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PRINCIPAL INVESTIGATOR: Dr. Richard L. Dunn

CONTRACTING ORGANIZATION: Atrix Laboratories, Inc.
2579 Midpoint Drive
Fort Collins, CO 80525

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Richard L. Norton, Ph.D., Stephen M.G. Knight, William E. Huffer, Ph.D., and Richard L. Dunn, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Atrix Laboratories, Inc.
2579 Midpoint Drive
Fort Collins, CO 80525

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After the revision of the statement of work, the objective of this contract was the development of a biodegradable bone wax. It would be used as a hemostatic agent for bone surfaces. Degradation of this material would lessen the interference with bone repair and other complications seen with Ethicon® Bone Wax which is considered minimally resorbable. Low molecular weight poly(caprolactone) (PCL) was combined with a range of biocompatible additives to prepare formulations with handling similar to Ethicon® Bone Wax. Several were found to be effective hemostatic agents in animal trials. Manufacturing and quality control procedures were developed for three formulations: 79% PCL with 21% poly(ethylene glycol), 85% PCL with 15% calcium carbonate, and 77% PCL with 23% poly(vinyl alcohol). These were compared to Ethicon® Bone Wax and untreated controls in a 12 weeks study. The hemostatic performance of all test formulations were comparable to that of Ethicon® Bone Wax. All test formulations were more biocompatible than Ethicon® Bone Wax. None of the formulations were completely degraded at 12 weeks. The formulation containing poly(ethylene glycol) was associated with the mildest foreign body response plus the most advanced defect healing and degradation.

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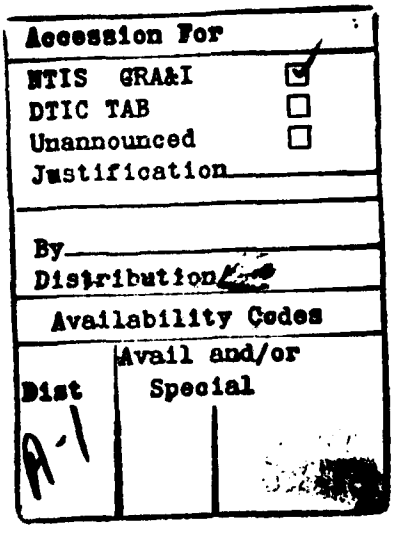

Richard L. Dunn 3/30/94
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1. INTRODUCTION

The initial objective of this contract was the development of a moldable, biodegradable bone repair material that would release osteoinductive proteins. During the course of this work, it was determined that the Army has a more urgent need for a biodegradable bone wax. A revision of the Statement of Work (SOW) was approved in January 1992, in which the objective of the last two years of the contract was changed to the development of a biodegradable bone wax. The goals of the revised statement of work are as follows:

Year 1 A moldable, biodegradable bone repair material will be developed and prototypes tested in animals for efficacy.

Year 2 A biodegradable bone wax will be developed and prototypes tested in animals for efficacy.

Year 3 The optimum biodegradable bone wax will be prepared under GMP, and sent to USAIDR for large scale safety and efficacy evaluations. Manufacturing and quality control procedures will then be established and a 510(k) submission prepared for the FDA.

Work relating to the initial objective is fully documented in the midterm report. This report details the bone wax development work done since the revision of the SOW.

Ethicon® Bone Wax consists of purified beeswax and isopropyl palmitate, a softening agent.[1] It is used as a hemostatic agent for bone that has been cut as a result of an injury or as part of a surgical procedure. These procedures include neurosurgery, oral surgery, thoracic surgery, and podiatric surgery.[2] Bone Wax achieves hemostasis by mechanical tamponade action.[1,2,3] It is firmly pressed into the bleeding bone surface, where it physically blocks blood flow and allows clotting to occur.

Bone Wax has several drawbacks that are outlined in the product insert.[1] These include statements such as: "Bone Wax is a minimally resorbable material.", "Bone Wax should not be used where rapid osseous regeneration and fusion are desired.", "Bone Wax may inhibit osteogenesis and may act as a physical barrier to the reparative process.", "Mild inflammatory reactions have been reported in tissues immediately adjacent to the site of implantation.", and

"Studies have suggested that Bone Wax as a foreign body may impair the ability of cancellous bone to clear bacteria." There are literature reports of Bone Wax's interference with bone healing[4,5] and clearance of bacteria.[6]

Efforts to produce materials that function as Bone Wax but with fewer drawbacks have focused on resorbable materials. These include compounds containing primarily fibrin, cellulose, collagen, and fatty acid salts.[3, 7-9] Use of resorbable polymer based formulations has also been investigated.[7, 10-12]

The objective of this project was to develop a biodegradable bone wax. Good biocompatibility as well as biodegradability would eliminate or diminish the above-mentioned drawbacks of Bone Wax.

The approach taken in this project was to blend resorbable polymers with resorbable or readily eliminated additives to produce formulations with handling characteristics similar to Bone Wax. A rabbit tibial defect model was then used to compare hemostatic efficacy and retention within the defect of these formulations to Bone Wax. Samples of the most promising formulations were sent to USAIDR for further testing in the same model. Preliminary storage stability tests were also conducted with these formulations.

Three formulations were chosen for further study. Pilot manufacturing and quality control procedures were developed and used to prepare samples for a more extensive animal trial. This trial used a modified version of the rabbit tibial defect model previously used, and was conducted under Good Laboratory Practices (GLP) guidelines. Bone Wax and an untreated group were tested as controls along with the three Atrix formulations. Hemostatic efficacy and retention in the defect were again evaluated. Animals were sacrificed at two, four, and twelve weeks to allow comparisons of the biocompatibility, degradation, and effect on bone regeneration of the treatment groups.

Three larger pilot manufacturing runs were conducted with one of the formulations. These batches were produced for an AAMI sterility validation and ongoing storage stability study (which will be continued at Atrix expense).

2. EXPERIMENTAL

A. Materials:

i. Biodegradable Polymers:

Poly(DL-lactide) (PLA) of molecular weight (MW) 2000 was purchased from Boehringer Ingelheim. Samples of this polymer were also autoclaved to produce lower molecular weight PLAs. Diols and triols of poly(caprolactone) (PCL) were purchased from Aldrich and Scientific Polymer Products. PCLs characterized by inherent viscosity and the poly(lactide-co-caprolactone)s (PLCs) were purchased from Birmingham Polymers, Inc.

ii. Chemicals:

Most poly(ethylene glycol)s (PEGs), calcium carbonate, and poly(vinyl alcohol) (PVA) were purchased from Aldrich. The PEG MW 3350, N.F. grade, was supplied by Dow Chemical. Airvol® 103, a poly(vinyl alcohol), was supplied by Air Products. U.S.P. grade calcium carbonate was supplied by Whittaker, Clark, and Daniels. Dextran and glycerol were purchased from Sigma. Glyceryl monopalmitate and calcium stearate were purchased from Pfaltz and Bauer. Ethyl lactate and Citroflex were supplied by Morflex. N-methyl-2-pyrrolidone (NMP) was purchased from ISP. Dyes were supplied by Warner Jenkinson.

B. Methods

i. Preparation of Formulations:

PLA-based formulations were prepared by weighing the polymer and solvent, or solvent/additive mixture, into glass vials and stirring. The formulations were placed in a 37°C environmental shaker overnight to ensure adequate mixing prior to evaluation.

PCL-based formulations were prepared by weighing the polymer and additive(s) into glass vials. The vials were then placed in a water bath at ~65°C to melt the polymer. The molten mixture was then stirred. The mixtures were placed in a 37°C environmental shaker overnight to ensure adequate mixture prior to evaluation. Selected formulations, including all batches used in animal testing, were irradiated with 20-25 or 30-35 kGy by either Cobe Labs (Denver, CO) or Isomedix Operations. (Morton Grove, IL)

ii. Preparation of Lower Molecular Weight PLAs:

The molecular weight of PLA was reduced by steam autoclaving. The polymer was weighed into a Teflon lined glass petri dish and autoclaved at 22 psi steam pressure for 20, 30, or 40 minutes. After cooling, the polymer was dissolved in approximately two parts dichloromethane, and the resulting solution was precipitated by pouring into methanol. The polymer was then air-dried.

iii. Determination of Formulation Swelling:

Three different swelling experiments were conducted. In the first, roughly spherical specimens of formulations were shaped by hand, and diameter measurements were taken. These samples were placed in phosphate buffered saline solution (PBS), pH 7.4, and kept at 37°C. To avoid possible fragmentation of the samples, further measurements were made with the specimen in its vial with the PBS removed. Percent changes in volume were calculated using the diameter measurements. In the second swelling experiment, 5 mm inner diameter glass tubes of measured length were filled with molten formulations by syringe. The tubes were then placed in PBS and kept at 37°C. At specific intervals, the tubes were removed, and the volume of formulation extruded from the tube ends was calculated. The design of this experiment was intended to provide better accuracy in measuring sample volumes, as well as to better simulate an in vivo use of the formulations. The third study measured volume change of disc-shaped samples of several formulations containing additives intended to increase fluid uptake of the formulation.

iv. In Vitro Degradation:

Two experiments were conducted to investigate in vitro degradation in the form of mass loss of several different formulations. The first experiment evaluated percent weight loss of samples over time in cell culture media (RPMI 1640) containing murine peritoneal macrophages (ATCC no. TIB 186) at 37°C. Control groups of samples in media were also run. A second study compared weight loss of samples of different formulations in PBS and in horse serum at 37°C.

A method to quantify molecular weight loss of PCL by gel permeation chromatography (GPC) was recently developed. This method, described later, was used in an experiment that looked at mechanisms of molecular weight degradation. This experiment measured mass and molecular weight loss of PCL, as well as the effects of certain additives on mass and molecular weight degradation.

v. In Vivo Testing:

In vivo testing of formulations' hemostatic potential was done using a rabbit tibial defect model. In this model, a dental burr was used to create 2-4 mm diameter full bone thickness defects in the medial proximal tibiae of 2-4 kg New Zealand White rabbits. Formulation was inserted in the defect, and its hemostatic potential was observed for a five minute period. This model was used for the initial in vivo study conducted by Atrix, ATS-52, and USAIDR's in vivo evaluation of Atrix formulations. In ATS-52, if a formulation was not effective as a hemostatic agent, it was removed and replaced with another test article.

A third in vivo trial, ATLS-64, was conducted by Atrix. The model used was the one utilized in the previous in vivo studies, with two exceptions: the defects were five millimeters in diameter, and the untreated control defects were left open until clot formation was evident.

vi. Histomorphometric Analysis

The following injection schedule was used for labeling bone growth in animals from ATLS-64: Seven and twelve days following surgery, all subjects received subcutaneous injections of 25 mg/kg of demeclocycline suspended in a sodium carbonate buffer. Animals euthanized 28 days postsurgery also received subcutaneous injections of 15 mg/kg of calcein suspended in a sodium carbonate buffer on days 21 and 26 postsurgery. Animals euthanized 84 days postsurgery received calcein injections on days 77 and 82 postsurgery.

Histomorphometric samples were taken at necropsy such that soft tissue surrounding the defect was not disturbed. Prior to analysis, this excess soft tissue was removed. Specimens were then x-rayed with a Faxitron Micro-X-Ray Processor to localize the defects and callus. Using the x-ray as a guide, serial 500 μm tibial cross sections encompassing the entire lesion were cut with a diamond wire saw. After taking another x-ray for histomorphometry, the central slab was infiltrated with glycol methacrylate monomer for 2-4 weeks, and imbedded in polymerized glycol methacrylate. Serial 3-5 μm sections were stained by von Kossa and sirius red methods to demonstrate mineralized bone and osteoid, tartrate-resistant acid phosphatase (TRAP) with azure B counter stain to demonstrate osteoclasts and osteoblasts, and an unstained section was used to demonstrate demeclocycline and calcein labeling by fluorescence microscopy.

Sections were measured by semi-automated video image analysis to measure parameters. The total bone in cross sections at the level of the center of the callus was measured in a video image of the x-ray of that section after appropriate contrast enhancement and thresholding. This area included the original cortical bone and all new bone forming around the defect, (callus bone) as well as reactive bone forming on endosteal and periosteal surfaces at a distance from the defect. The section x-rays were also used to measure the minimal distance across defects, or gap width.

The amount of labeled bone in the callus was measured in fluorescent images of unstained thin sections. Fluorescence of demeclocycline and calcein made it possible to precisely discriminate between new callus bone and preexisting bone on either side of the defect. After defining the callus area by fluorescence microscopy, total mineralized bone area and perimeter within the callus were measured in the same section viewed with transmitted visible light. The mineralized bone was black against an otherwise white background, and readily identified.

Osteoclasts and osteoblasts in the callus were counted as TRAP-positive cells and basophilic azure B-stained cells lined up on bone surfaces in sections adjacent to those used for measuring labeling area and mineralized bone respectively. Callus boundaries were identified by differences in cellular density in callus and adjacent noncallus bone.

In addition to the measurements above, the tissue area occupied by foreign body reaction to substances in the vicinity of the defect was measured by tracing von Kossa/hematoxylin and eosin-stained sections on monitor images.

vii. Preparation of 100g Batches of Formulations

Batches were prepared in a one pint double planetary mixer with a jacketed bowl (Charles Ross and Son Co.). Prior to mixing, the bowl was heated to 60°C using a circulating water bath connected to the bowl jacketing. Once the bowl temperature was 60°C, the PCL (and PEG if used) was placed in the bowl. Once the PCL was melted, the particulate additive (PVA or calcium carbonate) was added if used. The mixer was then run for at least an hour under vacuum. After this initial mixing period, the water jacket temperature was allowed to cool to 46°C while the mixer continued to run. After mixing for an hour at

46°C, the formulation was poured into a dispensing cartridge. The dispensing cartridge was kept at approximately 46°C by heating tape. A pneumatic dispensing device (EFD, Model 1500XL) was used to dispense 2-3 grams of formulation into polymer/foil laminate (Bell Fibre Foil-o-Rap) pouches. Pouches were immediately heat-sealed and placed in an oven set at 37°C. After 16-24 hours, the pouches were removed from the oven and stored at room temperature. Batches were irradiated with 30-35 kGy of gamma irradiation by Isomedix Operations (Morton Grove, IL).

viii. Preparation of Pilot Manufacturing Batches of Calcium Carbonate Formulation

The above procedure was used for the production of these batches with a few exceptions. The largest change was the cooling process used during mixing. Rather than allowing the bowl to cool passively from 60°C to 46°C, the bowl was actively cooled to 54°C using the refrigeration function of the circulating water bath. The refrigeration was turned off when the bowl temperature reached 54°C, and the water jacket temperature was allowed to passively cool to 46°C. This change was made to speed up the manufacturing process. These batches were manufactured in the controlled environment of Atrix's general manufacturing area, with the assistance of the manufacturing department.

ix. Gel Permeation Chromatography Procedure

GPC analyses of PCL and PCL-based formulations were conducted using a LDC/Milton Roy GPC system with a refractive index detector. The column used was a 300 x 7.8 mm Phenogel (Phenomenex) column with 5μm particle size and 500Å pore size. Samples were dissolved and run in chloroform at a flow rate of 2.0 mL/min.

x. Differential Scanning Calorimetry Procedure

DSC analyses were performed on a Perkin-Elmer DSC 7 with a TAC 7/DX thermal analysis controller, with data collection and analysis performed by computer using Perkin-Elmer software. Formulations were tested from -10°C to 70°C, at a scan rate of 10°C/minute. At least two scans were run for each sample, as the first scan differed from the second and subsequent runs of the same sample. The first scan of a sample was heavily influenced by the thermal history of the formulation. Second and subsequent scans differed

slightly from initial scans, and could be considered more accurate depictions of a formulation's thermal properties. All formulations tested exhibited two or three distinct peaks in their thermal scans. These peaks were analyzed for start, stop, onset, and peak temperatures, as well as areas. Peak areas were compared to a literature value for the heat capacity of 100% crystalline PCL to determine the percent crystallinity of formulations.[13] Differences in peak temperatures between first and subsequent scans were compared to observe changes in behavior.

xi. FTIR Procedure

Infrared spectral analysis of materials was performed on a Bio-Rad FTS-40 FTIR instrument. Samples were tested either by spreading on the ZnSe crystal of an ATR cell, or as cast films on NaCl windows. Cast films were usually prepared in chloroform, expect for PVA and formulations containing PVA, which were prepared in hexafluoroisopropanol.

xii. Calcium Content Determination

Weight percent calcium in samples of the calcium carbonate formulation was determined by Huffman Laboratories (Golden, CO) using inductively-coupled plasma spectroscopy following an acid digestion procedure.

3. RESULTS AND DISCUSSION

A. Formulation Development

i. PLA-Based Formulations

Bone wax is made from beeswax and a softening agent. We chose PLA as a biodegradable "replacement" for beeswax. Low molecular weight PLA, MW 2000, was used in an effort to limit the elasticity of the formulations when the solvents, intended to soften the polymer, were added. These solvents included N-methyl-2-pyrrolidone (NMP), ethyl heptanoate, butyl acetate, isopropyl myristate, butyryltri-n-hexyl citrate (Citroflex B-6), and ethyl lactate. Mixtures of solvents with glycerol or peanut oil were also investigated. Initial efforts yielded formulations varying in consistency from syrupy liquids to rubbery solids. Continued experimentation with these formulations resulted in

compounds with the consistency of model airplane glue. In an attempt to improve on these results, PLA 2000 was hydrolyzed to different extents, yielding lower molecular weight polymers. The rationale in doing this was to try to make the polymer more like beeswax. These hydrolyzed polymers were also mixed with many of the above solvents, resulting in formulations that in some cases were more viscous than those with PLA 2000, but were also extremely sticky.

ii. PCL MW 2000 and 1250 Based Formulations

After the lack of success with PLA-based formulations, our efforts turned toward using poly(caprolactone) (PCL) as the main component for a bone wax substitute due to its waxy consistency at low molecular weights. Initial formulations were made with a PCL diol of MW 2000, a hard, waxy solid, with various solvents as softening agents. It was difficult to dissolve the PCL in many solvents. Initial indications were that the solubility problems were related to difficulty in making a fine powder of the PCL, which is supplied as a fused mass. Different grinding methods were tested, with little improvement in the formation of solutions.

These problems led to the development of a new formulation method. Formulations were prepared by melting the ingredients together in a water bath, and then stirring the molten mixture. After stirring, the formulations were placed in a 37°C environmental shaker overnight before evaluation to ensure good mixture. Using this method, homogeneous mixtures were obtained with most of the solvents previously mentioned. After adjusting polymer-solvent ratios, several formulations with consistencies similar to Ethicon® Bone Wax were produced.

The same procedure was used to produce formulations using PCL of MW 1250 and nonsolvent additives. These nonsolvent additives included polymers that appeared to form blends with the PCL during formulation, nonmiscible polymers present as dispersed solids, and nonpolymeric additives present as dispersed solids. All additives chosen have some record of biocompatibility or use in pharmaceuticals. After experimenting with various amounts of these additives, many formulations were produced with handling properties similar to Ethicon® Bone Wax.

iii. Evaluation of Handling Characteristics

In order to rank formulations, a subjective rating system was developed based on the formulations' handling properties. The properties evaluated were:

- hardness
- stickiness to gloves
- cohesiveness
- smearability
- cohesion to a countertop after smearing
- effect of moisture on the above properties.

These criteria were chosen because of their importance to a surgeon using Bone Wax or a replacement product. The product must not stick to a gloved hand, nor be so slippery as to make handling difficult. It must be soft enough to be moldable by hand, and cohesive enough to avoid falling apart during molding and use. It must be of the proper consistency that it can be smeared across the surface of a cut bone and remain in place. The effect of moisture on these properties is important because of the presence of blood in the surgical field. A procedure was developed in which formulations were kneaded with a gloved hand to evaluate hardness, stickiness to gloves , and cohesiveness. A portion of the formulation was then smeared on a countertop to evaluate smearability and adhesion to a countertop. This procedure was then repeated after running water over the formulation and gloved hand. After conducting this procedure with samples of Bone Wax and PCL based formulations, formulations were assigned ratings from 1 to 7, using the following system:

1=Close to bone wax
2=Close after working by hand
3=Close, but with some decrease in handling properties during wet handling
4=Close, but with poor stability
5=Poor consistency
6=Not enough viscosity: describes consistencies from taffy to model airplane glue
7=Hard solid and/or no cohesion.

A rating of 4 says that the handling properties of the formulation were worse after being left at room temperature for several days.

Tables 1 and 2 list the components and ratings of several formulations out of over 160 that were rated using this system. Most of the additives were tried at various levels with only the best rated sample given in the tables.

TABLE 1: FORMULATIONS CONTAINING SOLVENTS AND/OR MISCIBLE ADDITIVES

POLYMER	SOLVENT AND/OR MISCIBLE ADDITIVE	RATING
79% PCL 1250	21% PEG 2000	1
89% PCL 1250	11% 25/75 PLC	1
86% PCL 2000	14% Ethyl Lactate	1
50% PCL 1250	50% PCL 2000	2
67% PCL 2000	33% PEG 1500	3
89% PCL 2000	10% NMP + 1% Glycerol	3
80% PCL 2000	10% Butyl Acetate + 10% Peanut Oil	4
82% PCL 1250	18% Isopropyl Myristate	5
85% PCL 1250	15% Sesame Oil	5
85% PLA 2000	7.5% NMP + 7.5% Glycerol	5
82% PLA 2000	18% NMP	6
84% PLA 2000	16% Ethyl Heptanoate	6
83% PLA 2000 (autoclaved 30 min)	17% PCL 1250	6
68% PCL 2000	19% NMP + 13% Glycerol	7
83% PLA 2000 (autoclaved 20 min)	17% PVA 14,000	7

TABLE 2: FORMULATIONS CONTAINING DISPERSED SOLIDS

POLYMER	ADDITIVE	RATING
77% PCL 1250	23% PVA 14,000	1
81% PCL 1250	19% Dextran 8000	2
82% PCL 1250	18% Calcium Stearate	2
74% PCL 1250	26% Cellulose	2
85% PCL 1250	15% Calcium Carbonate	2
85% PCL 1250	15% Carnauba Wax	3
80% PCL 1250	20% Carboxymethyl Cellulose	4
85% PCL 1250	15% Gum Tragacanth	4
83% PCL 1250	17% Gelatin	5
83% PCL 1250	17% Gum Xanthan	7
79% PCL 2000	21% Carnauba Wax	7
85% PCL 2000	15% Calcium Stearate	7
83% PLA 2000 (autoclaved 40 min)	17% PVA 14,000	7

Table 1 presents formulations of three types; formulations that incorporate solvents, formulations that incorporate other liquids, either alone or mixed with a solvent, and formulations that incorporate additives which melt at the same temperature as the PCL and are miscible in the molten state. Table 2 presents formulations in which the additives are present as dispersed solids. In general, PCL 1250 seemed very compatible with dispersed solid additives, whereas the PCL 2000 produced better formulations with solvents and/or miscible additives.

Many formulations tested showed little difference in handling properties between dry and wet latex gloves. The major weakness of any of the formulations was stability. This problem affected all of the PCL 2000 formulations that contained solvents.

Ethicon® Bone Wax is a sterile product. Gamma irradiation is the preferred method for sterilization of these biodegradable bone wax formulations for two reasons. Gamma irradiation sterilization does not require extreme heat that might melt and separate formulations, and it is relatively inexpensive. Thus, a group of promising formulations, as well as their component materials, were gamma irradiated at a dosage of 20-25 kGy. This dose should be sufficient to produce sterile product. NMP and ethyl lactate developed distinctive odors after irradiation. All formulations appeared to be unchanged or slightly better in consistency after irradiation.

These ratings were used in the selection of formulations for in vivo screening.

B. In Vivo Experimentation

i. Preliminary In Vivo Study: ATS-52

In preparation for ATS-52, new batches of the candidate formulations were prepared and irradiated with a dose of 29-32 kGy. Formulations tested were chosen to provide a good mixture of types of formulations. Obviously, formulations with good handling characteristics were selected. Also chosen were formulations with marginal properties, with the rationale that if one of these formulations was very effective as a hemostatic agent, the properties of the formulation could be optimized. Due to the design of the study, which specified a total of twelve test formulations, there were some formulations that were not tested. Criteria for success were:

- demonstrated hemostasis for 5 minutes
- workability prior to implantation
- implantability

The results for the candidate formulations can be summarized as follows:

SUCCESSES

76% PCL 1250 + 24% Glyceryl Monopalmitate
77% PCL 1250 + 23% PVA, MW 14,000
79% PCL 1250 + 21% PEG 2000
89% PCL 1250 + 11% 25/75 PLC
89% PCL 1250 + 11% PEG 1500

81% PCL 1250 + 19% Dextran 8800
83% PCL 1250 + 17% Baking Powder
82% PCL 1250 + 18% Calcium Stearate
85% PCL 1250 + 15% Calcium Carbonate
89% PCL 1250 + 11% 50/50 PLC

FAILURES

50% PCL 2000 + 50% PCL 1250
86% PCL 2000 + 14% Ethyl Lactate
80% PCL 1250 + 20% PEG 10000
89% PCL 2000 + 10% NMP + 1% Peanut Oil
80% PCL 1250 + 11% 25/75 PLC + 9% Calcium Stearate
80% PCL 2000 + 10% Ethyl Lactate + 10% Glycerol
89% PCL 2000 + 10% NMP + 1% Glycerol

NOT TESTED

82% PCL 1250 + 18% PEG 400 Monostearate
77% PCL 2000 + 23% PEG 1500
76% PCL 1250 + 24% Dextran 503,000

When a formulation failed to demonstrate hemostasis, it was removed, and another formulation was tested in the same animal and defect. Although some formulations, namely the 86% PCL 2000/14% ethyl lactate and the 50% PCL 1250/50% PCL 2000 formulations, were successful as hemostatic agents, they were eliminated from consideration due to poor handling characteristics. A synopsis of ATS-52 is given in Appendix 1.

ii. <u>USAIDR In Vivo Evaluation</u>

The promising results of ATS-52 prompted further in vivo study. Another two-week study using the rabbit tibial defect model was conducted by USAIDR. The ten treatment groups, with six treatment sites for each group, were:

76% PCL 1250 + 24% Glyceryl Monopalmitate
85% PCL 1250 + 15% Calcium Carbonate
89% PCL 1250 + 11% 25/75 PLC
89% PCL 1250 + 11% 50/50 PLC
77% PCL 1250 + 23% PVA, MW 14,000
79% PCL 1250 + 21% PEG 2000
89% PCL 1250 + 11% PEG 1500

81% PCL 1250 + 19% Dextran 8800
Ethicon® Bone Wax
Untreated

This trial included subjective ratings of each group's performance in several areas. These areas included handling properties, hemostatic ability, presence of hematoma at necropsy, evidence of test article extrusion from the defect site both during surgery and at necropsy, evidence of infection, general tissue reaction, and change in defect size between surgery and necropsy. The results of this trial showed that all of the Atrix formulations tested were generally equivalent to bone wax in overall performance for the parameters assessed. While these results did not indicate any exceptional formulations, they did allow other parameters to be considered in choosing which formulations to continue developing. It was decided to concentrate on three formulations. The 79% PCL 1250 + 21% PEG 2000 formulation was chosen for its excellent handling properties. The 85% PCL 1250 + 15% calcium carbonate formulation was chosen for its osteoinductive potential. This potential was observed in ATS-52, as animals treated with this formulation appeared to be further advanced in defect healing as compared to defects treated with other formulations. The 77% PCL 1250 + 23% PVA 14,000 formulation was chosen because it has the potential to be the most rapidly degraded formulation. When placed in an aqueous environment this formulation swells and then fragments. The resultant increase in surface area could lead to more rapid degradation of the implant and faster bone repair.

iii. ATLS-64

These three formulations were the subject of another rabbit tibial defect model study conducted by Atrix. The samples used in this study came from batches of 100g scale prepared using pilot manufacturing procedures and equipment. These formulations were made with some minor changes in components. These changes, described in a later section, were made to simplify regulatory issues. This study, ATLS-64, was conducted under Good Laboratory Practices guidelines in anticipation of using the results for the filing of a 510(k). The objectives of this study were to evaluate hemostatic potential and ability to be retained in the defect, as well as to observe biocompatibility and degradation behavior of these formulations over a twelve week period. Histomorphometric analysis was conducted to quantify new bone growth during the study. As previously described, three Atrix

formulations, Ethicon® Bone Wax, and an untreated group were included in the study. Three animals from each group were terminated at two, four, and twelve weeks postimplantation. One necropsy sample was evaluated by gross histopathology and two by histomorphometrics.

a. Hemostasis Results from ATLS-64

Observations were made one and five minutes after pressing the test article in place. If there was no bleeding from the defect, the test article received a hemostasis score of zero, if there was bleeding at the margins of the defect it received a score of one or two depending on the severity. If the formulation stayed in place it received a retention score of zero, if partially extruded it received a one, if fully extruded a two. Table 3 contains the average score for all the test articles plus the fraction of samples receiving a zero score.

The average hemostasis scores of all three test formulations are better or equal to the Ethicon® Bone Wax control. The retention of the formulations containing PVA and calcium carbonate was equal or better to the Ethicon® Bone Wax control. With the formulation containing PEG, the nonzero retention scores were all twos. These samples were rapidly extruded from the defect. These samples were pushed back into the defect by the surgeon prior to closure of the site.

TABLE 3: AVERAGE HEMOSTASIS AND RETENTION SCORES FOR ATLS-64

	PEG Formulation	PVA Formulation	CaCO3 Formulation	Ethicon® Bone Wax
Hemostasis 1 min.	0.8 3/9=0	0.3 6/9=0	0.4 5/9=0	0.7 3/9=0
Hemostasis 5 min.	0.9 3/9=0	0.4 5/9=0	0.4 5/9=0	0.9 2/9=0
Retention 1 min.	0.7 6/9=0	0.1 8/9=0	0 9/9=0	0.2 7/9=0
Retention 5 min.	0.9 5/9=0	0 9/9=0	0 9/9=0	0.2 7/9=0

b. Histological Results from ATLS-64 for the PCL/PEG Formulation

There was no day 14 specimen for gross histopathology for this formulation, as the test animal designated for this purpose suffered respiratory arrest during surgery. This did not appear to be test article related. In the histomorphometric analysis of day 14 samples, the foreign body response area was not much larger than seen in the untreated controls at this timepoint. The response was characterized by small granulomas containing basophilic material, possibly formulation, in various stages of fragmentation. Also seen at day 14 were large areas, lined by giant cells and fibrotic tissue, containing large amounts of cell remnants as well as viable capillaries, fibroblasts, histiocytes, and foreign body cells.

At day 28, gross histopathology revealed test article in the tissue surrounding the defect area. When this tissue was removed to reveal the defect, test article was evident in the defect itself. Cutting the defect by cross-section also revealed test article in the medullary cavity. In all cases, the test article exhibited minimal encapsulation by fibrous tissue. The cross-section also revealed that test article in the defect was surrounded by hard tissue in an open cup-like formation. Histomorphometric analysis of day 28 samples showed significantly better defect healing than the other treatment groups, and there was no evidence of the formulation in the defect. This defect healing was almost as complete as the untreated controls at day 28. This formulation continued to exhibit a very mild foreign body response, in terms of both area and characterization. The foreign body response at this time consisted solely of the large spaces containing viable capillaries, fibroblasts, histiocytes, and foreign body cells as seen in the day 14 specimens.

The day 84 gross histopathology specimen showed about 80% closure of the defect. While no test article was evident in the defect or medullary cavity, a portion of test article was found in the tissue near the defect site. This test article exhibited minimal encapsulation. The histomorphometrics results were the same as in the 28 day samples. No formulation was detected in the defect and the gap width was the about the same as at 28 days.

c. Histological Results from ATLS-64 for the PCL/PVA Formulation

The gross histopathological evaluation of the 14 day specimen revealed test article present in the defect. This test article was minimally encapsulated by fibrous tissue. Histomorphometric analysis of day 14 specimens showed a very mild foreign body response in terms of area. This response was characterized as large and small portions of homogeneous basophilic material surrounded by histiocytes and foreign body giant cells in relatively dense fibrotic tissue.

Gross histopathology of the 28 day specimen revealed test article both in the defect and in the tissue surrounding the defect site. In both instances, the test article was minimally encapsulated. Histomorphometric analysis of day 28 specimens indicated that the foreign body response seen in day 14 specimens was persistent. In addition to the type of response seen previously, larger cystic spaces were also present. Unlike the PCL/PEG formulation, no vascularization or fibroblastic invasion was evident.

Gross histopathology of the day 84 specimen revealed the most significant visual evidence of foreign body response seen for any Atrix test article. A mass of dense fibrotic tissue was found near the defect site. When probed, this mass was found to contain crystals of PVA. The tissue immediately over the defect site was adhered to the defect. When this adhesion was cut, more crystals of PVA were evident in the defect. Also evident were small flakes of PCL. Relatively unfragmented test article was evident in the medullary cavity. While this test article was significantly more encapsulated than seen with the other Atrix formulations, the encapsulation was still rather mild. Histomorphometric analysis of day 84 specimens showed significant foreign body response consisting of large cysts.

d. Histological Results from ATLS-64 for the PCL/Calcium Carbonate Formulation

Gross histopathological evaluation of the day 14 specimen revealed the presence of test article in the defect and medullary cavity. The test article was minimally encapsulated by fibrotic tissue. Histomorphometric analysis showed this formulation to induce the greatest area of foreign body response. This response

was characterized by a moderately dense fibrous stroma containing variously sized spaces of giant cells and histiocytes. These spaces contained granular calcified material, which was also evident in the medullary space.

Gross histopathology at day 28 revealed minimally encapsulated test article present in the medullary cavity. Histomorphometric analysis produced some interesting results. While the bone area to callus area ratio was much higher for this formulation than any other group at this timepoint, the defect healing was not as advanced as seen with the other Atrix formulations. Osteoclast and osteoblast counts were also higher tnan the other groups at this timepoint. The foreign body response was characterized by large cysts containing calcified material and medium sized cysts containing cellular remnants.

Gross histopathology at day 84 showed about 60% defect closure. Test article was evident in the defect and medullary cavity, and was minimally encapsulated. The portion of test article in the medullary cavity appeared to have promoted bone ingrowth, as an outcropping of hard tissue extending from the medullary cavity wall into an adjacent portion of test article was seen. Histomorphometric analysis showed about 50% defect closure and continued foreign body response of the type and area seen in day 28 specimens.

e. Histological Results from ATLS-64 for Ethicon® Bone Wax

Gross histopathological evaluation at day 14 revealed the presence of test article in the defect. The test article exhibited moderate fibrotic tissue encapsulation. Histomorphometric analysis showed a much larger foreign body response area than seen for the PCL/PEG and PCL/PVA formulations at this timepoint. This response consisted of cysts containing unstained material surrounded by histiocytes, giant cells, neutrophils, and dense fibrous tissue. These cysts were found in the defect area, adjacent periosteal tissue, and the medullary space.

Gross histopathology of the day 28 specimen found the defect area covered by hard tissue. When the specimen was cross-sectioned, moderately encapsulated test article was found in the medullary cavity. Also, the hard tissue covering the defect was not

fluorescent under UV light, suggesting that this tissue was not new bone growth. This tissue also surrounded a band of dark, soft material. Histomorphometric analysis showed only about 20% defect healing, which supports the hypothesis that the hard tissue seen in the gross histopathology specimen was not new bone growth. Foreign body response was essentially the same as seen in day 14 specimens.

The gross histopathology specimen for day 84 showed almost no defect closure, as the defect was filled with a large portion of test article that extended into the medullary cavity. This test article was completely compartmentalized from the marrow by dense fibrous tissue. While histomorphometric analysis did not show a large area of foreign body response, the response was clearly more severe than seen for any of the Atrix formulations tested. This response consisted of cysts containing only unstained material and no cellular remnants. In one specimen, prominent lymphoid nodules with germinal centers were present in the wall of a large cyst. Both specimens exhibited prominent polymorphonuclear cells surrounding the foreign material contained in the cysts.

f. Histological Results from ATLS-64 for the Untreated Control

Gross histopathology evaluations showed the defect to be completely healed by day 28, with no apparent change between days 28 and 84. Histomorphometric analysis correlated with these visual observations. Defects were completely closed by day 28. While a very minimal foreign body response was seen at day 14, most likely due to bone fragments from the surgery, there was no measured foreign body response area at days 28 and 84. Osteoblast and osteoclast counts were comparable to the treatment groups at each timepoint, which shows that none of the treatments inhibit the cellular processes of bone regeneration.

g. Summation of ATLS-64 Results

This animal trial has shown all three formulations to be at least comparable to Ethicon® Bone Wax in hemostasis in this model. All three formulations were more biocompatible than Ethicon® Bone Wax. The formulation containing PEG appeared to be the most biocompatible and interfered the least with defect healing.

The PEG formulation also seemed to be more rapidly degraded in the defect than the other formulations.

The poor retention in the defect of five of the nine PEG formulation samples and the presence of a large piece of formulation outside the defect at 84 days makes it unclear as to whether the superior histology results with this formulation are due to the formulation not being retained in the defect rather than being degraded in place. The samples used for the 28 day histomorphometrics analysis were well retained after five minutes. Retention in the defect after this length of time may not be important to hemostasis, and may actually slow the healing process. The untreated controls showed the best bone repair and least foreign body response.

The other complication when assessing the performance of the PEG containing formulation is the poor handling of this batch of formulation that is probably responsible for the poor retention of some samples. The lack of cohesion and nonuniform hardness of these formulations is most likely due to lack of sufficient control of temperature during the filling process. This will be discussed in the section "Packaging of Formulations." If this formulation was made again with proper control of temperature, a better handling material with better retention in the defect would probably result.

Undegraded material was found in the 84 day samples with all formulations. The identity of the material as formulation was confirmed by FTIR analysis. Because of the low molecular weight of the PCL used, which is known to be a degradable polymer, it was expected that degradation would have been complete in this time frame. The major mechanism of degradation of PCL, and related polyesters, is hydrolysis of the ester bonds. The rate of hydrolysis can be affected by several factors. The water content and pH of the immediate environment plus the dimensions, water uptake and crystallinity of the implant are among the most important. It is possible that the PEG containing formulation was rapidly degraded in the defect but not outside the defect due to differences in the environment. The presence of crystals of PVA, a water soluble polymer, suggests that there are not large amounts of water coming in contact with the formulation, perhaps as a result of the foreign body response. It may be that the

crystallinity of the PCL in these formulations causes it to be resistant to degradation even when used with these hydrophilic additives. In this model, a rather large plug of formulation is forced into a drilled out defect. In most actual uses, a fairly thin film of the formulation would be smeared across a cut bone surface. The greater ratio of surface area to volume in the smear versus the plug would almost certainly increase the rate of degradation.

A longer study will be needed to show the degradation of the formulations. If this future study were to use a model more like the intended use of the formulations than the current model, there would be several advantages. It would be a more relevant test of hemostasis and bone healing, and may show that the formulations can be more rapidly degraded than in the model previously used.

C. Product Development Work

This section describes the work done in developing the manufacturing procedure and other related processes that will be used for actual production of any of the formulations from ATLS-64.

i. Manufacture of Formulations

Production-scale manufacture of these formulations by Atrix would be conducted in a large Ross planetary mixer. Thus, a procedure for producing 100 gram size batches of formulation in a benchtop Ross mixer, which is identical to the large Ross mixer in all aspects but maximum batch size, was developed. The first step necessary in developing the manufacturing process was to develop a method for heating the mixing bowl to about 60°C, which is the approximate temperature used in making small 1-5 gram batches in the lab. This was accomplished with a circulating water bath, as the mixer bowl is built with jacketing. Fittings were arranged such that the water temperature is measured at the bowl inlet and outlet, as well as from the bath itself. Once the bowl was adequately heated, the mixing time for the formulations needed to be determined. By visual observation, it was determined that a minimum mix time of one hour was sufficient to ensure good mixture. This manufacturing procedure was used to produce the formulations tested in ATLS-64.

ii. Changes in Formulation Components

Several minor changes were made in the components used for the formulations to be tested in ATLS-64. PCL was obtained from a new supplier, which is certified to produce polymers for medical use. This PCL was prepared using intrinsic viscosity (IV) as the measure of molecular weight. This PCL had an IV of 0.10 dL/g, which matches that of the PCL MW 1250 used in the previous experiments. The polymerization was initiated with propylene glycol, which makes the resultant polymer a diol. Propylene glycol was used because of its known biocompatibility. The molecular weight of the PEG used was changed to 3350 from 2000. This change was made as PEG 3350 is available in N.F. Grade, which is approved for use in pharmaceuticals. A supplier of U.S.P. Grade calcium carbonate, which is also approved for use in pharmaceuticals, was also identified. A new supplier of PVA who has a record of dealings with the FDA was also identified. These new components were all tested for suitability in small lab-scale batches prior to the production of any pilot batches.

iii. Packaging of Formulations

After experimenting with different packaging designs, it was determined that a foil package similar to the Ethicon® Bone Wax packaging was ideal. Several different types of foil packaging material were tested before a suitable material was found, as initial materials tested were not stiff enough. It was determined that the packaging needed to maintain the formulation's thickness at a certain level during cooling to yield an optimal product. Packaging is most easily accomplished by filling the package with the formulation in a molten state, then slowly cooling the formulation. A pneumatic dispensing device, the EFD 1500XL, was used for the filling operation. This device is essentially a computer-controlled pressurized gas regulator. The EFD can dispense material for a set amount of time, and thus be used to repeatedly fill packages with a desired amount of liquid material. The difficulty with using this method of packaging lies in keeping the formulation warm while filling packages. After several tests, it was determined that heating tape could be used to heat the dispensing cartridge, thus maintaining the formulation at a desired temperature and viscosity while dispensing it into packages. Experiments with the filling equipment showed that the temperature at which the formulations were prepared, 60°C, was not suited to dispensing the formulations. At this temperature, the formulations were not viscous enough to allow sufficient control of the dispensing process. Thus, a gradual cooling of

the water bath was added to the manufacturing process. The formulations were allowed to cool to 46-48°C prior to packaging. At this temperature, the formulations are viscous enough to dispense in a controlled fashion. Also, this gradual cooling keeps the formulations that contain solid fillers well mixed, whereas the solid fillers tend to settle out when the formulation is kept at 60°C without mixing.

When the formulation containing PEG was prepared for ATLS-64, the temperature and hence the viscosity of the formulation was not well controlled. This made it difficult to get the proper amount of formulation (2.0 to 3.0 g) into the pouch. The time between the start of filling a pouch and the placement of the pouch in the annealing oven (37°C) also varied. In general, samples from this batch were less cohesive than samples from small scale batches. There was also variation from sample to sample and within a given sample in hardness. When the PVA and calcium carbonate containing formulations were prepared, the filling temperature and viscosity were fairly constant, and all samples were quickly filled and placed in the annealing oven. The handling properties of these two formulations were consistent from sample to sample and were comparable to those made at the small scale.

iv. Sterilization of Formulations

Gamma irradiation is the preferred method for sterilizing the formulations. Steam or dry heat sterilization is not an option, as the formulation could melt and separate. During formulation development, promising formulations were subjected to a gamma irradiation dose of 20-25 kGy to observe any potential changes in the materials. This dosage level did not have any adverse effects on the materials. Prior to ATLS-64, an experiment was conducted which compared the effects of a 30-35 kGy dose to that of a 20-25 kGy dose on the three formulations to be tested in the study. It was hoped that the higher irradiation dose might eliminate the need for slow cooling of these formulations. The results of this experiment showed that 30-35 kGy did produce better handling formulations from sample to sample than 20-25 kGy, but that room temperature cooled formulations still exhibited poorer handling properties in comparison to annealed formulations. Differential scanning calorimetry (DSC) testing was also conducted on these samples. The results of this testing showed that annealed samples of formulations had clearly lower degrees of crystallinity than room temperature cooled samples of the same formulation.

A preliminary validation of sterilization, an AAMI (Association for the Advancement of Medical Instrumentation) Method 1 validation, was recently conducted. This validation is designed such that the minimum level necessary to produce sterile product can be determined with only three batches of 100 samples each. To conduct this validation, three 400 gram batches of the calcium carbonate formulation were produced. Prior to this production, a pilot 400 gram batch was produced using the manufacturing and packaging processes developed for 100 gram batches. The pilot run showed that the previously developed manufacturing processes were suitable for this larger batch size, and these methods were utilized for the production of the three lots for sterility validation. After the three lots were finished, twelve samples from each batch were analyzed to quantify the microbial load, or "bioburden," in each batch. The batch with the highest initial bioburden, in this case 11.8 CFU/unit, was subsequently treated with a dose of gamma irradiation sufficient to sterilize at least 98 of 100 samples with this bioburden. This level is called the 10^2 sterility dosage, and is available in reference tables. Sterility testing was then conducted on the irradiated batch to detect any bacterial growth. All samples were sterile in this testing. This result means that the 10^6 sterility dosage for the initial bioburden, as taken from the same table, will sterilize 999,999 out of 1,000,000 samples with the same initial bioburden that receive the 10^6 sterility dosage. The 10^6 dosage can then be used for sterilization of the commercial product. Based on the results of this testing, a dosage of 17.9-18.1 kGy will be sufficient for sterilization of the commercial product. As we intend to use a dose of 30-35 kGy, sterility of product should not be a problem in production. Batches used in the validation procedure were manufactured in the controlled environment of Atrix's general manufacturing area with the assistance of this department. This involvement represents further progress in scaling up the production process.

Samples from these three batches were also tested for handling properties and calcium content. These samples were taken from various time points during each run, to check for consistency of properties and composition throughout the process. Handling properties remained rather constant throughout the production of the three batches. The material made in these lots was somewhat harder and less cohesive than that used in ATLS-64. This is probably due to less exact temperature control in the large oven. The results of this testing did not show any significant changes in calcium levels during the filling procedure, and

the average calcium content of 6.08 ± 0.32% for the 19 samples tested from these three lots agrees with the expected value of 6.00%.

v. Quality Control and Stability Testing

Performing ATLS-64 under Good Laboratory Practice guidelines required the development of a quality control and stability testing protocol for the formulations in the study. Infrared spectral analysis proved to be a useful tool. IR spectra were generated for all the raw materials of the formulations in ATLS-64. These spectra were found to match well with reference spectra for these materials. Formulations were then tested, and for each formulation, there was an easily identifiable peak that was not in the spectrum of the PCL, but could be matched to a peak from the spectra for the additive in the formulation. A qualitative method for evaluating handling properties was developed as the primary quality control and stability test, as handling properties seem to be the strongest indicator of a formulation's performance as a hemostatic agent. This method is presented in the appendix of procedures developed. For the calcium carbonate formulation, a contract laboratory was identified which was able to analyze samples of this formulation for calcium content.

Several other tests were evaluated for potential use in quality control and stability testing. Inherent viscosity measurement, ultraviolet spectral analysis, and differential scanning calorimetry were judged unsuitable. The results of these tests varied over the course of several months of stability testing, whereas the handling properties of the formulations were unchanged.

The initial testing of the three pilot batches of the calcium carbonate formulation consisted of handling tests, calcium content determination, infrared spectroscopy, and molecular weight by GPC. The remaining samples from the two batches not used in the AAMI validation are being used in a room temperature stability test. Handling properties of samples from each lot will be tested every three months, with other testing conducted every six months.

vi. Regulatory Requirements

An opinion from the FDA has been obtained as to what would be required for a 510(k) approval of a biodegradable bone wax. These requirements are subject to change, due both to a general increase in

requirements for device approval, and a change in FDA reviewers for bone wax and related products expected by mid-1994.

For approval of a 510(k), a new product must be proven to be substantially equivalent to a product already on the market. In this case it would be Ethicon® Bone Wax. The label claims of the new product would be essentially the same as the predicate device. The FDA opinion stated that a claim of biodegradability would be allowable. If our material is not substantially equivalent to Ethicon® Bone Wax, or we want to make different claims, a Premarketing Approval (PMA) would be required instead of a 510(k). A PMA requires more extensive safety and efficacy testing.

The specific current requirements for a 510(k) approval of a biodegradable bone wax are a test of biochemical effect on blood clotting and an animal study which shows complete degradation of the product and complete bone healing. The FDA opinion specified the Lee-White in vitro coagulation assay for clotting testing. This test consists of exposing whole blood to a device or formulation and measuring the time needed for coagulation to occur. This time is compared to a control sample of whole blood exposed to air. As Ethicon® Bone Wax claims to have no biochemical effect on coagulation, an Atrix formulation should not biochemically affect clotting either. If a formulation affected clotting in this assay, it would not be approvable via a 510(k), and additional issues of safety and efficacy would need to be addressed. An animal study conducted to support a 510(k) filing would need to run a minimum of six months, perhaps longer. This study must include histological evidence that bone healing is complete and that the formulation is completely degraded. Additional safety testing may also be required. The FDA could not say if a biodegradable bone wax would be considered a significant risk device. It is likely that it would be classified as such, as it is an implant. In this case, tripartite testing, which consists of a battery of toxicity tests, would likely be required. Human clinical tests are not currently required, but this situation could change with the change in FDA reviewers.

D. Other In Vitro Testing

i. PCL 830 Based Formulations

PCL diol of MW 830 is much softer and less cohesive than MW 1250 or 2000 PCLs at room temperature, and thus was not included in the initial development of PCL-based formulations. However, during initial in vivo testing done by Atrix, it was noted that although several Atrix formulations were easy to apply and effective as hemostatic agents, a softer formulation would be even easier to apply. This observation led to the development of formulations based on PCL MW 830. An important advantage to PCL 830 MW formulations is that they are much less sensitive to cooling rate than the higher molecular weight PCL formulations. This property allows the 830 MW formulations to be cooled at room temperature and still have acceptable handling properties. After testing the effect of cooling temperature on PCL 830 itself, all formulations were prepared without a special annealing period. Several promising formulations were produced using the same additives for the higher molecular weight PCL formulations. In preliminary formulation work, miscible and semi-miscible polymer additives, such as PEG and PVP, were more suitable with PCL 830 than nonmiscible additives such as PVA and calcium carbonate. However, given previous experience with PCL 1250, further formulation development should produce acceptable formulations with many of these additives.

These lower molecular weight PCL formulations were developed too late in the contract period to be adequately tested in vivo. However, there are several advantages to these formulations that warrant future investigation. The lower sensitivity to cooling rate would make these formulations much easier to manufacture, as the logistics of quickly getting filled pouches into an oven, as well as keeping that oven at 37°C while filling it, are eliminated. The softer consistency of PCL MW 830 formulations makes them easier to apply than the higher molecular weight PCL formulations. Finally, the lower crystallinity of PCL 830 could potentially lead to more rapid degradation of these formulations.

ii. Coloring of Formulations

During the first in vivo study, it was noted that the formulations were difficult to see once placed in the defect. To alleviate this problem, the feasibility of adding coloring to these formulations has been investigated. The following dyes were added to the formulation of 77%

PCL 1250 and 23% PVA: FD & C Blue #1, D & C Green #6, and D & C Violet #2. Blue #1 is approved for use in food, drugs, and cosmetics, excluding use in the eye area. Green #6 is used in sutures, and is approved for externally applied drugs and cosmetics. The formulations did exhibit a relationship between dye load and dissolution rate. The blue formulation (about 0.1% dye) fell apart in PBS at about the same time a specimen without dye would. However, the green (about 0.12% dye) and the violet (around 0.14% dye) specimens took slightly longer to fall apart. The green and violet specimens also left some coloration on latex gloves following handling. Another interesting observation was that the blue dye appeared to be released from the formulation during dissolution, while the violet and green dyes appeared to be bound up in the formulation fragments in the vial.

iii. In Vitro Degradation Experiments

An experiment was conducted to see if the presence of macrophages would increase the rate of mass loss of several PCL based formulations. The following formulations were exposed to murine peritoneal macrophages in cell culture media:

76% PCL 1250 + 24% Glyceryl Monopalmitate
85% PCL 1250 + 15% Calcium Carbonate
89% PCL 1250 + 11% 25/75 PLC
89% PCL 1250 + 11% 50/50 PLC
77% PCL 1250 + 23% PVA, MW 14,000
79% PCL 1250 + 21% PEG 2000

Control groups of samples in media without cells were also tested. Percent mass loss was determined by removing samples, lyophilizing, and weighing weekly for one month. The results of this study were inconclusive, as there were no clear trends seen in the samples exposed to macrophages or the control specimens.

Another in vitro degradation study was conducted to compare mass loss of formulations immersed in PBS to mass loss of formulations immersed in horse serum. This experiment tested the formulations that were tested in the macrophage trial. The results indicated that there was no significant difference in mass loss between immersion in PBS and horse serum. While two formulations showed significantly higher degrees of mass loss in serum, the other four showed no significant

difference. In general, mass loss ran from 15-40% loss over a four week period.

The suitability of gel permeation chromatography (GPC) to quantify the molecular weight of PCL was evaluated. Using the appropriate column and poly(styrene) standards, it is possible to generate values for the molecular weight of the PCLs used in this project. GPC was used in an experiment that investigated some of the mechanisms of PCL degradation in vitro. This experiment involved keeping samples in PBS, pH 7.4, at 37°C for various lengths of time. The samples were then removed, lyophilized, weighed, and analyzed by GPC. Two PCLs were tested; the 0.10 dL/g inherent viscosity polymer used in ATLS-64, and a 0.13 dL/g inherent viscosity polymer, which has one carboxylic acid end group because the polymerization was initiated using water. The acid group is expected to catalyze degradation. In addition, the effect of two additives on the molecular weight of PCL was studied. Small amounts of capric acid and stearic anhydride were added to the 0.10 dL/g inherent viscosity PCL to observe any potential catalytic effect that these compounds might have on the degradation of the polymer. Also, the PCL/calcium carbonate formulation was tested. At this time, the experiment has run for one month. There were no significant trends in the molecular weight degradation at the one month timepoint. The calcium carbonate did exhibit a greater amount of mass loss than any other formulation at the one month timepoint, losing an average of 40% mass, with the next highest average mass loss being 25%.

iv. <u>In Vitro Swelling Experiments</u>

Initially, a crude study of in vitro swelling was conducted on some polymer blends, as well as the more stable of the PCL 2000/solvent formulations. Portions of the formulations were hand-formed into approximately spherical shapes, and placed in vials containing 10 milliliters of PBS. The vials were kept at 37°C in an environmental shaker, and approximate volume measurements were made after one, two, four, and six days in these conditions. Overall swelling rates for the experiment ranged from +52% to -45%. The most interesting data gathered from this experiment was that one formulation, 77% PCL 1250 and 23% PVA, crumbled into small flakes in less than 24 hours.

Another in vitro swelling test was conducted with the formulations considered for animal study. This test was intended to more accurately

represent the conditions that formulations would encounter in the animal study. Molten formulation was injected by syringe into glass tubes (o.d.=6 mm, i.d.=5 mm) of known length. The tubes were then immersed in PBS and kept in an environmental shaker at 37°C. Volume measurements were made by measuring the amount of formulation extruded from the ends of the tube, and were taken after one, two, and five days in these conditions. The results of this experiment were consistent with those obtained from the sphere swelling test.

A third study was conducted which looked at swelling of disc-shaped samples of several different formulations. These formulations were made with additives commonly used in tablet formulation to promote fluid intake. These additives include sodium starch glycolate, starch, and maltodextrin. The swelling rate at 24 hours was determined by calculating initial volume and change in volume after 24 hours in PBS, pH 7.4 at 37°C. Formulations containing sodium starch glycolate fragmented in a manner similar to those containing PVA. Formulations containing starch and maltodextrins swelled from 80 to 200% on average, while still maintaining their original shapes. These results are promising; if fluid intake is a critical factor in degradation of the PCL, these formulations could degrade much faster than those previously tested in vivo.

v. Quantitative Physical Testing

The handling properties of a formulation are a combination of several physical properties such as hardness, elasticity, cohesion and adhesion. These four properties, and others, can be quantified by a texture profile analysis on a TA-XT2 texture analyzer (Texture Technologies, Inc., Scarsdale, NY). This instrument is used to test physical properties of gels, creams, paste and ointments. It has significant potential to be used as a research instrument and a quality control instrument since it can do quantifiable and objective testing of properties relevant to the handling and hemostatic performance of bone wax formulations.

The texture profile analysis is a double compression test. A probe compresses the sample a set amount, returns to the starting position, recompresses the sample and then returns again to the starting position. This is done at a controlled rate. The output is a graph of force versus

time. Several different parameters can be obtained from the data using established equations.

Four samples were used in preliminary testing at Texture Technologies, Inc. The operator kneaded the material until it was smooth and pliable, as is done before actual use of a formulation, and then formed it into a sphere for testing. The formulations tested and the testing results are given in Table 4.

The formulations with the particulate additives (PVA and calcium carbonate) have very similar results in this test that correlate well with the similarity in the handling characteristics of these two formulations. The calcium carbonate formulation is actually somewhat harder and less adhesive than the PVA formulation as is shown in this testing. This test indicates both PEG-containing samples are less elastic and cohesive than the other two samples. This indicates that the PEG, which is at least partially miscible with the PCL, is not as effective a reinforcing agent as the particulate additives, which is expected. The PEG formulation annealed at 37°C is considered a good handling sample, whereas the one annealed at room temperature is not. This testing found the room temperature sample to be harder, less elastic, less cohesive and especially less adhesive than the 37°C sample. These results correspond very well with the qualitative assessment of these formulations' handling properties.

This was a very preliminary test, but the ability of this equipment to differentiate between a good handling and a poor handling sample of the same formulation indicates that this equipment has potential to be very useful in quality control testing.

TABLE 4: TEXTURE PROFILE ANALYSIS RESULTS

FORMULATION COMPONENTS	HARDNESS (g)	ELASTICITY (no units)	COHESION (no units)	ADHESION (g-sec)
77% PCL + 23% PVA	258	.417	.197	-162
85% PCL + 15% $CaCO_3$	294	.491	.199	-130
79% PCL 21% PEG 3350 (annealed at 37°C)	276	.213	.161	-133
79% PCL i.v.=0.10 + 21% PEG 3350 (annealed at room temp.)	291	.169	.142	-62

4. CONCLUSIONS

The goals for the biodegradable bone wax development outlined in the revised Statement of Work were as follows:

Year 1 A moldable, biodegradable bone repair material will be developed and prototypes tested in animals for efficacy.

Year 2 A biodegradable bone wax will be developed and prototypes tested in animals for efficacy.

Year 3 The optimum biodegradable bone wax will be prepared under GMP, and sent to USAIDR for large scale safety and efficacy evaluations. Manufacturing and quality control procedures will then be established and a 510(k) submission prepared for the FDA.

Significant progress was made toward the year one objective. Formulations were prepared that in laboratory testing had the desired properties. The formulations were easily molded into a desired shape and hardened to rigid, porous implant upon contact with an aqueous environment. Model proteins and enzymes were incorporated into these formulations and were released for periods of one to two tive form. The in vitro degradation of these materials was seen in less than four weeks. Formulations were sent to USAIDR, where they were evaluated in a bone wax model. The details of this work are given in the midterm report. This report contains the results of the work done since the revision of the statement of work toward the year two and three goals. Many of these obje ctives were met. A large number of formulations with acceptable handling characteristics was produced using low molecular weight poly(caprolactone) (PCL) as the main component. Several of these formulations have demonstrated effective hemostasis and the ability to remain in a bone defect in animal studies. The results of these studies indicate that acceptable handling properties are an excellent indicator of hemostatic ability. Several formulations were sent to USAIDR for confirmation of efficacy in animals. The results of this trial indicated that all of the Atrix formulations were essentially equivalent to Ethicon® Bone Wax in terms of hemostatic performance. These results led to the selection of three formulations for further study: 79% PCL (IV 0.10) with 21% PEG (MW 3350), 77% PCL (IV. 0.10) with 23% PVA, and 85% PCL (IV. 0.10) with 15% calcium carbonate. Preliminary manufacturing (100g scale), quality control, and stability testing procedures were developed for all three of these formulations. These formulations were tested again by Atrix in a twelve-week animal study conducted under Good Laboratory Practices (GLP) guidelines.

This study indicated that Atrix formulations are comparable to Ethicon® Bone Wax in hemostasis and retention in the rabbit tibial defect. Also, all three formulations induced a much less severe foreign body response than Ethicon® Bone Wax. Visual observations of necropsy specimens suggested that Atrix formulations caused less inhibition of bone regrowth than Ethicon® Bone Wax. Histomorphometric analysis was conducted on samples from this study to quantify this behavior. This analysis showed that all three Atrix formulations are more biocompatible than Bone Wax, which elicited acute inflammatory and immunological responses in addition to a foreign body response. The PEG formulation stood out as the formulation with the most complete defect healing, the mildest foreign body response, and possibly the most advanced degradation.

Three 400g pilot manufacturing runs were done with the calcium carbonate formulation. The samples from these runs were used in a successful AAMI sterility validation, and are being used in an ongoing stability study conducted at Atrix's expense.

The main objective not accomplished by this research was the filing of a 510(k) with the FDA. The project was held up for several months while waiting for USAIDR to first perform in vivo testing and then perform histology on specimens from that testing. This histological analysis was never done. This delay significantly shortened the time available for formulation optimization and further in vivo study. An opinion from the FDA was obtained which stated that for an absolute minimum for 510(k) approval, a six-month animal study that shows complete defect healing and formulation degradation and the Lee-White coagulation test must be done. This FDA opinion was received too late in the contract for this to be done. It is likely that a human clinical trial, which is beyond the scope of this contract, will be required for future approval of a 510(k).

The formulation with PEG has to be considered the most promising due to the histomorphometric data from the final in vivo study. The particular batch of this formulation used in the study was less than ideal in terms of handling properties and hemostatic potential. These problems were unexpected, as this formulation had excellent handling properties and hemostatic potential when prepared on a small scale. The shortcomings of the batch used in the final in vivo study are likely due to problems with temperature control during the manufacturing process. As this batch was the first larger scale batch of this formulation manufactured, we are confident that better temperature control will yield a product with the desired handling and hemostatic properties.

5. FUTURE PLANS

In the short term, Atrix will supply test articles (at Atrix expense) to Dr. Galloway of USAIDR for testing of hemostatic performance in a new animal model. This model, which involves trephining out a thin disc from the rabbit iliac crest, is similar to how a biodegradable bone wax would be used clinically. This long-term study will also look at degradation of the formulation.

Plans are being developed for a contract proposal to USAIDR. The contract to be proposed would involve further formulation optimization and animal testing. An Investigational Device Exemption (IDE) would be filed with the FDA. This contract would culminate with a human clinical trail and filing of a 510(k) for a biodegradable bone wax.

Acknowledgements

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APPENDIX 1: ATS-52 SYNOPSIS

GENERAL NOTES:

For all necropsies, the bone surrounding the defect site was excised. Tissue and polymer samples were also taken where noted. Animal #1385 was resutured on 8-11 with stainless steel, and was again resutured on 8-12 with Vicryl® and silk sutures. The sutured area was wrapped with Vet-Wrap®. Animals #1412 and #1400 were also resutured on 8-12, using silk sutures.

FORMULATION 6-22-AG: 77% PCL 1250, 23% Poly(vinyl alcohol), 99% hydrolyzed, MW 14000

This formulation was first tested on 7-23 in animal #1383. It demonstrated effective hemostasis, and the consistency was excellent. The formulation performed just as well in its second use on 8-7, in animal #1392, with no change in consistency.

Animal #1392 was sacrificed on 8-14, seven days after surgery. A mass of tissue was found near the defect site. Some formulation was present in the tissue surrounding the defect. The defect itself had partially closed up.

Animal #1383 was sacrificed on 8-6, fourteen days after implantation of the formulation. The defect was not evident, and a nodule of hard tissue was present which appeared to correspond to the defect site. There was no apparent evidence of gross irritation, but there was a slight redness of the tissue within several millimeters of the defect.

FORMULATION 59-A: 83% PCL 1250, 17% Baking Powder

This formulation was first tested on 7-23 in animal #1407. The formulation was an effective hemostatic, and had a consistency slightly harder than bone wax. 59-A was also effective in animal #1402 on 8-7, with no difference in consistency between the two test dates.

Animal #1402 was sacrificed on 8-14, seven days post-surgery. A nodule of hard tissue, appearing to correspond to the defect site, was present.

Animal #1407 was sacrificed on 8-6, 14 days after implantation. No external irritation was evident. A nodule of hard tissue which appeared to correspond to the defect site was present.

FORMULATION 6-16-C: 80% PCL 2000, 10% Ethyl Lactate, 10% Glycerol

This formulation was first tested on 7-23 in animal #1417. The

formulation had poor cohesion, and failed to stop blood flow in the defect. The formulation was removed from the defect after about fifty seconds.

FORMULATION 6-17-AB: 89% PCL 2000, 10% NMP, 1% Glycerol

This formulation was also tested in animal #1417 on 7-23.. The formulation had little cohesion, was ineffective as a hemostatic, and was removed after about two minutes.

FORMULATION 6-17-AE: 89% PCL 2000, 10% NMP, 1% Peanut Oil

This formulation was tested in animal #1417 on 7-23. It was initially hard, but working by hand yielded a good consistency. The formulation failed as a hemostatic, and was removed after about forty-five seconds.

FORMULATION 50-C: 89% PCL 1250, 11% PEG 1500

This formulation was first tested in animal #1417 on 7-23. The formulation required some hand working to achieve a good consistency. Effective hemostasis was demonstrated.

The second test for this formulation was on 8-7 in animal #1403. The formulation was still an effective hemostatic, and there was no change in consistency.

Animal #1403 was sacrificed on 8-14, seven days after surgery. A fragment of formulation was loose near the defect site, and was removed for analysis. The defect was still present, and some formulation could be seen in the defect.

Animal #1417 was sacrificed on 8-6, fourteen days post-implantation. There was redness in the general vicinity of the defect site. The defect itself appeared to be covered by a nodule of hard tissue.

FORMULATION 6-23-D: 86% PCL 2000, 14% Ethyl Lactate

The formulation was first tested in animal #1410 on 7-23. The formulation had a grainy texture, but was an effective hemostatic agent.

The second test of 6-23-D occurred on 8-7, in animal #1408. While still effective, the consistency was poor, as the formulation was non-homogenous and sticky.

Animal #1408 was sacrificed after a period of seven days, on 8-14.

Animal #1410 was sacrificed on 8-6, after a period of fourteen days. A fragment of formulation was present in the connective tissue, and was saved for analysis. The defect appeared to have been covered with a nodule of hard tissue.

FORMULATION 6-22-AE: 79% PCL 1250, 21% PEG 2000

6-22-AE was first tested in animal #1413 on 8-5. The formulation had excellent handling characteristics, comparable to 6-22-AG. The formulation was effective as a hemostatic.

The second test of this formulation was on 8-10, in animal #1411. The formulation again demonstrated effective hemostasis, with no change in consistency.

Animal #1411 was sacrificed on 8-17, seven days post-surgery. There appeared to be some formulation still present in the defect.

Animal #1413 was sacrificed on 8-19, fourteen days post-surgery. A nodule of hard tissue was present that seemed to correspond to the defect site.

FORMULATION 51-G: 89% PCL 1250, 11% 50/50 PLC

This formulation's first test use was on 8-5, in animal #1381. The formulation had a good consistency, but was slightly sticky, leaving the surgeon's gloves tacky. The formulation was effective as a hemostatic.

This formulation was re-tested on 8-10, in animal # 1393. The surgeon noted that the formulation became slippery when wet, but effective hemostasis was achieved.

Animal #1393 was sacrificed on 8-17, seven days post-implantation. The defect was partially healed.

Animal #1381 was sacrificed on 8-19, fourteen days post-surgery. A large nodule of hard tissue was present distal to the defect site. The defect itself was partially healed.

FORMULATION 51-E: 89% PCL 1250, 11% 25/75 PLC

51-E was first tested in animal #1400 on 8-5. The formulation was hard, and required extensive manipulation to soften it. It was successful as a hemostatic.

The formulation was tested again on 8-10, in animal #1385. Effective hemostasis was demonstrated, with no change in handling properties.

Animal #1400 was sacrificed on 8-14, nine days post-surgery. It was originally planned for this animal to be sacrificed on 8-19, fourteen days post-surgery, but the animal was sacrificed early due to problems with the animal chewing its sutures and favoring the leg which was operated on. The defect appeared to have healed, with a hard nodule of tissue present. Also, a mass of tissue which resembled a fibrotic capsule was found near the defect. This mass was excised for further analysis. The defect itself was still evident, and was filled with a rust-colored viscous fluid.

Animal #1385 was sacrificed on 8-24, fourteen days post-surgery. A large

mass of tissue was found covering the defect site. This tissue was cut open to expose the defect site, and was excised intact with the bone. The defect was still present under this tissue mass.

FORMULATION 6-22-AF: 80% PCL 1250, 20% PEG 10,000

This formulation was first tested on 8-5, in animal #1398. The formulation failed as a hemostatic, and was removed after about three minutes.

FORMULATION 48-C: 82% PCL 1250, 18% Calcium Stearate

This formulation was first tested on 8-5, in animal #1398. The formulation required a small amount of hand working to yield a good consistency. 48-C was effective as a hemostatic in this test.

The formulation was retested on 8-10, in animal #1397. Effective hemostasis was again demonstrated, with no change in formulation consistency.

Animal #1397 was sacrificed on 8-17, seven days post-surgery. The defect was mostly healed, leaving a smaller, more shallow hole present in the bone.

Animal #1398 was sacrificed on 8-19, fourteen days post-surgery. A nodule of hard tissue was present distal to the defect site. The defect had virtually disappeared, with only a small depression in the bone left behind.

FORMULATION 6-22-AA: 50% PCL 1250, 50% PCL 2000

6-22-AA was first tested in animal #1382, on 8-5. The formulation was not homogenous, with hard chunks present. It was difficult to work the formulation into a homogenous mixture by hand. The formulation did demonstrate effective hemostasis.

The formulation was tested again on 8-10, in animal #1380. The formulation was still effective as a hemostatic, with no change in consistency.

Animal #1380 was sacrificed on 8-17, seven days post-surgery. The defect was mostly healed.

Animal #1382 was sacrificed on 8-19, fourteen days post-surgery. A mass of tissue was present distal to the defect site, while the defect itself was mostly healed.

FORMULATION 59-C: 85% PCL 1250, 15% Calcium Carbonate

This formulation was first tested on 8-5, in animal #1420, and it proved effective as a hemostatic.

59-C was retested in animal #1412 on 8-11. The surgeon noted that the formulation had a good consistency, with a good level of tack. Effective hemostasis was demonstrated.

Animal #1412 was sacrificed on 8-18, seven days post-surgery. A mass of tissue was present. The defect was almost completely healed.

Animal #1420 was sacrificed on 8-19, fourteen days post-surgery. Before the necropsy began, it was noted that the animal was missing hair lateral to the surgical site. A small wound was also present distal to the surgical site. It did not appear that either of these phenomena were related to the test article. The defect was not apparent.

FORMULATION 51-F: 80% PCL 1250, 11% 25/75 PLC, 9% Calcium Stearate

51-F's first test use was in animal #1409 on 8-6. It failed to achieve hemostasis, and was removed after about one minute.

FORMULATION 53-B: 81% PCL 1250, 19% Dextran MW 8800

This formulation was first tested in animal #1409 on 8-6. It was effective as a hemostatic.

53-B was retested on 8-11, in animal #1388. This trial was also successful, with the formulation showing no changes in handling properties.

Animal #1388 was sacrificed on 8-18, seven days post-surgery. A loose portion of formulation was found and removed for further analysis. The defect was significantly smaller than its original size.

Animal #1409 was sacrificed on 8-20, fourteen days post-surgery. A mass of tissue covered the defect site; this tissue was cut open to reveal the defect, and excised along with the bone. The defect had almost completely disappeared.

FORMULATION 48-D: 76% PCL 1250, 24% Glyceryl Monopalmitate

48-D was first tested on 8-6 in animal #1421. The formulation was slippery, but still implantable, and it demonstrated effective hemostasis.

Effective hemostasis was also shown in the second test of 48-D, in animal #1399 on 8-11. No change in formulation consistency was noted.

Animal #1399 was sacrificed on 8-18, seven days post-surgery. A mass of tough tissue was present near the site, as well as a Vicryl™ suture. It was not possible to expose the defect site without significant disruption of the surrounding tissue mass, so the defect site was not seen.

Animal #1401 was sacrificed on 8-20, fourteen days post-surgery. A tissue mass covered the defect site. This tissue was cut into to expose the defect site, and was excised with the bone. The defect itself was still present, but appeared to be somewhat smaller than its original size.

CONTROL FORMULATION: ETHICON BONE WAX

Bone wax was used in animals #1421 and #1394, on 8-6 and 8-14 respectively. Effective hemostasis was demonstrated for both trials. Animal #1394 was anesthetized with Metofane, due to a lack of isofurane.

Animal #1394 was sacrificed on 8-21, seven days post-surgery. The defect was still quite apparent, and was filled with a rust-colored viscous fluid.

Animal #1421 was sacrificed on 8-20, fourteen days post-surgery. A mass of spongy tissue was found distal to the defect site. This tissue was attached to the bone by hard tissue. The defect itself appeared to have healed completely.

APPENDIX 2: SUMMARY OF RESULTS FROM ATLS-64

The following paragraphs are the histological observations of the histomorphometric samples from ATLS-64:

79% PCL i.v.=0.10 / 21% PEG MW 3350, N.F. Grade

Two types of foreign body response were seen at day 14. Small foreign body granulomas contained uniform small (30-60μm) round spherules of basophilic material surrounded by flattened mononuclear histiocytes and more rarely by fused foreign body giant cells. The basophilic material was in various states of fragmentation and so appeared to be breaking down. Within the granulomas were a few larger spaces, 2-3 mm in diameter surrounded by large foreign body giant cells. These spaces contained only cell remnants. The second type of reaction consisted of much larger spaces, up to 10 mm in diameter, lined by foreign body giant cells and fibrous tissue, but containing no identifiable foreign material. They contained large amounts of cell remnants along with viable capillaries, fibroblasts, histiocytes, and foreign body cells. Only the latter type of response was present at 28 and 84 days. These persistent foci were small, but were localized in the small gaps still present in both 84 day specimens.

77% PCL i.v.=0.10 / 23% Airvol® 103

At 14 days, large and small pieces of homogenous basophilic material surrounded by histiocytes and foreign body giant cells were present in relatively dense fibrous tissue. There was little evidence of fragmentation of the foreign material. At 28 days, a similar reaction was even more prominent, and had not diminished at day 84. At both the 28 and 84 day timepoints, some larger cystic spaces, surrounded by fibrous tissue and foreign body giant cells, containing only cellular remnants were present. Unlike the large cysts seen for the PEG formulation, there was no vascularization or fibroblastic invasion of these spaces. Foreign body reactions with and without foreign material occupied large persistent defects and adjacent periosteal and endosteal sites at both 28 and 84 days.

85% PCL i.v.=0.10 / 15% Calcium Carbonate, U.S.P. Grade

At 14 days, small, moderate, and very large spaces surrounded by foreign body giant cells and histiocytes in a moderately dense fibrous stroma were present. These spaces contained granular, slightly azureophilic material which stained for calcium with von Kossa stain. Calcified material could also be seen in the medullary space in specimen x-rays. Many of the larger spaces appeared to have lost this material, either mechanically or as a result of solvents used in

processing, since the spaces were completely clear except for small residues of foreign calcified material around the edges. At 28 days, in addition to large cysts containing calcium-positive foreign material, medium sized spaces without foreign material but full of cellular remnants and surrounded by histiocytes and foreign body giant cells were present. One of the 84 day samples consisted entirely of this type of reaction. The other 84 day specimen had small areas of a similar reaction near the defect, and very large cysts containing silve-staining foreign material in the medullary cavity at a distance from the defect. Regardless of reaction type, persistent large defects with foreign body reactions within and around them were present at 28 and 84 days.

Ethicon® Bone Wax

At day 14, very large unilocular cysts containing unstained broad strands of birefringent material filled most of the medullary space, and were surrounded by histiocytes, foreign body giant cells, neutrophils, and dense fibrous tissue. Dense fibrous tissue with smaller cysts containing similar material occupied the defect and the adjacent periosteal tissue. The 28 and 84 day samples were essentially the same. There was some variation in the size of the unilocular intramedullary cysts, which in two specimens extended into the defect. Multiple smaller cysts with birefringent material were more prominent in the defect and periosteal sites. Mo cystic spaces containing cellular remnants were seen. In one 84 day specimen, prominent lymphoid nodules with germinal centers were present in the wall of the large cyst. Polymorphonuclear cels were quite prominent along the junction with the foreign material in both 84 day cysts.

UNTREATED CONTROL

There was essentially no foreign body response. A small cyst containing precipitated protein and calcified debris was present in the defect of one of the 14 day specimens, but no foreign material was identified. At 28 and 84 days, the defects were closed without any histologic abnormalities in or adjacent to the original defect area.

COMMENTS

The PCL/PEG formulation was clearly the best treatment with the least foreign body response and best healing of the defect. The other three treatments appeared to significantly impair bone repair due to persistent foreign body reactions. Quantitatively, the PCL/Airvol®, PCL/$CaCO_3$, and Ethicon® Bone Wax were similar. Qualitatively, Ethicon®Bone Wax was the worst treatment, as it elicited both a polymorphonuclear and a lymphoid response at 84 days. Also,

the foreign body response initiated by this treatment was poor in resolution.

It is of interest that none of the treatments appeared to interfere with or prolong bone formation per se. This was shown by the similar measurements of cross-sectional bone area, labelled bone area, and osteoblasts and osteoclasts per callus unit area in all groups at all timepoints. Foreign body material and the associated responses seemed to spatially misdirect the bone formation so that the defect was not filled as quickly as the control defects. These abnormalities may be related to effects of the foreign materials and/or reactions to them on the movement of fibroblasts and osteoblasts into the defect. Normally, such movement is facilitated by the physical and chemical characteristics of blood clots and the influx of inflammatory cells into them. Thus, it is not surprising that treatments inhibiting clotting and altering the inflammatory infiltrate might also alter the healing process.

Foreign materials used in the treatment groups all elicited foreign body responses demonstratable by day 14 as an influx of mononuclear histiocytes which elongated and fused to form multinucleated giant cells. These giant cells segrated and walled off the foreign materials into aggregates or cysts of varying sizes. Differences in size were probably related to the ability of histiocytes to penetrate the foreign materials. The smallest cysts were present in the small granulomas seen in PCL/PEG treated specimens, followed by the PCL/Airvol®specimens, which contained intermediate sized cysts. The PCL/$CaCO_3$ and Ethicon® Bone Wax specimens both contained very large cysts of foreign material surrounded but not penetrated by histiocytes and foreign body giant cells at day 14. The PCL/PEG specimens at day 14 also contained some large cysts at day 14, but these cysts contained only large amounts of cellular remnants and no foreign material. These large cysts were also penetrated by fibroblasts and blood vessels in distinction to the other treatment groups. These characteristics probably reflect increasd breakdown of the foreign material as discussed further below.

Breakdown of foreign materials was judged by whether it was visible and how uniform and intact pieces within cystic spaces were. With the PCL/PEG specimens, at 14 days the small spherules contained in granulomas contained material that appeared to be disintegrating. Large cysts contained only cellular debris, indicative of rapid breakdown and dissapearance of the foreign material. This was substantiated by absence of foreign material in 28 and 84 day specimens, which had a reduced extent of foreign body reaction associated only with residual cellular debris. Whether rapid disappearance of foreign material was due to a formulation that gave an increased non-cellular hydrolysis rate, or to a composition that facilitated compartmentalization by histiocytes and invasion by fibroblasts and capillaries is not clear. Perhaps enhanced hydrolysis favors penetration by histiocytes, mesenchymal cells and capillaries, or conversely, such penetration may hasten breakdown of the material. In any case, more rapid breakdown was associated with better bone repair.

Based on the appearance of 28 and 84 day specimens, breakdown of foreign material was faster in the PCL/PEG formulation than the other Atrix formulations. PCL/Airvol® seemed to break down less rapidly than PCL/$CaCO_3$, as judged by a greater proportion of foreign body cysts containing cellular remnants in the PCL/$CaCO_3$ specimens. Ethicon® Bone Wax seemed to persist more than any other treatment, and elicited acute inflammatory and immunologic responses, as well as a foreign body response.

In summary, the study showed that all treatments impaired bone repair, but none had noticable effects in enhancing or prolonging bone formation. Instead, foreign body reactions appeared to spatially prevent bone formation to heal the defects. The worst results were with the Ethicon®Bone Wax, which was the least well penetrated by reactive cells, persisted in tissues, and elicited several types of inflammatory responses. Best bone repair occured with the PCL/PEG formulation, which was the most rapidly broken down. Bone repair in the presence of this material did not differ markedly from that in the untreated control group.

APPENDIX 3: METHODS AND PROCEDURES

The following documents are examples of some of the operating procedures developed for the production and testing of formulations. These documents are representative of the style used in similar documents which are Atrix SOPs.

LABORATORY MANUFACTURING RECORD FOR PROJECT 2070
FORMULATION SMK-226-59-C

To be performed in the ATRIX General Manufacturing Facility at 1901 Sharp Point Drive

BATCH SIZE: 400 grams

MANUFACTURED BY: _____ DATE: _____

_____ DATE: _____

_____ DATE: _____

NOTEBOOK REF. #: _____

INGREDIENT	LOT #	TARGET WT.	ACTUAL	%W/W
Poly(caprolactone) (PCL)		340g		
Calcium Carbonate Light, U.S.P. ($CaCO_3$)		60g		
Total				100

Variation from Required Weights ≤ 0.5%

MAJOR EQUIPMENT

Small Ross Mixer
Small Ross Mixer Bowl No. ____
Plumbing and Temperature Assemblies
Vacuum Pump
Haake Circulating Water Bath
Top Loading Balance Accurate to 0.1 gram

PROCEDURE	VERI-FICA-TION	DONE BY	CHECKED BY
1. If necessary, attach the plumbing assemblies to the outlets on the mixer bowl such that the barbed fittings are pointed downward. Place mixer bowl onto the Ross Mixer, attach quick release pins to secure the mixer bowl. Close Ross Mixer using the lift handle.			
2. Slide tubing from the circulating water bath over the barbed fittings on the bottom of the plumbing assemblies. The outlet tube from the water bath should be attached to the higher assembly on the mixer bowl. The inlet tube to the water bath should be attached to the lower, or right-hand side, assembly of the mixer bowl.			
3. Turn on the Haake bath, and set temperature to 60° C. This is done by holding the "Set" button in while turning the white "Temperature Set" knob such that the LCD display reads 60° C.	Haake Set Temp ___°C		
4. Weigh out the PCL into a suitably sized container.	Cont. Weight _____g PCL Weight		

	____g		
5. Weigh out the calcium carbonate into a suitably sized container.	Cont. Weight ____g $CaCO_3$ Weight ____g		
6. When the thermometers at the mixer bowl inlet and outlet read 60 ± 2°C, open the Ross Mixer using the lift handle, and transfer the poly(caprolactone) to the Small Ross Mixer Bowl. Close Ross Mixer using the lift handle.	Inlet Temp:__ Outlet Temp:__		
7. Observe the poly(caprolactone) through the site glass every 5-10 minutes, using a flashlight to aid vision. When the poly(caprolactone) is completely melted, open the Ross Mixer, and add the calcium carbonate. Close the Ross Mixer, and turn the mixer on. Slowly increase the mixer speed to 16 Hz by turning the knob on the control box clockwise.	Start Time _____ PCL Melted Time _____ Speed ____Hz		
8. Attach vacuum pump to vacuum assembly of Ross Mixer. Turn on vacuum pump. Open Vacuum Valve No. 1, and close Vacuum Valve No. 2 on mixer to achieve a vacuum of 21-23" Hg. Once	Vacuum ____"Hg _____	_____	_____

vacuum has been achieved, close Vacuum Valve No. 1, and open Vacuum Valve No. 2 on the mixer to maintain the vacuum pressure. Turn vacuum pump off. Record Mix Start Time.	Mix Start Time ____			
9. Using the procedure in Step 3, adjust the set temperature of the Haake bath to 54° C. Turn on the cooling unit in the bath with the "Cooling" switch. Record the Active Cool Start Time.	Haake Set Temp. ____°C Active Cool Start Time ____			
10. When the mixer bowl thermometers read 54 ± 2°C, adjust the set temperature of the Haake bath to 46° C. Turn off the "Cooling" switch. Record the Passive Cool Start Time.	Inlet Temp:__ Outlet Temp:__ Haake Set Temp. ____°C Passive Cool Start Time ____			
11. Slowly turn the mix speed to 0, and turn off mixer. Release vacuum in the mixer by opening Vacuum Valve No. 1.	____	____	____	

Open the Ross Mixer. Using a spatula, scrape any unmixed material off the sides of the bowl and paddles. Close the bowl, and slowly turn mix speed to 16 Hz. Reestablish vacuum as per Step 8. Observe the bowl thermometers about every 15 minutes. When the thermometers read 46-48°C, continue mixing for at least one hour.	Mix Speed: ____Hz Vacuum ____"Hg Inlet Temp:__ Outlet Temp:__ Start Time _____ Stop Time _____		
12. At the end of the mix time, slowly reduce mixer speed, and turn the mixer off.			
13. Release vacuum in the mixer by opening Vacuum Valve No. 1.			
14. Open Ross Mixer, and observe the formulation. It should be a bright white viscous solution, with no lumps of undispersed calcium carbonate evident. If further mixing is required, proceed with Step 15. If not, proceed with Step 16.			
15. Close mixer, and mix for 15 minutes at 16 Hz under vacuum applied as per	Vacuum ____"Hg	_____	_____

Step 8.	Start Time _____ Stop Time _____		
16. When proper mixture is achieved, proceed with filling using the Filling Procedure for ATRIX Formulation SMK-226-59-C.			

EVALUATION OF HANDLING PROPERTIES OF PROJECT 2070 FORMULATIONS

GENERAL PROCEDURES

1. Put on latex examination gloves prior to handling formulation

2. Remove foil pouch from outer pouch.

3. Remove formulation from foil pouch.

4. Spend 60-90 seconds kneading the entire sample of formulation prior to evaluating the handling properties. This kneading action should consist of repeated squeezing and rolling of the sample between the thumb and fingers. Formulation may be brittle and uncohesive at first, but continued hand working should yield the desired consistency. Special attention should be paid to working any portions of formulation which may be harder than the rest of the sample into a homogenous mass.

5. Proceed with evaluation when the sample has been formed into a single mass.

APPEARANCE

1. Look at the color of the formulation

2. Observe the texture of the formulation, both visually and by handling. Note if the formulation appears and feels homogeneous, and describe any nonhomogeneous texture.

HARDNESS TEST

1. Pinch the formulation between the thumb and forefinger, using firm and even pressure.

2. Observe the ease of yielding of the formulation, as well as how much the formulation spreads.

STICKINESS TEST

1. Observe the level of tack between the latex gloves and the formulation during hand working. Note any residue which the formulation may leave on the gloves.

COHESIVENESS TEST

1. Roll the formulation into a roughly cylindrical shape approximately 1-2 inches in length.
2. Grab the formulation at each end and slowly pull apart. Note the degree of force needed to pull the formulation apart, as well as the yielding behavior of the formulation- Does it form "strings" when coming apart?

SMEAR TEST

1. Form a portion of the formulation into a roughly spherical shape about 1 inch in diameter.

2. Place this sample on a countertop surface.

3. Using your thumb, press down firmly on the sample and pull the formulation towards you.

4. Observe how much the formulation spreads, and the degree of force needed to spread the formulation.

WET HANDLING TEST

1. If desired, a large portion of the sample may be separated and set aside for other testing before proceeding, and this test may be conducted on a small portion of formulation.

2. Dip fingertips in gently running water, shaking off excess. Gloves should be noticeably wet befor proceding.

3. Knead and work formulation as described in Step 4 of the General Procedures, making an effort to get the formulation moist.

4. Observe any changes in handling properties of the formulation in comparison to its dry properties.

SPECIFICATIONS FOR PROPERTIES OF FORMULATION 59-C

APPEARANCE

The formulation is bright white in color. Small portions of extremely hard material may be evident in the formulation after hand working.

HARDNESS

The formulation yields fairly easily when pinched between the thumb and forefinger. The formulation does not spread significantly when subjected to this test.

STICKINESS

The formulation is slightly sticky to latex examination gloves. A fine residue may be deposited on the gloves after several seconds of hand working.

COHESIVENESS

The formulation breaks rather abruptly when pulled apart, and thus, undergoes almost no elongation.

SMEARABILITY

The formulation requires some effort to smear on the laboratory benchtop.

WET HANDLING PROPERTIES

The formulation will maintain its consistency for several minutes of vigorous hand working after becoming wet.

SUBMITTED BY:

Stephen M.G. Knight
Scientist I

Richard L. Norton Ph.D.
Senior Polymer Scientist

APPROVED BY:

Richard L. Dunn Ph.D.
Vice President of Drug Delivery Research