decision variable the aforementioned experiments for both the 45 and 60 experiment decision will be performed in addition to two more additional experiment types. These additional experiments include: evaluation of the degradation rate of the polymer versus the cell tissue

ingrowth and evaluation of the long-term success rate of the procedure on male Wistar rats.

4.1.1.9 Degradation versus Tissue In-growth

The main purpose of this experiment will be to evaluate the relationship between the polymer construct degradation versus the cartilage tissue growth throughout the construct. These two rates will need to be approximately equal for the construct to maintain integrity and provide for an adequate platform for tissue regeneration.

The way this experiment will work is similar to previous experiments (constructs will be implanted into rats and evaluated at certain time points). Tissue histologies and percent mass lost and swelling will be evaluated simultaneously, in addition to mechanical stability of excised constructs.

4.1.1.10 Long-term Success

Essentially this experiment will deal with performing the procedure as a whole and allowing the construct to remain in the defect site for 3 months. The cartilage tissue regenerated will be compared with rats that had only polymer, polymer with only cells, and polymer with only growth factors injected. This will give a long-term result for the procedure in male Wistar rats and gives some preliminary results which will ensure a greater chance of success in the FDA approval process

These experiments for the 70 experiment set are estimated to cost approximately $200,000 (see Figure 13). The amount of time required for these experiments will depend on the number of laboratory technicians working, which will be 90 days for 10 lab technicians and 450 days for 2 lab technicians (see Figure 13).

In order to determine which of these first stage decisions would be the most profitable and contain the least amount of risk, N.K.O.B.® formulated a risk simulation. For each stage of the FDA approval process N.K.O.B.® created decision trees with assigned probabilities, times, and costs, in order to create a variety of various paths that are possible to occur during the process.

4.1.2 PMA Application Filing

The first part of the FDA approval process deals with the filing of a PMA application. This costs $250,000 and takes approximately 180 days. Figure 17 illustrates the different events that may occur during this process. For each first stage decision variable the probabilities are the same since the filing process depends mainly on paperwork and organization.

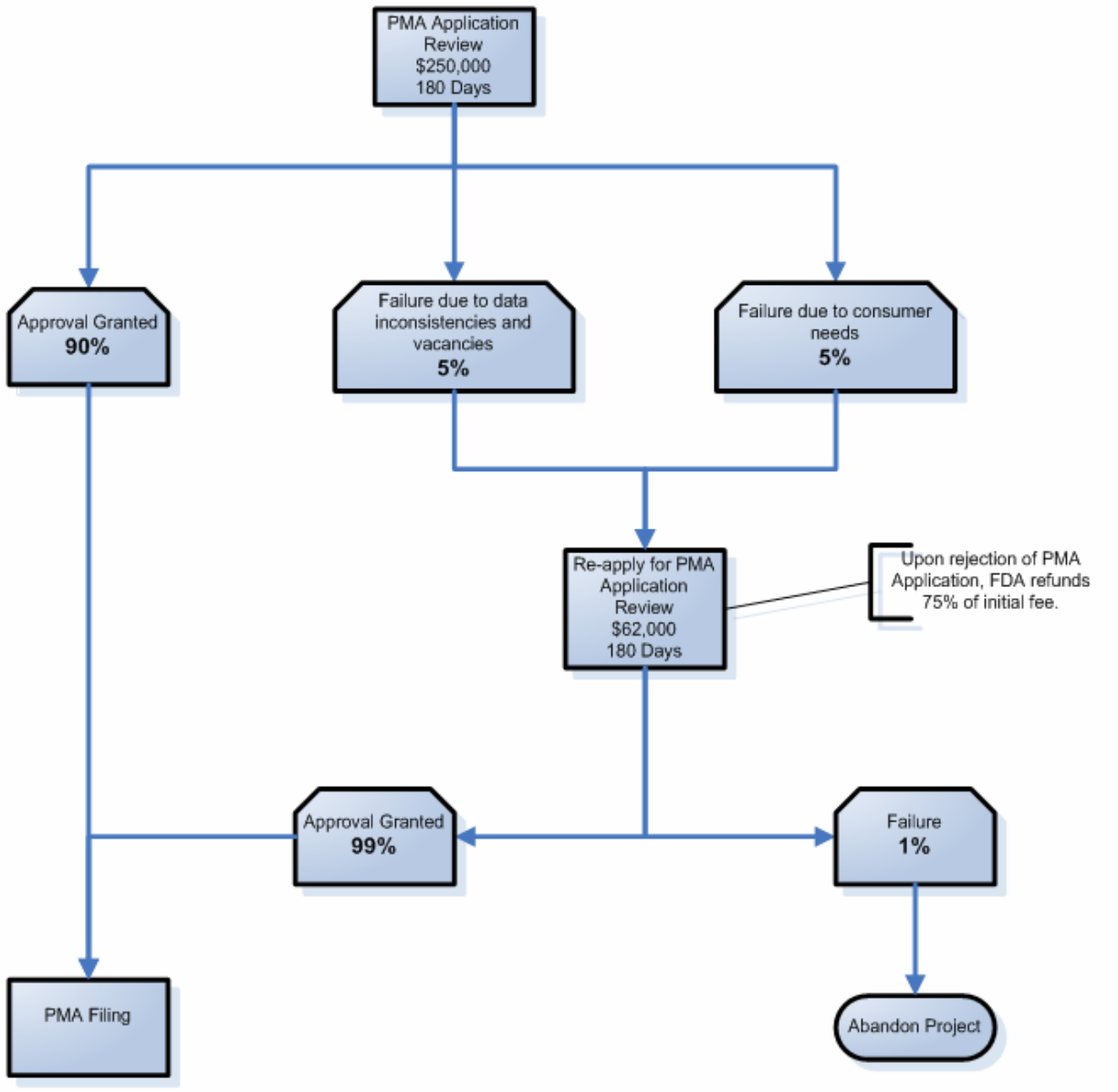

Figure 17. PMA Filing Flowchart

If the PMA application is rejected initially, FDA will refund 75% of the application fee, therefore to reapply it would only cost 25% of the original fee, $62,500.

4.1.3 Modular PMA

Once the PMA application is approved and is filed the FDA testing process begins. Since N.K.O.B.® is going through the Modular PMA process, which consists of three modules, each module will have to be approved before the process can be moved forward to the next module, or to the medical market. If a failure occurs, due to any reason, the reason for the failure must be identified, and the cause of the failure must be fixed. Fixing these failures that are possible will cost N.K.O.B.® more time and more money, and additionally, the module testing must then be performed again, which also costs more time and money. The probability of these failures is different for each of the various first stage decisions that N.K.O.B.® makes. It will not change with the amount of workers that N.K.O.B.® employs for the pre-FDA testing process because the number of experiments will still be performed, but, the number of experiments performed affects the probability of failure of the same or similar tests performed during FDA approval. Next, we describe these decision trees in detail.

4.1.3.1 Module 1

Module 1 consists mainly of laboratory and bench scale testing. It is estimated that this process will take approximately 3 years and have an expected cost $500,000 to complete if all goes without failure. During this module all formulations of the polymer, the gelatin microparticles, and the growth factor microspheres will be tested in great detail. Failure could result in any of these three parts of experimentation, leading to a cost and a time to fix each problem. After a failure has occurred, it must first be fixed, and Module 1 testing must be repeated. If failure again occurs due to any reason the project should most

likely be abandoned, which would result in a total loss in money invested into the project. Figure 18 details the various branches and their associated probabilities and each processes cost and time. It should be noted that the probabilities listed correspond to 70, 60 and 45 experiments respectively. Also, these flowcharts are the same for 10, 5, or 2 lab technicians, since this variable does not affect probability, only cost and time. This tree has eighteen possible paths each one with different probabilities. Of these paths, thirteen paths lead to failure, the other five allow Module 2 to start.

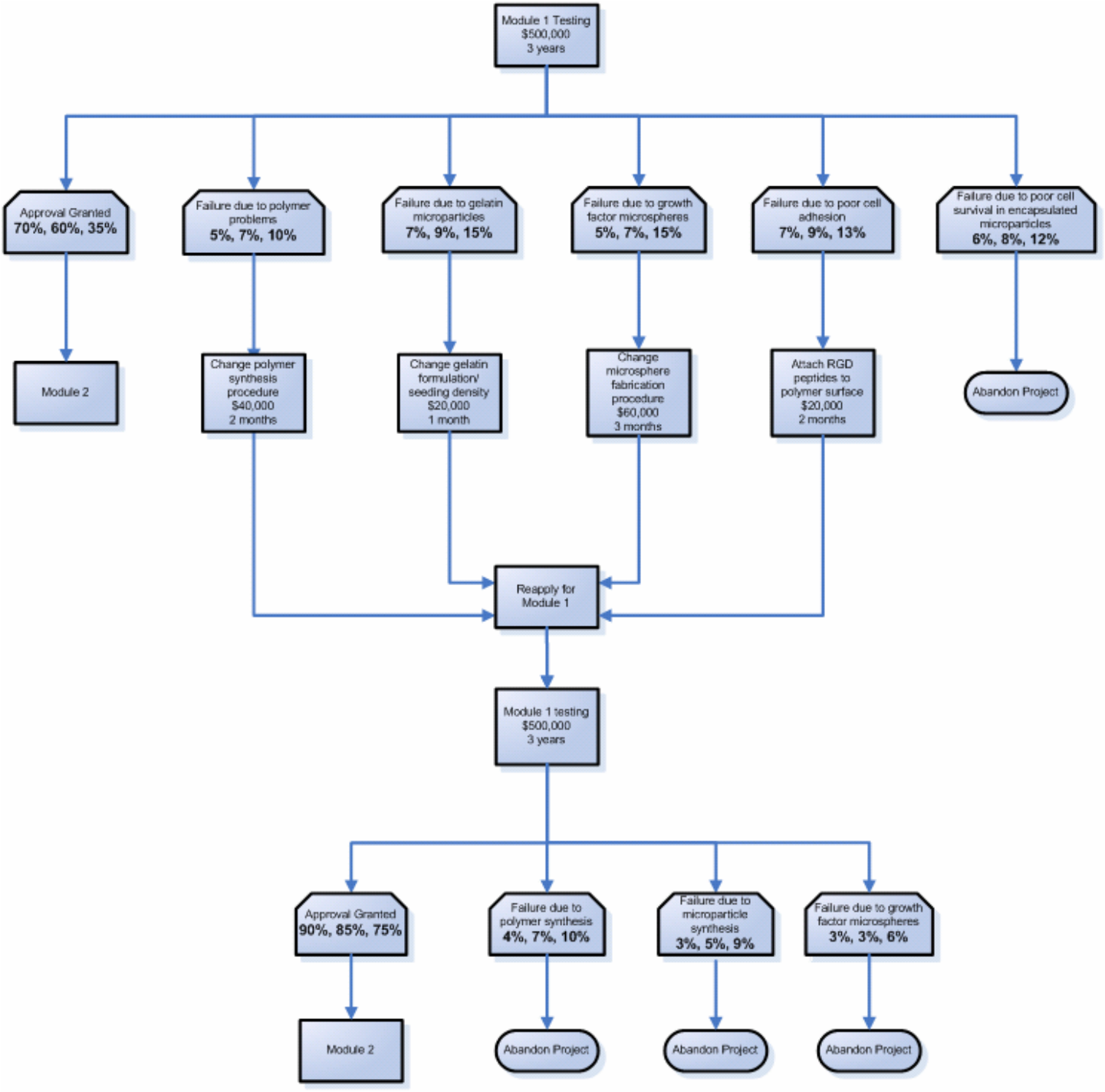

Figure 18. Decision tree for Module 1 testing

4.1.3.2 Module 2

Once Module 1 has been passed and approved the project moves on to Module 2. Module 2 will consist of mainly non-clinical animal testing. This is

done prior to clinical human trials to first evaluate the proposed treatments biocompatibility and effectiveness. It also consists of refining the surgical procedure and process. It is estimated that Module 2 will also have an expected cost of $500,000 and take an additional 3 years to complete if all goes without failure. Figure 19 illustrates the decision tree for Module 2. The animal studies will first begin with small animals such as mice, and guinea pigs. Then the testing will proceed to evaluation son larger animals, such as rabbits, dogs, and even monkeys. Once Module 2 testing has been approved by the FDA, Module 3 testing will begin. This decision tree has twenty-six possible paths each one with different probabilities. Seventeen of these seven paths lead to failure, the other nine allow Module 3 to start.

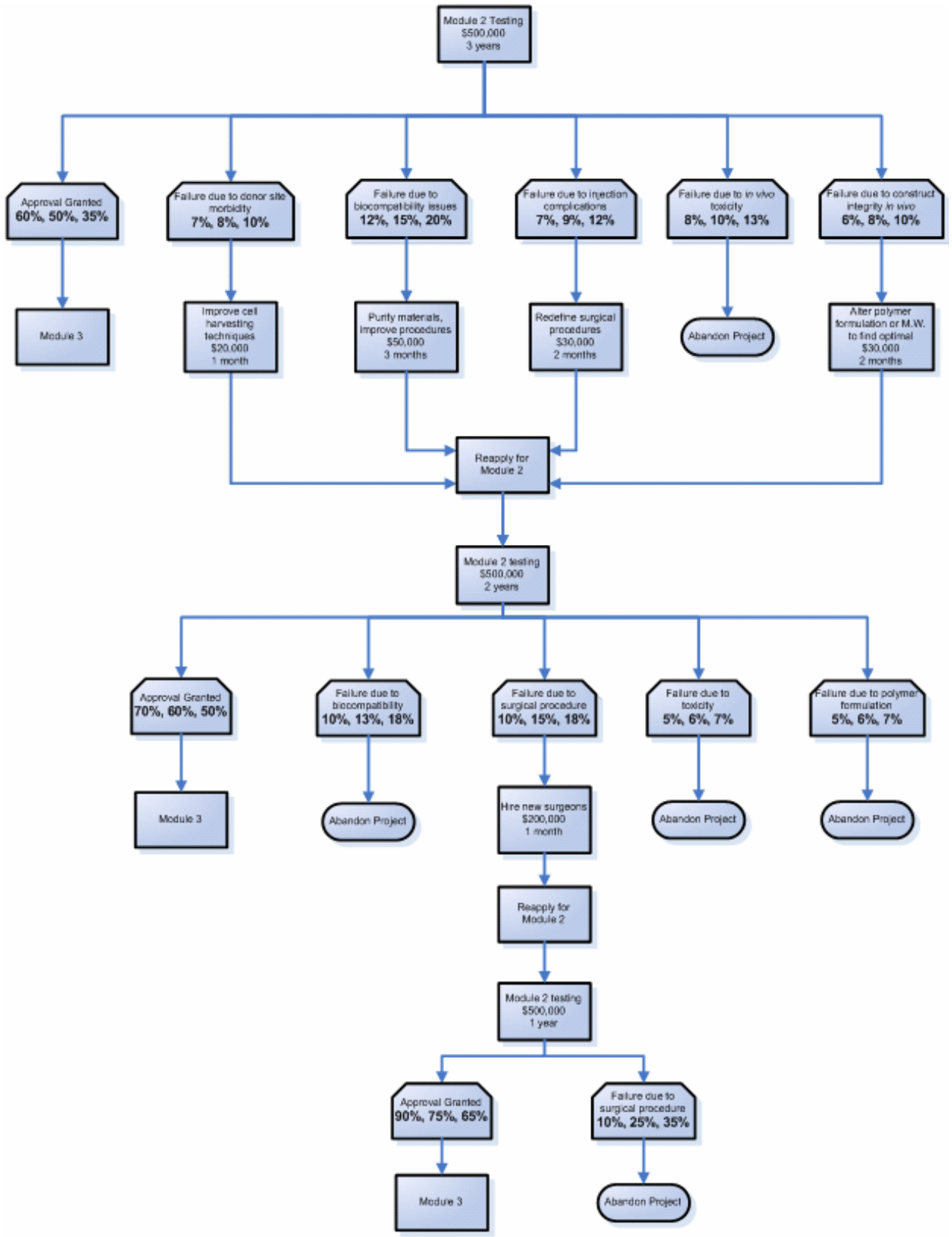

Figure 19. Decision tree for Module 2 testing

4.1.3.3 Module 3

Module 3 is the most expensive and time consuming part of the FDA approval process. It consists of clinical human trials, which is critical to the success of the project and to receive approval for the medical market. Since results from this module will be held to high standards there is a certain amount of risk involved, and many points during the process where failures may occur to a variety of reasons. The costs of the clinical trials not including the FDA costs represent the bulk of this module's cost, including doctors fees, hospital fees, etc., as estimated in the business plan section, section 5, to have an expected cost of $100,000,000. However, through the risk analysis this cost was ignored until the end of each path, therefore, the only costs associated in Figures 18 through 19 are those related to FDA approval. Figures 20 and 21 represent the various paths that could occur throughout the process. Module 3 was estimated to have an expected cost of $4,000,000 and take 10 years if all goes without failure. This tree has thirty seven possible paths each one with different probabilities. Fifteen of these paths lead to failure, the other twenty two allowing market approval.

Figure 20. Decision Tree for Module 3, Part a

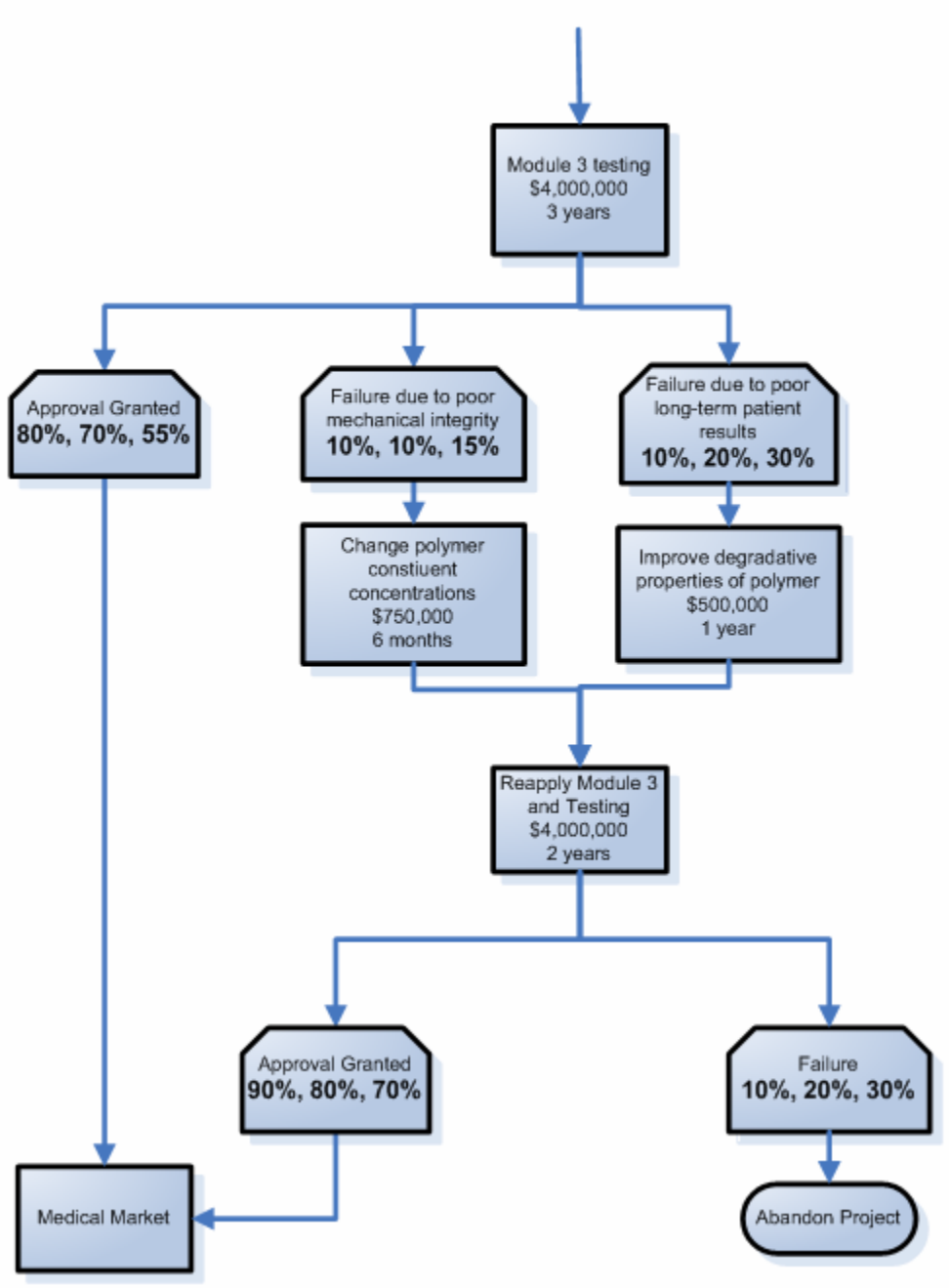

Figure 21. Decision Tree for Module 3, Part b

4.1.4 Risk Analysis Results

From these flowcharts 5,290 different paths or scenarios, 2,970 that lead to success and 2,320 that lead to failure were generated that have a certain probability, cost, and time attached to each. For each path, the net present value was determined and the cumulative distribution for all paths was plotted for each of the 9 first stage decisions. Figure 22 represents the results obtained for 10 lab technicians and for 70, 60 or 45 experiments performed.

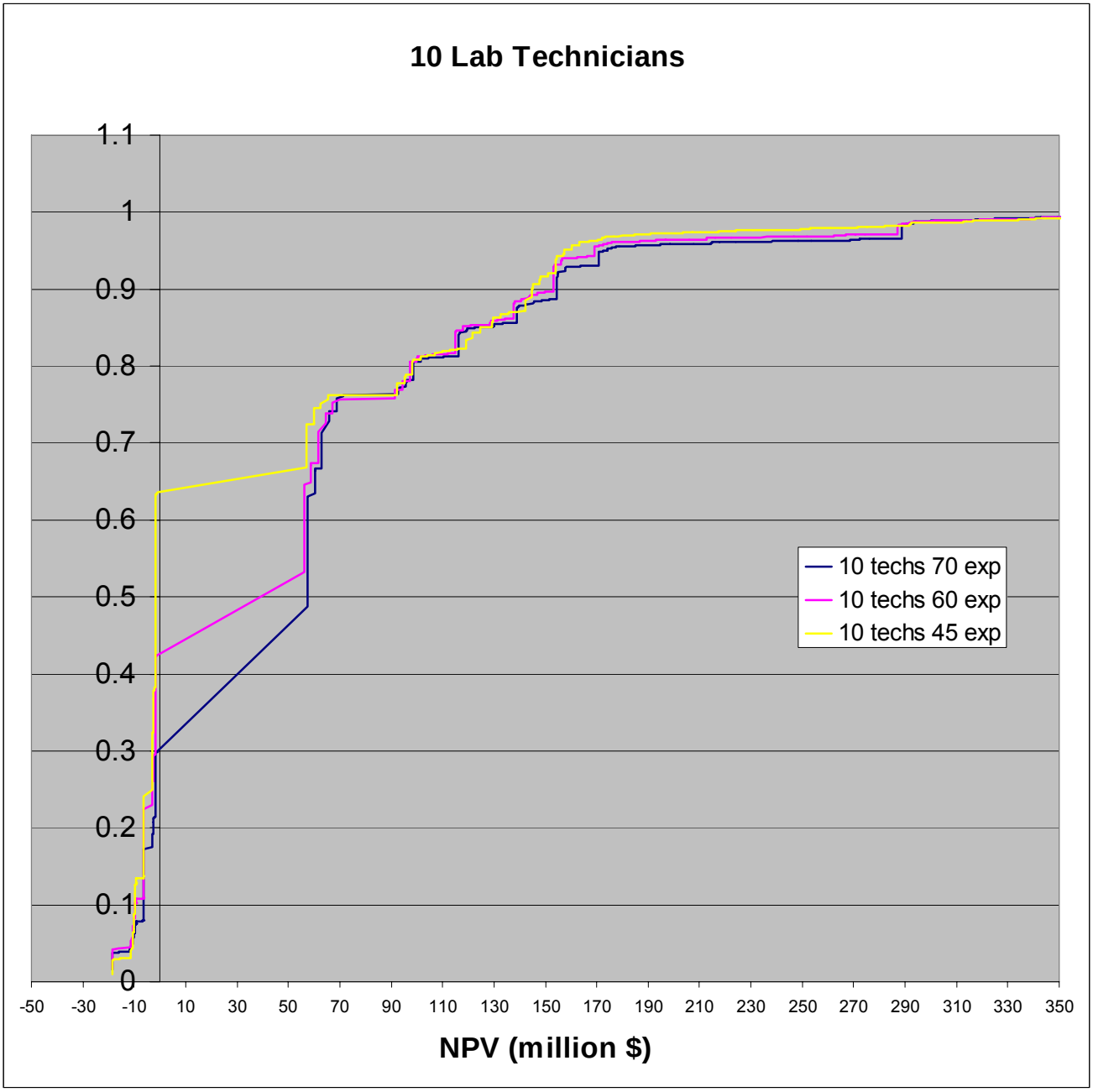

Figure 22. Risk Curves for 10 Lab Technicians

Figure 22 tells that utilizing fewer experiments, i.e. 45 experiments, leads to a distribution with higher upside frequencies, that is the probabilities of higher profits are larger. However, there is also a larger probability of losses. Indeed, the probability of loosing money with this decision is close to 64%, whereas for the

other curves this number is lower, indicating that more upfront spending reduces the risk of losing such investment, at a cost of also reducing the overall expected profit of the venture. The curves for 5 lab technicians and 2 lab technicians are similar and can be seen in Figures 23 and 24.

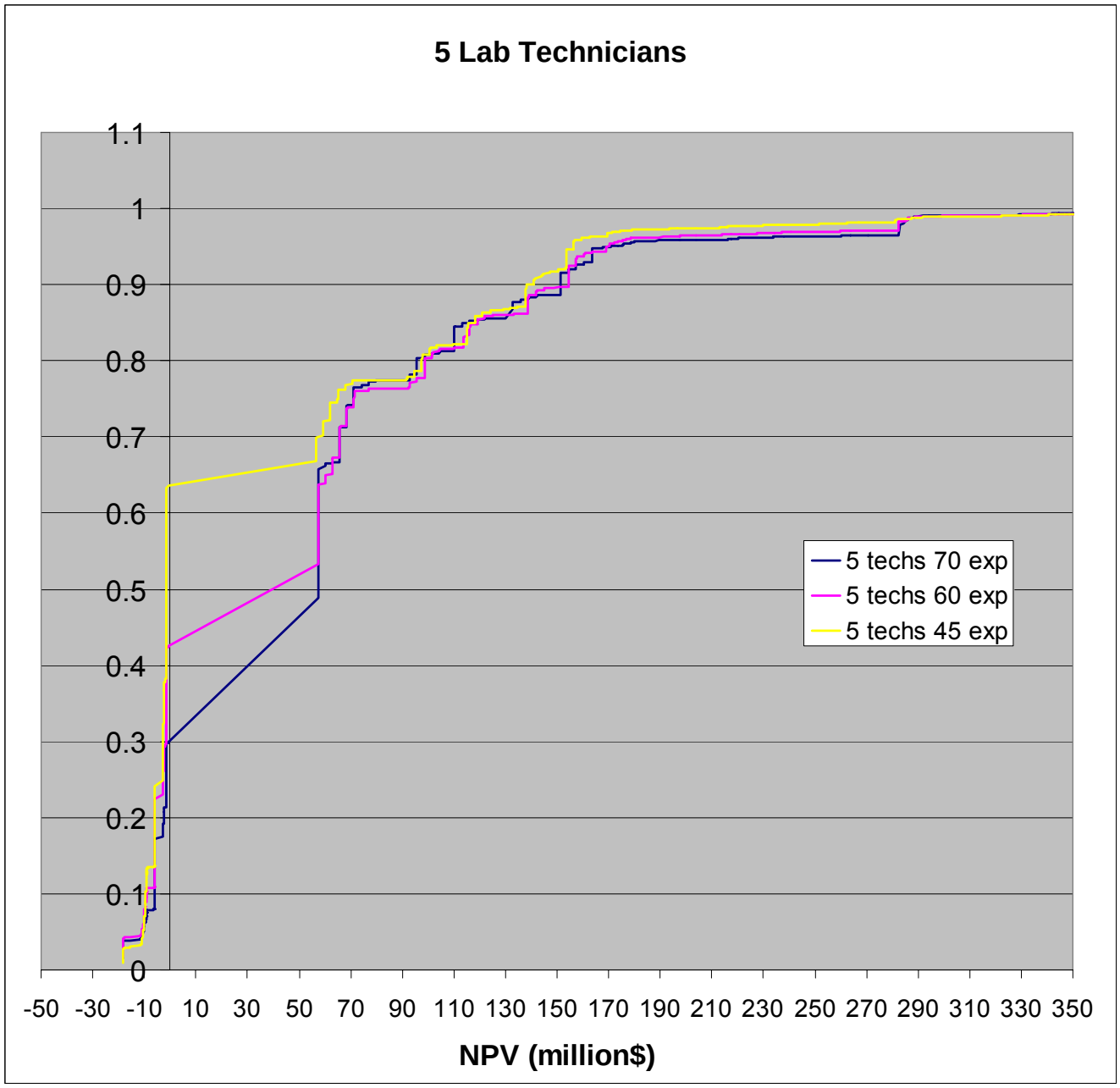

Figure 23. Risk Curves for 5 Lab Technicians

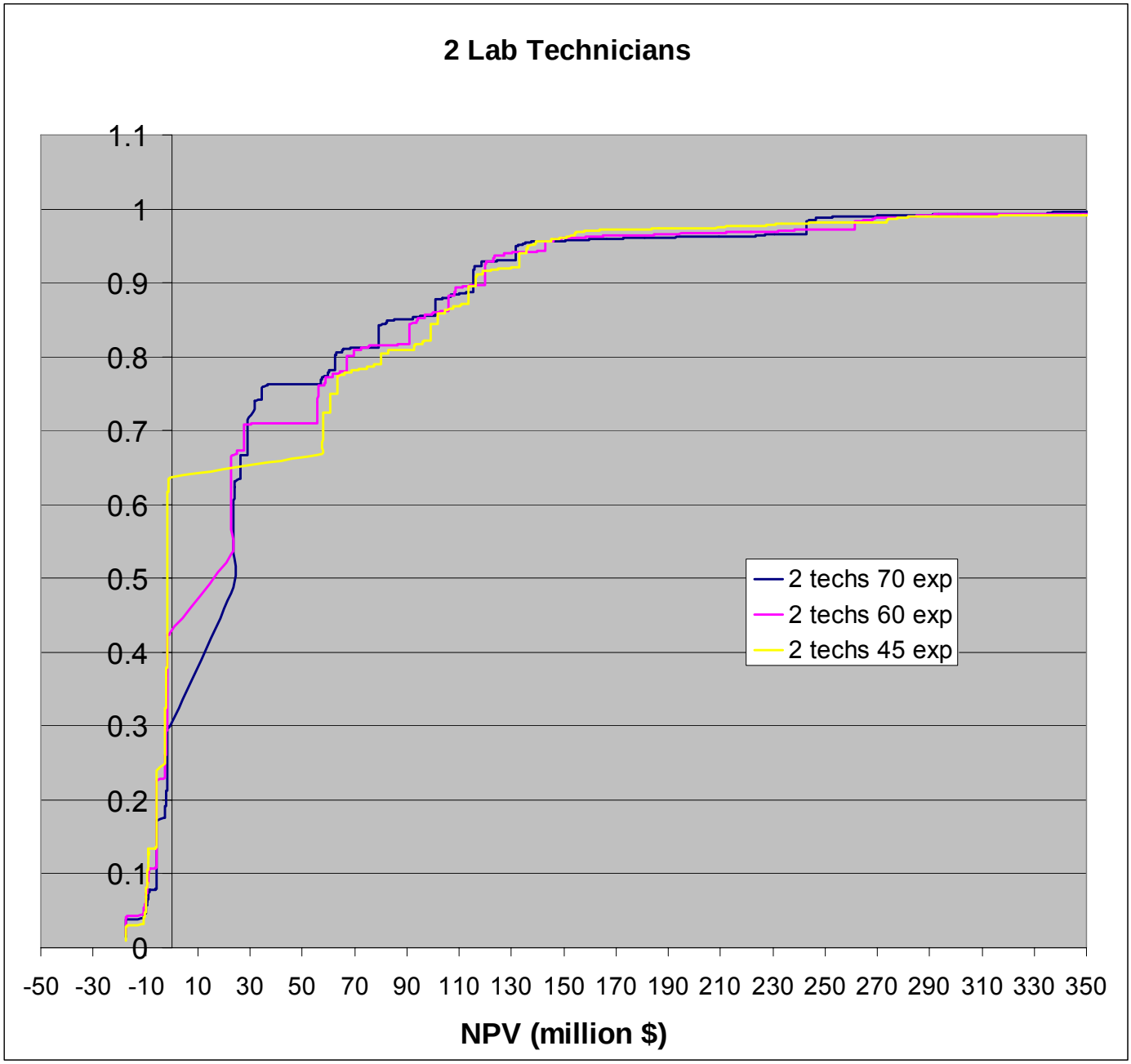

Figure 24. Risk Curves for 2 Lab Technicians

These curves produced illuminate much as to the inherent risk of the FDA approval process. The choice of which decision to make lies in the hands of the decision makers, and their attitude toward risk.

4.1.5 Risk Analysis Summary

If one adopts a risk averse attitude, one should choose to perform the maximum number of pre-FDA experiments run in the simulation, 70 experiments. Even though this will reduce N.K.O.B.®'s chances of making more money, it will also lower the chances of losing money.

However, still the decision must be made as to how many lab technicians will be employed. Based on N.K.O.B.®'s decision to perform 70 experiments in pre-FDA a comparison of the different decisions with this number of experiments versus the number of workers was formulated, and can be seen in Figure 25.

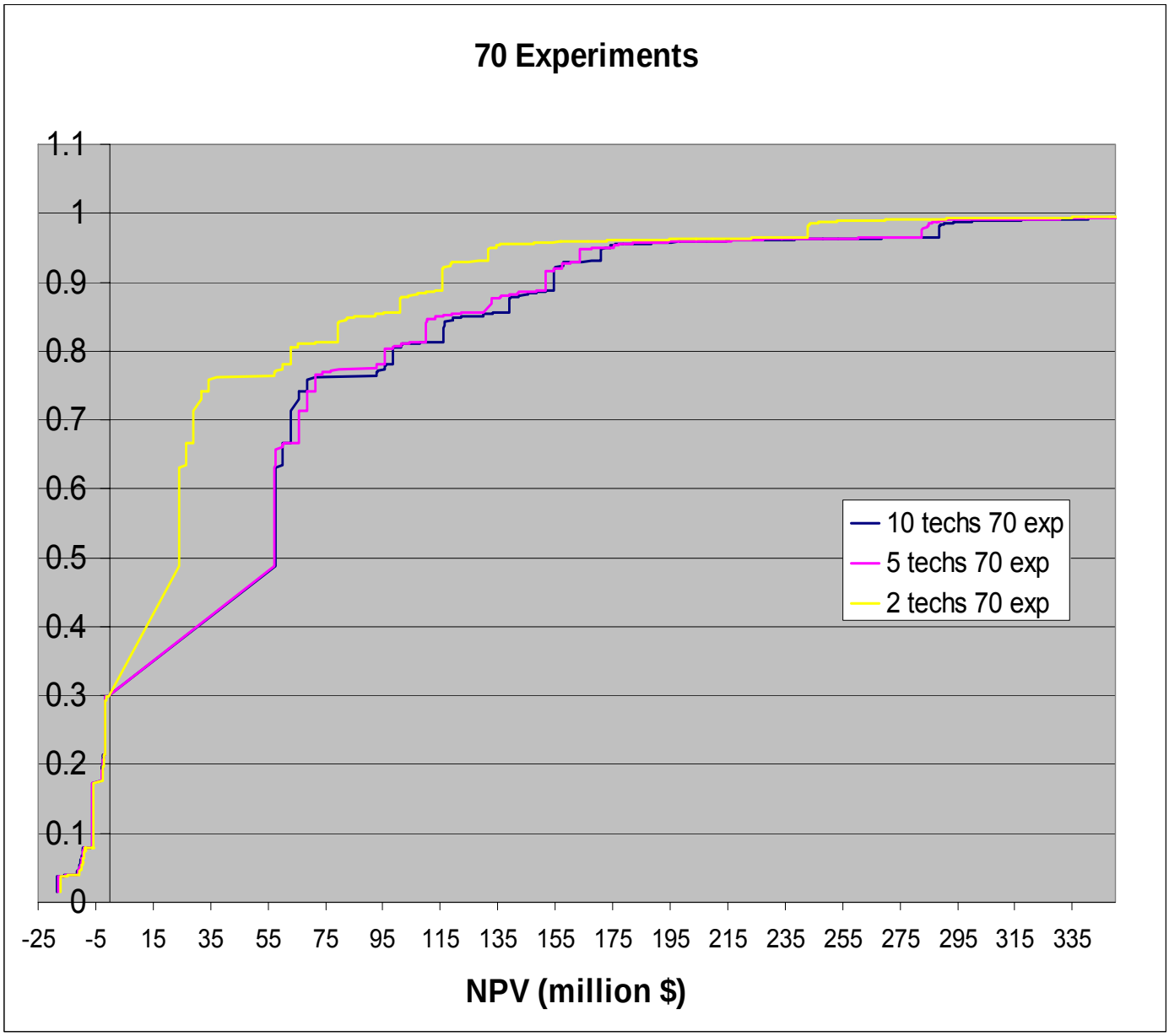

Figure 25. Risk Curves for 70 experiments

From Figure 25 it appears that the risk associated with each different number of lab technicians is almost the same, therefore N.K.O.B.® will choose to utilize 10 lab technicians for the pre-FDA testing phase. This is mostly due to the decreased amount of time that 10 lab technicians can perform experiments,

therefore leading to a shorter time for pre-FDA testing. All of the generated risk curves and their method of generation can be found in the risk simulation file.

The following gives the shortest, longest, cheapest, and most expensive path:

- **Shortest path**
  - Path in which approval is met with no failures using 10 lab techs and 45 experiments
    - Total Time: 4250 days or 11.6 years
    - $ENPV_{cost}$: $4.4 million
    - ENPV: $1130.4 million
- **Cheapest Path**
  - Path in which approval is met with no failures using 2 lab technicians and 45 experiments
    - Total Time: 4470 days or 12.2 years
    - $ENPV_{cost}$: $3.96 million
    - ENPV: $1012.8 million
- **Longest path**
  - Path in which every failure possible is met using 2 lab techs and 70 experiments
    - Total Time: 11483.5 days or 31.5 years
    - $ENPV_{cost}$: $8.7 million
    - ENPV: -$8.7 million
- **Most Expensive Path**

- Path in which every failure possible is met using 10 lab technicians and 70 experiments
  - Total Time: 9060 days or 24.8 years
  - $ENPV_{cost}$: $18.6 million
  - ENPV: -$18.6 million

5. Cost Analysis

In analyzing the costs for development and manufacturing of N.K.O.B.®, there are several costs that need to be estimated. From the equipment and facility costs, a raw capital investment can be estimated. FDA fees must also be included in the overall cost since this will be the major contributor to total cost. The FDA costs will include fees and costs for non-clinical and clinical studies. The production costs will include all raw material costs, labor and utilities that will be included in the production and manufacturing of the N.K.O.B.® polymers and microspheres.

5.1 Investment

Equipment

The equipment costs needed for the production of the materials, including the polymer and microspheres, and the culturing of the cells, were estimated by using information from product sellers as VWR™ International and Fisher Scientific International Inc[18,19]. The following table lists approximate costs for major equipment pieces.

Table 2. Equipment Costs

Equipment	Quantity	Unit Price	Price
Sterile Scalpels	10	$1.00	$10.00
Culture Flasks	100	$1.50	$150.00
Incubator	2	$13,440.00	$26,880.00
Cell Strainers	50	$1.97	$98.50
Centrifuge	1	$5,251.00	$5,251.00
Hemacytometer	1	$174.68	$174.68
Scale	1	$3,950.00	$3,950.00
Falcon tubes	500	$0.36	$180.00
Water Purification System	1	$3,523.00	$3,523.00
Refrigerator/Freezer	1	$1,348.70	$1,348.70
Autoclave	1	$7,448.00	$7,448.00
Pasteur Pipettes	1000	$0.08	$80.00
Eppendorf Pipettes	5	$63.00	$315.00
Needles	100	$0.10	$10.00
Stirrer/Hot Plate	2	$366.50	$733.00
Media Bottles	20	$9.80	$196.00
Ice Bath	1	$55.90	$55.90
Vortexer	1	$260.00	$260.00
-80 Freezer	1	$7,115.00	$7,115.00
Reaction Vessel	2	$350.00	$700.00
Mechanical Stirrer	2	$160.00	$320.00
Cold Water Condenser	1	$150.00	$150.00
Thermometer	25	$14.60	$365.00
Hot Oil Bath	1	$79.85	$79.85
Laminar Hood (UV)	1	$4,200.00	$4,200.00
Glassware			$2,000.00
Misc.			$2,000.00
Office Supplies			
Computer	4	$1,000.00	$4,000.00
Microscope	2	$500.00	$1,000.00
Scanner/Fax	2	$500.00	$1,000.00
Plate Reader	1	$700.00	$700.00
Misc.			$2,000.00
		Total	**$76,293.63**

The total equipment cost is estimated to be $76,300. Several of these pieces of equipment will be purchased continuously (pipettes, syringes, miscellaneous glassware, etc.); however, the larger pieces of equipment will only be purchased once unless unexpected damage occurs (laminar hood, incubators, autoclave, etc.). The

continuous costs of equipment are assumed to be insignificant when compared to the costs of the major pieces of equipment.

Facilities

For the research and production of N.K.O.B.®, a new facility is to be built containing both laboratories and offices. The building will be constructed with 2 major laboratory facilities, 1 animal storage room, 1 cellular storage room, and a business section that will include 3 offices and 1 conference room. Based on research facilities at major universities[20], the facility is approximated to be 10,000 square feet, with a cost of $300 per square feet[21], which is an approximation and will be dependent on location of the facility. This cost includes basic furnishing of the facilities such as water and gas lines, shelves, and desks, etc. This cost was estimated using the cost per square feet of newly constructed research facilities at major universities. Therefore, the cost of the facility is estimated to be $3 million. This cost will vary depending on location and actual square feet needed for the facility.

The facility cost is assumed to be a major contributor to the fixed capital investment (FCI). However, the highest contributor will be the FDA approval costs, which are calculated in detail in the next section. Other investment costs, such as equipment, are insignificant when compared to the facility costs; therefore, the FCI is assumed to be unaffected by the cost of the equipment.

FDA Process

For pre-FDA, the labor is determined from a risk simulation that models the risk involved with different scenarios with varying number of personnel. These simulations are presented and discussed in the FDA approval process section. Using

N.K.O.B.®'s attitude toward risk, it was decided to proceed with research and development with 10 lab technicians, 1 Ph.D, and 3 office assistants. By increasing the number of lab technicians, this will decrease the time spent in each module while also allowing the best chance for FDA approval. However, this also will introduce the greatest cost of labor. The average salary for a lab technician was found to be $40,000, for a Ph.D was $70,000, and for an office assistant was $30,000. This number of employees will be used throughout pre-FDA and Module 1 of the FDA application process, and will be used as a minimum for non-clinical and clinical studies. Table 3 lists a summary of all the labor involved with the FDA approval process.

As research enters non-clinical studies, the salary for a surgeon and costs for the various animals to be used is also taken into account. The number of employees will remain constant. The average salary for a part-time surgeon is $70,000. The costs for animals, part-time surgeon, and other expenses involved with non-clinical studies are summarized in Table 4.

In clinical studies, the salaries will be similar to non-clinical studies and is estimated to last about 10 years. This will allow for time to perform a sufficient number of surgeries and to also evaluate the results of the surgery. About 1,500 patients are to be treated during the 10 years, with the cartilage deficiencies ranging from minor osteoarthritis to more severe damage to the bone. The cost to perform a surgery in clinical studies is estimated to be $50,000, with hospital fees of $10,000. The total cost of the clinical studies is summarized in Table 5.

Table 3. Summary of Labor Costs

Pre-FDA (Module 1)		
	Cost	Cost/3 yrs
10 Lab Technicians	$400,000	$1,200,000
1 Ph.D.	$70,000	$210,000
3 Office Assistants	$90,000	$270,000
Non-Clinical		
	Cost	Cost/3 yrs
10 Lab Technicians	$400,000	$1,200,000
1 Ph.D.	$70,000	$210,000
3 Office Assistants	$90,000	$270,000
1 Part-Time Surgeon	$70,000	$210,000
Clinical		
	Cost	Cost/5 yrs
Stage 1		
10 Lab Technicians	$400,000	$2,000,000
1 Ph.D.	$70,000	$350,000
3 Office Assistants	$90,000	$450,000
1 Part-Time Surgeon	$70,000	$350,000
Stage 2		
15 Lab Technicians	$600,000	$3,000,000
1 Ph.D.	$70,000	$350,000
5 Office Assistants	$150,000	$750,000
1 Part-Time Surgeon	$70,000	$350,000
3 Marketers	$60,000	$300,000
	Total	$11,470,000

Table 4. Non-Clinical Studies Cost

	Cost	Cost/3 yrs
Labor	$630,000	$1,890,000
Equipment (syringes, pipets)	$5,000	$15,000
Small Animals	$5,000	$15,000
Large Animals	$8,000	$24,000
Utilities (Refrigeration)	$12,000	$36,000
Misc. Operating Costs	$5,000	$15,000
	Total	$1,995,000

Table 5. Clinical Studies Cost

	Cost	Cost/5 yrs
1st Stage		
Labor	$630,000	$3,150,000
750 Patients	$50,000	$37,500,000
Hospital Fees ($10,000 per patient)	$10,000	$7,500,000
Utilities (Refrigeration)	$12,000	$60,000
Misc. Operating Costs	$5,000	$25,000
2nd Stage		
Labor	$950,000	$4,750,000
750 Patients	$50,000	$37,500,000
Hospital Fees ($10,000 per patient)	$10,000	$7,500,000
Utilities (Refrigeration)	$12,000	$60,000
Marketing	$10,000	$50,000
Misc. Operating Costs	$5,000	$25,000
	Total	$98,120,000

The FDA approval process will constitute a major bulk of the costs in developing N.K.O.B.®. However, these costs do not include all the fees for failure in any module submission. A more complete analysis of all possible paths and failures are included in the FDA Risk Analysis section. The average costs for the FDA application and approval is approximately $8 million. The cost for clinical studies, which include the surgeon fees, material production, and cell culturing, is estimated to $100 million. This is an approximation of costs that are summarized in Table 5. By introducing more or less experiments, the clinical costs will be affected and this number is only assumed to be an average depending on N.K.O.B.®'s expectations of the studies.

5.2 Production Costs

Raw Material

The raw material cost was estimated using an average implant size of 2 cm^2 x 7 cm. This implant size is an estimate based on defect sizes, and can be changed with

amount of material injected into the defect size. This size will be used to find an average cost for raw materials needed. The following table lists the costs of individual materials and the total cost for the implant.

Table 6. Raw Materials Cost for Cell harvesting and Culture for Chondrocytes and MSC's

Material	Amount		Unit price		Cost per Implant
Chondrocytes					
DMEM	150	mL	$22.00	per 500mL	$6.60
Collagenase	8	Mg	$76.00	per 500mg	$2.82
DNase	10	Mg	$132.00	per 25mg	$52.80
Ascorbic Acid	7500	µg	$29.10	per 10g	$0.02
Gentamicin	7500	µg	$41.00	per 10mg	$30.75
Amphotericin	375	µg	$24.60	per 50mg	$0.18
FBS	30	mL	$74.60	per 100mL	$22.38
PBS	5	mL	$32.40	per 10L	$0.02
Trypsin	1	mL	$22.70	per 500mL	$0.05
Mesenchymal Stem Cells					
α-MEM	150	mL	$26.90	per 500mL	$8.07
gentamicin	7500	µg	$41.00	per 10mg	$30.75
Ascorbic Acid	7500	µg	$29.10	per 10g	$0.02
bFGF	0.15	µg	$118.95	per 10µg	$1.78
FBS	15	mL	$74.60	per 100mL	$11.19
PBS	5	mL	$32.40	per 10L	$0.02
Trypsin	1	mL	$22.70	per 500mL	$0.05
				Total	**$167.49**

Table 7. Raw Materials Cost for Polymer Production and Crosslinking

Material	Amount		Unit Price		Cost per Implant
DEF	0.401111	g	$81.20	per 1000g	$0.0727
PG	0.531813	g	$23.20	per 1000mL	$0.0119
ZnCl2	0.003175	g	$52.70	per 10g	$0.0167
Hq	0.000513	g	$22.30	per 500g	$0.0000
Methylene chloride	0.28	mL	$22.12	per 100mL	$0.0619
N-VP	0.0364	g	$25.10	per 500mL	$0.0017
BP	0.000546	g	$54.70	per 500g	$0.0001
DMT	0.91	µL	$73.20	per 500g	$0.0001
NaCl	0.1	g	$42.98	per 1000g	$0.0043
Ethyl Ether	10	mL	$65.62	per 1000mL	$0.6562
β-TCP	0.12012	g	$305.00	per 10g	$3.6637
PEG	0.33488	g	$22.08	per 250g	$0.0296
Chloroform	10	mL	$31.10	per 500mL	$0.6220
P. Ether	10	mL	$43.30	per 2000mL	$0.2165
				Total	**$5.3575**

Table 8. Raw Material Cost for Gelatin Microparticles and Growth Factor Microspheres

Material	Amount		Unit Price		Cost per Implant
Gelatin Microparticles					
Gelatin	10	g	$16.60	per 100g	$1.66
DMEM	60	mL	$22.00	per 500mL	$2.64
Mineral Oil	400	mL	$35.85	per 1000mL	$14.34
DSP	0.080884	g	$136.00	per 1g	$11.00
PBS	400	mL	$32.40	per 10L	$1.30
Growth Factor Microspheres					
PLGA	0.02296	g	$589.00	per 50g	$0.27
PEG	0.00023	g	$22.08	per 250g	$0.00002
Methylene Chloride	2	mL	$22.12	per 100mL	$0.44
BSA	0.004592	g	$226.00	per 100g	$0.01
G.F.	2.3E-06	g	$89.00	per 1 µg	$204.34
PVA	117	g	$89.00	per 1000g	$10.41
IsoP alcohol	100	mL	$21.20	per 500mL	$4.24
				Total	**$ 250.66**

The total cost for an average implant is estimated to be $423.52. The actual selling price for the implant will obviously be a lot higher. The selling price will include influences from demand and competitor's prices. This cost is analyzed with a pricing model later.

Labor

The labor varies with each stage of the FDA process and also into the production of N.K.O.B.®. As the demand for treatment increases, the labor will also need to be increased accordingly. A complete discussion of salaries and number of workers needed for each stage of the FDA process was included previously in FDA Process of the Cost Analysis Section. Depending on the success of the company, appropriate expansions will be made to the production and therefore, the labor required.

Utilities

For the operating costs of the process, several utilities must be estimated such as electricity, refrigeration, and water. The cost of electricity is found to be $0.045/kWh in *Plant Design and Economics for Chemical Engineers*[22]. The cost of refrigeration is also found to be $60 per GJ, and the cost of water is $0.53 per 1000 kg. By estimating the required utilities for a single building covering 10,000 square feet, the total utility cost was calculated to be $12,803 per year.

By taking into consideration the raw materials, labor, and utilities the total production can be estimated. The labor costs will increase over time, but for approximation, clinical studies will be used as a basis for the costs. The utilities will also increase with production, but can be assumed to be a constant for estimation. The raw materials will be relatively constant, but the production rate will also increase as the demand for the treatment increases. Therefore, the total production costs will only be applicable for the first few years. By using an estimate of 3,000

implants per year for the first few years, the cost for raw materials will be $1.3 million. This corresponds to a total operating cost of $5.7 million, which includes a small amount dedicated to miscellaneous fees and costs. Table 9 lists the cash flow as a function of production costs and revenue over time. Figure 26 also represents the cash flow graphically versus time. More work will be done to determine a better approximation of costs, which will be dependent on both the path taken and the risk involved with each step.

Table 9. Cash flow versus time as a function of revenue and production cost

Year	Raw Material Cost (2% inflation rate)	# of implants	Revenue	Total Production Cost	Cash Flow
1	$423.50	3000	$33,000,000	$2,025,500.00	$30,974,500.00
2	$431.97	3450	$38,709,000	$2,137,296.50	$36,571,703.50
3	$440.61	3968	$44,515,350	$2,395,117.79	$42,120,232.21
4	$449.42	4563	$51,192,653	$2,697,542.17	$48,495,110.33
5	$458.41	5247	$58,871,550	$3,052,285.97	$55,819,264.41
6	$467.58	6034	$67,702,283	$3,468,400.44	$64,233,882.49
7	$476.93	6939	$77,857,625	$3,956,502.72	$73,901,122.65
8	$486.47	7980	$89,536,269	$4,529,046.69	$85,007,222.49
9	$496.20	9177	$102,966,710	$5,200,640.77	$97,766,068.79
10	$506.12	10554	$118,411,716	$5,988,420.62	$112,423,295.37

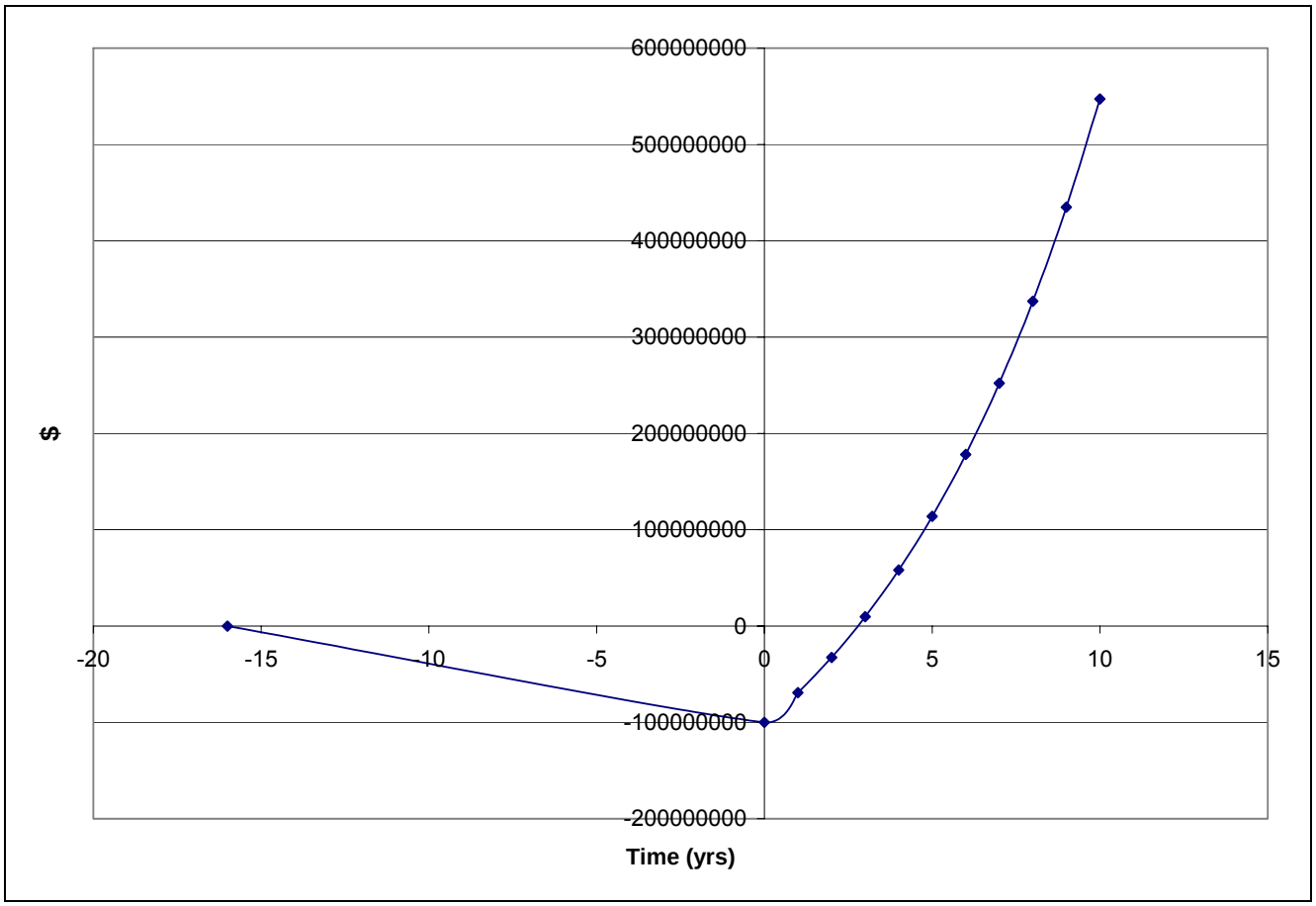

Figure 26. Cumulative cash position versus time

6. Business Plan

6.1 Mission Statement

The goal of N.K.O.B.® is to treat cartilage defects through the autologous culturing of chondrocytes. The treatment includes the culturing and implantation of the chondrocytes with the intent of regenerating the cartilage in place of the deficiency. By providing an extremely noninvasive surgery, this new approach will reduce time spent in the surgery and therefore decrease the cost of the surgery. Another main goal of N.K.O.B.® is to allow patients with a variety of cartilage

injuries, including osteoarthritis, to afford these treatments with the aid of insurance companies.

6.2 Marketing Plan

The main business plan includes charging a fee for the culturing of the cells and the production of the implantation materials. Prior to surgery, the patient will undergo a minor biopsy of cartilage from a low load-bearing zone of the knee. These cells will be cultured in a N.K.O.B.® facility, where the cells will be harvested to an appropriate amount. The cells will then be packaged and shipped along with the materials needed for the polymer and microcapsules. Surgeons will be properly trained to handle the cells and prepare the polymer and microcapsules for injection into the patient.

The actual cost charges will entail the culturing of the cells and the production of the implantation materials. The cells obtained from the patient will be cultured in N.K.O.B.® facilities and shipped in the appropriate manner to the hospital for implantation. The packaging and shipping will involve shipping the cultured cells in incubators in order to maintain cell survival. The raw materials will be used to produce the polymer used for injection, which could be made in bulk. The microspheres containing the growth factors could also be made in bulk and frozen until required for implantation. The microcapsules containing the cells can only be made prior to surgery, but the gelatin would still be sold and shipped along with the other necessary materials. The training of surgeons would involve the production of the gelatin microcapsules containing the cultured cells and the implantation of both the microcapsules and the growth factor microspheres into the injectable polymer.

Education of the chemistry and polymerization is also necessary in order for the surgeon to appropriately supervise the efficient polymerization and temperature rise involved with the implantation. In case of any adverse side effects involved with the polymerization or microcapsules, the surgeon should also be trained on the appropriate measures to take to prevent harm or injury to the patient.

The cost of the selling price will be determined based on competitor's prices and demand for the new treatment. The major obstacle for the market is training the surgeons or hospital assistants to handle and prepare the injections. In order to market and promote this new treatment, several conferences will be held prior to FDA approval in order to inform the surgeons and insurance companies of this treatment. It is the goal of N.K.O.B.® to begin training of surgeons before FDA approval, and within the first two years to have successfully trained 2,000 surgeons. This number is based on the number of surgeons trained in Carticel® treatments in their first two years.

6.3 Market and Demand

In 2000, approximately 17 million people reported having knee problems, not including osteoarthritis[23]. The demand for total knee replacements has risen to 250,000 a year, which alone costs insurance companies $41 billion annually[24]. Osteoarthritis, resulting from the degradation of the cartilage, is the leading chronic condition reported by the elderly, and current methods are unable to fully repair this deficiency[23]. The demand is evident, and the market for new treatments has been

extremely limited to invasive surgeries which only provide temporary treatment or alleviation of pain.

Although there are several current forms of treatment available, few offer the ability to completely regenerate the cartilage, while none can offer this in addition to the benefit of being completely noninvasive. The main competitor in autologous cultured chondrocytes is Genzyme Tissue Repair which manufactures the cell therapy Carticel®. This treatment includes chondrocyte implantation into the knee; however, this involves an invasive surgery and has not been proven to treat osteoarthritis. Carticel® was the first autologous cell treatment to receive FDA approval and has helped set the standards for further developments in this area of treatment[23]. Therefore, the market for N.K.O.B.® will be based on the similar market as Carticel®.

Many of the largest insurance companies in the U.S. have added Carticel® treatment to their policies, and many patients have opted for this treatment as an alternative to other knee surgeries. After N.K.O.B.® has been appropriately marketed and the demand for this new treatment has become evident, it is a goal for insurance companies to also carry this treatment. This will allow more patients to receive the treatment despite costs of surgery and fees.

6.4 Strengths and Weaknesses

The obvious strength in N.K.O.B.® treatment involves the reduced cost of surgery and hospital fees as a result of the noninvasiveness of the surgery. Hospital stays will be decreased as a result of the increased recovery time of this noninvasiveness. A successful regeneration of cartilage in the defect site will allow

for fewer revisits to a physician, and the new cartilage will be expected to last longer than a total knee replacement or other arthroscopic treatments. Also this implant will allow for the treatment of osteoarthritis, which until now has no permanent treatments, only temporary alleviations of pain. For more severe damage to the cartilage, there is often damage to the underlying bone. N.K.O.B.® treatments will be the only treatment that will allow regeneration of both cartilage and bone in a single defect site without multiple surgeries or major increases in cost.

There is a high probability that insurance companies will adopt this treatment on policies, based on their adoption of previous cell therapeutics. With insurance companies covering the treatment, the availability to a broad range of patients will increase. More patients will have the option to receive the latest treatments at the cost of their insurance companies. In addition, insurance companies will be able to afford the new treatment, as N.K.O.B.® fees will be highly competitive and possibly much less than existing treatments, depending on the severity of the defect.

The weaknesses that are involved with this treatment involve common drawbacks that are usually associated with new treatments. Although the clinical studies will have proven a degree of success and decreased risk of injury or harm, initially it is expected that the treatment be accepted with a large degree of speculation. This speculation is common for new technologies, and is described as an inferiority function that will be described in more detail in the pricing model. Another aspect of the inferiority involves the initial lack of consumer base and advertising. In order to reduce some of this inferiority, the N.K.O.B.® treatment will

be promoted and marketed well before FDA approval in order to increase acceptance and awareness among medical associations and interested patients.

6.5 Costs for Treatment

Current treatments for total knee replacement is approximately $25,000[24], while costs for other arthroscopic treatments can range from $5,000 to $10,000. Carticel® treatment averages about $26,000, which includes the cost for surgery fees, hospital fees, and Genzyme Tissue Repair fee of $10,360[24]. Although this cost is as expensive as a total knee replacement, this new treatment has proven to be extremely profitable. Carticel® treatments brought in $29 million in sales their first year after FDA approval, and more than 136 million people have insurance policies that now include this treatment in their plan[24].

Since N.K.O.B.® will be a less invasive surgery; this will decrease the cost of surgery, which will allow the cost of the treatment to remain extremely competitive. The cost of the fees will be determined by the demand and prices of Carticel® in order to achieve a reasonable but highly profitable cost.

6.6 Pricing Model

The cost of N.K.O.B.® treatment will be determined from a pricing model which will take into consideration the current demand for treatment and the prices of competitors (Carticel®). The following equation describes the relationship between price and demand in a competing market:

$$p_1 d_1 = p_2 d_2 \qquad \text{Eq. 6.1}$$

In this equation, p_1 is the selling price of N.K.O.B.®, p_2 is the selling price of Carticel®, and d_1 is the production rate based on demand for N.K.O.B.®, where d_2 is D-d_1. The total demand, D, is the total demand for the treatment and is assumed not to be a function of time, and rather a constant.

This equation assumes that the production rate is inversely proportional to the selling price for each company. It also assumes that at an equal demand, the selling price should lead to equal market share. This assumption will be corrected through an in depth pricing model that takes into consideration the difference in equal demand. Since Carticel® has been on the market for a longer time, they have an advantage initially, as N.K.O.B.® has yet to achieve a stable consumer base. The inferiority to existing competitors will be represented by α(t), which is a function of inferiority over time.

On the other hand, since this new treatment offers more benefits and technological advances then current competitors, a superiority function must also be introduced, β(t). The following equation represents the price model with the superiority and inferiority functions.

$$\beta(t) \cdot p_1 d_1 = p_2 (D - d_1) \cdot \alpha(t) \qquad \text{Eq. 6.2}$$

The following graph represents the plots of α versus β as a function of time. The plots of both functions are approximations based on information of market and demand for similar companies.

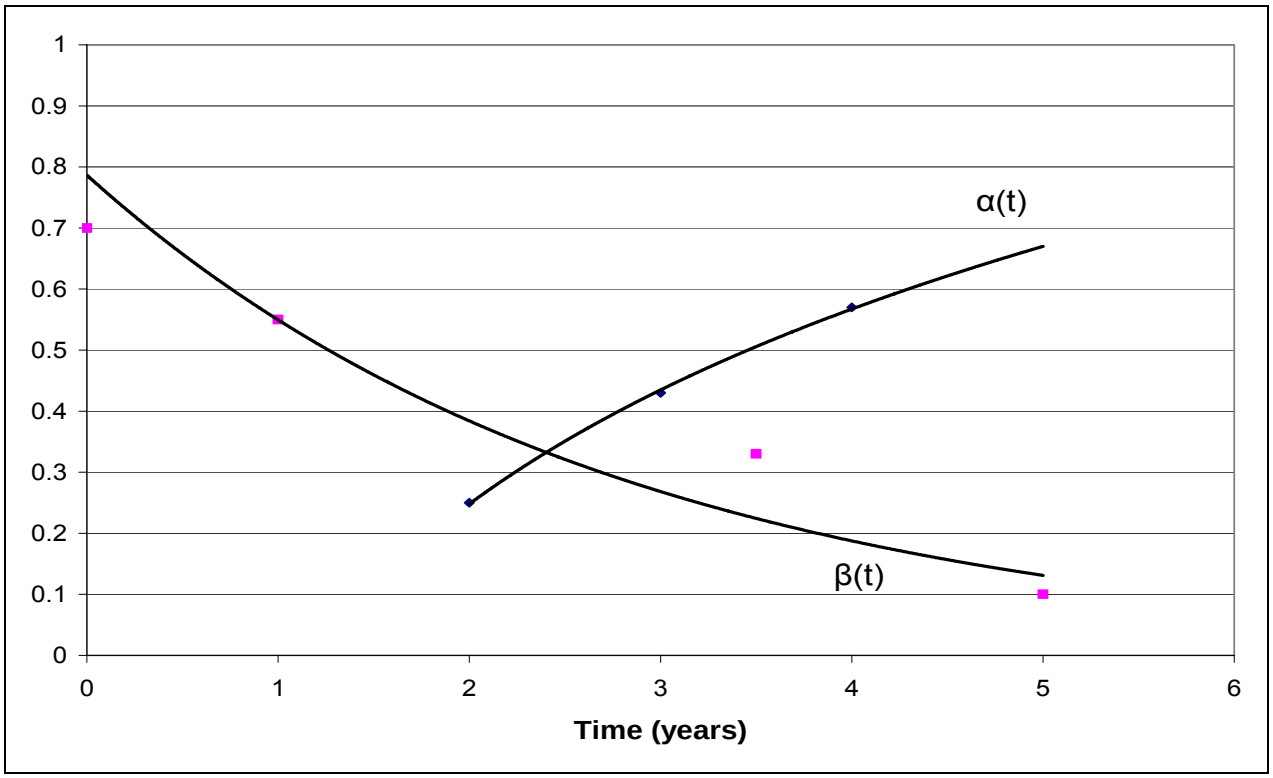

Figure 27. Inferiority and Superiority Functions

The inferiority function α was approximated using Carticel® data on number of patients treated and number of surgeons trained in the first two years of their production. The following charts were used when estimating the function α(t).

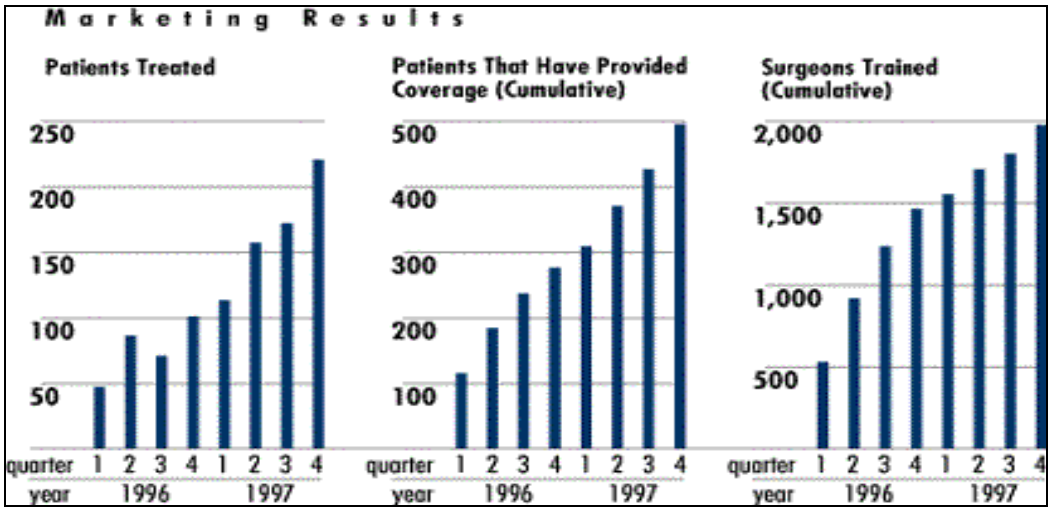

Figure 28. Market Analysis of Carticel®[24]

The superiority function β was approximated from existing information on the development of new technologies. It is assumed that initially, N.K.O.B.® treatments will offer a high level of superiority (β=0.8) and will decrease over time. In tissue engineering, it is estimated that current treatments will be replaced by superior treatments over a short period; therefore it is assumed that β will decrease and plateau after 4 years, after which new advanced treatments will be available.

Using the following equation, the selling price for N.K.O.B.® based on the demand and competitor's price can be determined.

$$\sum_{i=1}^{3}\left[p_1 d_i - PC\right] = FCI + FDA \qquad \text{Eq. 6.3}$$

In this equation, *PC* is the production cost of the implant, *FCI* is the fixed capital investment, and *FDA* is the cost for the FDA approval process, determined from the risk simulations. The investment is desired to be recovered in the first three years. Solving for d_1, the following equation can be derived and solved for p_1.

$$\sum_{i=1}^{3}\left[\frac{p_1 p_2 D\alpha_i}{\beta_i p_1 + \alpha_i p_2} - \frac{pc \cdot p_2 D\alpha_i}{\beta_i p_1 + \alpha_i p_2}\right] = FCI + FDA \qquad \text{Eq. 6.4}$$

For Carticel®, over 2,000 patients were treated the first few years, with 136 million people having insurance policies that covered Carticel® treatment in 1998. Using this information, and the existing number of knee surgeries and osteoarthritis cases in the U.S., it is assumed that the demand will be for 25,000 implants. The following numbers were used in the calculation:

p_2=$10,360/implant

D=25,000 implants

pc=$423.50/implant

FCI = $3 million

FDA+clinical trials = $100 million

Solving for the p_1, the selling price of the N.K.O.B.® implant is calculated to be $11,000. By charging $11,000, which is higher than the competitor, we are obviously making more of a profit. However, in order to remain competitive in the market, the entire cost of the surgery must be reasonable. Since the surgery is less invasive, this will allow for the total cost of the surgery to be much less than the Carticel® treatment. By maintaining an average cost of $21,000 per surgery, this allows approximately $10,000 for surgery and hospital fees, which is comparable to a normal arthroscopic surgery cost of $5,000. The cash flow over the first ten years is discussed and presented in the Cost Analysis Section of the report.

7. Future Work

In order to maintain a level of superiority on the market, research will be continued in order to advance in the tissue engineering aspect to possibly eliminate hospital times or culturing times. The growth of the company will involve expansion into new locations and larger numbers of employees. For future analysis of this project, an exact location will be determined using a simulation that will take into consideration living costs and medical demands of the area. Also the labor increases can be estimated over time as a result of the growth and expansion.

Furthermore, it may also be necessary to develop a model explaining the *in vivo* degradation of poly(propylene fumarate) and consequential regeneration of cartilage tissue in the defect site. This would determine the proper formulation of the polymer, i.e. molecular weight desirable, and also justify the plausibility of the project as a reparative approach to cartilage tissue regeneration.

8. References

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Supporting Documents

These Documents can be found on the M: Drive.

1. Costs Analysis.xls
2. FDA Risk Analysis.xls
3. Pricing Model.xls
4. Percent Cell Survival With Temperature Heating.xls
5. Stage One Variables.vsd
6. PMA Application.vsd
7. Module1.vsd
8. Module2.vsd
9. Module3.vsd