

Copyright
by
Terry Wayne Hudson
2003

**The Dissertation Committee for Terry Wayne Hudson certifies that this is
the approved version of the following dissertation:**

**ENGINEERING OF AN OPTIMIZED ACELLULAR
PERIPHERAL NERVE GRAFT**

Committee:

Christine Schmidt, Supervisor

Gregory Evans

George Georgiou

Charles Mullins

Wesley Thompson

Engineering of an Optimized Acellular Peripheral Nerve Graft

by

Terry Wayne Hudson, B.S.

Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

May, 2003

Acknowledgements

I want to express my sincere appreciation for all those who have helped me achieve this accomplishment. First and foremost, I must thank Christine for the opportunity, guidance, and mentoring that she provided as my supervisor. I would also like to thank my committee members George Georgiou, Wesley Thompson, Gregory Evans, and Charles Mullin for their invaluable contributions to this work.

I must thank my wife Allison, and thank God for her, because the joy, love, kindness, and patience that she has demonstrated are truly a reflection of Him. I also want to thank my parents for their support and instruction. They always encouraged me to pursue my goals and helped in any way possible. The rest of my family also provided constant encouragement.

Throughout my graduate career many people have provided essential support, and to those individuals I want to extend my deepest gratitude. Those who helped teach me how to perform research: Beth, Joel, Julie, and especially

Tyrell. Fellow researchers who have provided assistance, suggestions, and moments of jocularly: Jennie, Jessica, Aruna, Prachi, Mike, Archit, Curt, Kate, Kiko, Scott, and Natalia. Dedicated laboratory assistants: Steve, Kim, Andy, Laura, Scott, Char, and Kate. UT employees that have been extremely charitable with their time and wisdom: Hyma, “T” Stockman, Kay, and Berto. Professional guidance: Chris and Phil. Finally, the friends who kept me sane and made my years in Austin unforgettable: Paul, Drew, Karl, Travis, Shon, Kade, Seth, Tom, and Tim.

Engineering of an Optimized Acellular Peripheral Nerve Graft

Publication No. _____

Terry Wayne Hudson, Ph.D.

The University of Texas at Austin, 2003

Supervisor: Christine E. Schmidt

The long-term goal of this work is to engineer a nerve graft for therapeutic applications in peripheral nerve repair. Currently, surgeons use an autologous nerve graft when attempting to repair a peripheral nerve injury with a defect longer than a few millimeters. This approach, however, has several significant limitations, including loss of function at the site from which the donor nerve is extracted. This dissertation describes the creation of an acellular nerve graft with a well-preserved extracellular matrix (ECM), a significant step toward the creation of a replacement for the autologous nerve graft. To create the acellular graft, the effects of various detergents on peripheral nerve structure and protein composition were examined. That knowledge was subsequently used to develop a chemical process for deriving an acellular graft with a well-preserved ECM from native nerve tissue. The success of this process was demonstrated through histological and Western analysis of the graft. Subsequently, the graft was

implanted into the sciatic nerve of a rat, and immunological tolerance to the graft was examined. The cellular response to the graft was compared to a positive control that was immunologically tolerated and a negative control that was rejected. The number of macrophages and T-cells present in the graft after 28 days illustrated that the optimized acellular graft was immunologically tolerated. The regenerative capacity of the optimized acellular graft was also examined in vivo. Acellular grafts created with the most common thermal and chemical decellularization methods were also tested. In initial studies, axon density at the midpoint of the optimized graft was 96% higher than in the thermally decellularized model and 42% higher than in the chemically decellularized model. The results imply that a well-preserved ECM and the removal of cellular material are both important for optimizing regeneration through an acellular nerve graft. In addition to serving as a model for studying the role of the ECM in regeneration, cells, growth factors, or both, can be incorporated into the optimized acellular graft. Thus, the graft may act as a natural scaffold upon which a clinical alternative to the autologous graft can be built.

Table of Contents

List of Tables	xi
List of Figures.....	xii
Chapter 1: Introduction.....	1
Chapter 2: Background on Engineering Peripheral Nerve Grafts	5
2.1 Physiology of the Peripheral Nervous System	5
2.2 Nerve Injuries	7
2.3 History of Surgical Approaches	8
2.4 State of the Art.....	9
2.5 Nerve Guidance Channels	10
2.6 Physical Properties of Nerve Guidance Channels	10
2.7 Next Generation Nerve Guidance Channels.....	12
2.8 Allografts and Xenografts	13
2.9 Acellular Nerve Grafts.....	14
2.9.1 Thermal Decellularization	14
2.9.2 Radiation Decellularization	15
2.9.3 Chemical Decellularization	15
2.9.3.1 Detergents	16
2.9.3.2 The Sondell Protocol	16
2.10 Key Factors in Regeneration through Acellular Grafts	17
2.10.1 The Role of Cellular Components	17
2.10.2 The Role of Growth Factors in Regeneration.....	18
2.10.3 The Role of the Extracellular Matrix (ECM) in Regeneration.....	18
2.10.4 The Importance of Removing Cellular Material	19
2.11 Research Goals	20

Chapter 3: Materials and Methods.....	27
3.1 Introduction	27
3.2 Selection of Detergents for Chemical Treatment	27
3.3 Chemical Treatment of Nerve Tissue	28
3.4 Histological Analysis of Nerve Grafts.....	29
3.5 Analysis of Cellular Removal and Structural Preservation in the Grafts	31
3.6 Gel Electrophoresis and Western Analysis	32
3.7 Optimized Graft Decellularization Protocol	34
3.8 Creation of Grafts for In Vivo Experiments	36
3.9 In Vivo Evaluation of Graft Immunogenicity and Regenerative Capacity	37
3.9.1 Immune Response to Grafts Analyzed Histologically	38
3.9.2 Regeneration in Grafts Analyzed Histologically	39
3.10 Statistical Analysis	39
Chapter 4: Results.....	45
4.1 Introduction	45
4.2 General Trends in Decellularization.....	45
4.3 Most Effective Detergents Identified	46
4.4 Optimized Process Preserves the ECM and Removes Cellular Material.....	47
4.5 Gel Electrophoresis and Western Analysis Confirm Cellular Removal.....	48
4.6 Grafts are Immunologically Tolerated	49
4.7 Optimized Grafts Support Regenerating Axons	50
4.8 Regenerative Capacity of Optimized Grafts Surpasses other Acellular Models	51
4.9 Conclusions	51
Chapter 5: Discussion.....	70
5.1 Introduction	70

5.2 Impact of Detergents on Nerve Tissue	70
5.3 Selection of Detergents for Decellularization Treatment	71
5.4 Optimized Protocol Achieves Goals of Cellular Removal and ECM Preservation	72
5.5 Immunological Tolerance of Optimized Grafts Was Confirmed	73
5.6 Optimized Acellular Grafts Support Regeneration	75
5.7 Regenerative Capacity Correlated to Graft Structure and Content	75
5.8 Conclusions	76
Chapter 6: Conclusions and Recommendations	78
Appendices	81
Appendix A: Hematoxylin and Eosin Staining of Tissue Sections	81
Appendix B: Immunostaining of Tissue Sections	83
Appendix C: Gel Electrophoresis and Western Analysis Protocol	86
Appendix D: Detergent Effectiveness Based on Histological Analysis of Post-Treated Nerve Tissue.....	93
Glossary	94
References	96
Vita	111

List of Tables

Table 2.1:	Nerve Conduit Materials	21
Table 2.2:	Cells and Growth Factors Used in NGCs.....	22
Table 2.3:	Nerve Conduit Internal Matrices	22
Table 3.1:	Detergents and Concentrations Used in Initial Experiment	41
Table 3.2:	Creation of Nerve Grafts	42
Table 3.3:	Implants to Examine Immunological Tolerance of Optimized Grafts	42
Table 4.1:	Most Effective Detergent Treatments, Based on Histological Analysis of Treated Nerve Tissue	53
Table 4.2:	Cellular Removal by Anionic Detergent Treatments with Highest Total Effectiveness Scores, Based on Western Analysis of Myelin Basic Protein Concentration.....	54
Table 4.3:	Implants to Examine Immunological Tolerance of Optimized Graft.....	54

List of Figures

Figure 2.1: Schematic of the nervous system.....	23
Figure 2.2: Illustration and transmission electron microscopy (TEM) image of a cross section of a typical peripheral nerve	24
Figure 2.3: Schematic of myelinated axons and the surrounding ECM	25
Figure 2.4: Schematic of cell-mediated immune rejection of an allograft.....	26
Figure 3.1: Preparation of grafts used for in vivo experiments.....	43
Figure 3.2: Illustration of trimming of the graft prior to implantation.....	44
Figure 4.1: Effectiveness of detergents at structural preservation, based on histological evaluation	55
Figure 4.2: Effectiveness of detergents at cellular removal, based on histological evaluation	56
Figure 4.3: Removal of MBP by each category of detergent, based on a Western analysis	57
Figure 4.4: Chemical structures of the detergents selected for the optimized decellularization protocol	58
Figure 4.5: Harris Hematoxylin and Eosin stained cross-sections.....	59
Figure 4.6: Preservation of basal laminae visualized by the staining of laminin protein.....	60
Figure 4.7: Removal of Schwann cells visualized by staining of S-100 protein.....	61
Figure 4.8: Removal of myelin and cell membranes evaluated by staining of lipids	61

Figure 4.9: Proteins from homogenized tissue samples visualized with a Colloidal Blue stain	62
Figure 4.10: MBP concentration in tissue samples before and after decellularization, based on Western analysis	63
Figure 4.11: Cell-mediated immune response in grafts 28 days after implantation visualized by staining CD8a+ cells	64
Figure 4.12: Level of cell-mediated immune response, based on percentage of tissue covered by CD8a+ cells.....	65
Figure 4.13: Grafts stained for macrophages 28 days after implantation	66
Figure 4.14: The level of macrophages in grafts, based on percentage of tissue covered with staining.....	67
Figure 4.15: Axonal regeneration through acellular nerve grafts demonstrated by staining longitudinal tissue segments for neurofilaments.....	68
Figure 4.16: The regenerative capacity of four nerve graft models evaluated by measuring axon density 28 days after implantation	69

Chapter 1: Introduction

Peripheral nerve injuries can result from thermal, chemical, mechanical or pathological damage. In such injuries, the nerve may be pinched, crushed, partially cut, or even completely severed. Of these, a severed nerve is by far the most serious because it has the least chance of repairing itself without surgical intervention, resulting in permanent loss of sensory or motor function. The work detailed here specifically addresses severed nerves and their repair.

Ideally, it is desirable to have a readily available, off-the-shelf graft that can be used clinically to treat severed nerves. Currently, surgeons use an autologous nerve graft (i.e., autograft) to bridge the gap between severed nerve ends and to guide the regenerating axons back toward the site they previously innervated. Unfortunately, this approach requires the removal of a functional nerve from another site in the patient, resulting in donor site morbidity, a longer surgery, and the increased potential for complications or infection. Despite decades of research, no alternative nerve graft has proven as effective as the autograft. We believe that the lack of success in this endeavor is in part the result of an incomplete understanding of the critical factors needed to create a superior nerve graft.

Thus, we engineered a simplified acellular nerve graft that can be used to study the critical factors in nerve regeneration. This graft was created from native

nerve with a chemical treatment specifically designed to remove cellular material (i.e., decellularize) while preserving the extracellular structure of the tissue. By removing cellular material, the graft could be used to treat a severed nerve without inducing cell-mediated rejection. In addition, the graft can serve as a model system for studying the regenerative process and the role of the extracellular matrix (ECM) in regeneration.

This dissertation is organized into six chapters. **Chapter 2** provides an overview of peripheral nerve repair and past research aimed at developing an alternative to the current clinical treatment. A description of this clinical procedure (i.e., the autograft) is given, as well as a discussion of its benefits and limitations. The state of the art in nerve guidance channels and acellular grafts is addressed. The roles of cellular and extracellular components in regeneration are discussed, with specific attention given to their importance in acellular nerve grafts. Finally, the fundamental goal of this work is presented: the creation of an optimized acellular nerve graft.

Chapter 3 details the methods used in the development of the optimized decellularization protocol, analysis of the acellular grafts, implantation of the grafts into animals, and the evaluation of the grafts after 28 days in vivo. Nerve tissue was treated with various detergents, and the impact of the detergents on cellular material and tissue structure was examined. An optimized chemical decellularization protocol was derived from that work, and the details of that process are explained. Optimized acellular grafts created with the new decellularization protocol and acellular grafts created with a published chemical decellularization protocol (i.e., the Sondell protocol) were both examined with histological and Western analysis. Finally, fresh and acellular grafts were

implanted into the sciatic nerve of rats to test the immunogenicity and regenerative capacity. Details are provided for the implant procedure and subsequent analysis of the grafts.

Chapter 4 provides results from the experiments described in Chapter 3. The detergents most capable of removing cellular material from tissue while still preserving the extracellular structural were identified. These detergents included Triton X-200, SB-10, and SB-16, the chemicals selected for the optimized decellularization protocol. Both the optimized acellular grafts and the Sondell grafts contained low levels of cellular material, but the natural extracellular structure in the optimized acellular grafts was more conserved than in the Sondell grafts. In vivo experiments demonstrated that after 28 days, the optimized acellular grafts had not elicited cell-mediated immunological rejection. Additional in vivo experiments provided evidence that the regenerative capacity of the optimized acellular grafts was higher than the regenerative capacity of two published acellular graft models.

Chapter 5 contains a thorough discussion of the experimental results. A discussion of the rationale for the selection of detergents in the optimized decellularization protocol is also found in this chapter. Based on evaluations of the grafts before and after implantation into animals, the results suggest that by removing cellular material from nerve tissue, an immunologically benign graft was created (i.e., the graft did not elicit cell-mediated immune rejection). Removal of cellular debris also appeared to improve the regenerative capacity of the acellular nerve grafts. A correlation between preservation of the natural extracellular structure and regenerative capacity was also implicated.

Finally, **Chapter 6** summarizes the work presented in this dissertation and recommends future studies using the optimized acellular graft. We believe this graft can serve as a simple, natural platform with which to study the role of other variables (e.g., angiogenesis) in nerve regeneration.

Chapter 2: Background on Engineering Peripheral Nerve Grafts

2.1 PHYSIOLOGY OF THE PERIPHERAL NERVOUS SYSTEM

According to the National Center for Health Statistics, in 1995 over 50,000 peripheral nerve repair surgeries were performed in the U.S. [1]. The actual number of injuries far exceeds this figure because most crush injuries do not require surgery. In addition, limitations associated with the current clinical treatment, the autologous nerve graft, cause some of the most severe cases to go untreated because the probability of repair is too remote to warrant the procedure [2]. Even when surgery is used to treat nerve damage, patients rarely regain complete functional control, and without surgical intervention the odds of regaining function are negligible [3]. Thus, an improvement in the treatment for peripheral nerve injuries is actively being sought.

When the term “nerve” is used, one is usually referring to a bundle of nerve cell extensions rather than a single nerve cell. A peripheral nerve cable is actually composed of hundreds or thousands of extensions from individual nerve cells, called axons. Each peripheral nerve axon innervates a specific site in the body, providing signals to a muscle, transporting sensory information back to the central nervous system (CNS), where the cell body is located, or both. The peripheral nervous system (PNS) is composed of all nerves outside the CNS (**Figure 2.1**). Besides location, other physiological differences exist between the PNS and CNS. For the purposes of this work, the most important difference is that nerves can regenerate through PNS tissue, but will not regenerate through native CNS tissue (e.g., the spinal cord) using currently available surgical techniques [4].

The two most common cell types found in a nerve cable are nerve cells and Schwann cells. A nerve cell carries electrical signals between its cell body, located at or near the spinal cord, and the site innervated by the cell's axon. Schwann cells wrap around axons, forming an insulating layer called a myelin sheath (**Figure 2.2**). Myelination allows electrical signals to travel rapidly along the length of the axon. In addition to improving electrical conduction, Schwann cells also provide neurotrophic factors to axons during stages of initial development and regeneration. Other cell types can also be found in nerve tissue, including resident macrophages and endothelial cells. Macrophages are phagocytic cells of the immune system; they recognize, ingest and destroy foreign antigens and cell debris. Endothelial cells are a component of the blood vessels; therefore, they play a role in providing nutrients to nerve tissue.

Nerve cables also possess an intricate extracellular structure. Myelinated axons are located within tubes of structural proteins called basal laminae. Laminin, collagen IV, fibronectin, and other proteins comprise the basal laminae, with laminin being primarily found on the internal surface [5, 6](**Figure 2.3**). An interstitial endoneurium provides structural support between the basal laminae. The endoneurium is composed of proteins including collagen I and collagen III [7]. The basal laminae and interstitial endoneurium are contained within a layer called the perineurium. This complex is called a fascicle, and numerous fascicles may be found within a single nerve cable. As the nerve cable extends from the spinal cord (proximal end) to the innervation site (distal end), the cable branches as nerves lead to different endpoints. It is important to understand this structure when considering how to develop nerve regeneration treatments.

2.2 NERVE INJURIES

If a peripheral nerve is crushed or compressed, the individual may lose sensory or motor function along the entire path of the nerve cable distal to the injury. For example, a pinched nerve in the neck can result in numbness to the entire arm. In such cases, the axons within the nerve may only be temporarily traumatized, halting the transmission of signals. In most cases, function is soon restored as the axons return to normal operation. However, in more severe cases, the axons may be so severely damaged that the axons die from the site of injury all the way to the point of innervation. In these cases, function is lost until the axons can re-grow along their previous path and re-innervate their target site [8].

The process of an axon dying and the cascade of events that follow are called Wallerian degeneration [9, 10]. The axon seals off its cell membrane at the site of the injury, completely disconnecting the distal end of the axon from the rest of the cell and the cell body. Without contact with the cell body and the nutrients it supplies, the distal end of the axon degrades. As this happens, the Schwann cells that were wrapped around the axon unravel (demyelinate) and shed a portion of their lipid composition. New Schwann cells infiltrate, joining the Schwann cells that have demyelinated, and they begin to engulf axon and lipid debris. The Schwann cells also produce neurotrophic factors that provide nutrients and neurotropic factors that provide guidance cues to regenerating axons. Macrophage cells are recruited to help in the debris clearance, and they also produce neurotrophic factors. The living portions of the axons sprout growth cones and begin to extend. If the extensions find a desirable substrate, growth factors, or both, the axons extend in that direction. In this manner, axons will grow back down the basal laminae in which they previously resided. As the axons extend, Schwann cells will again form myelin sheaths around the axons. The

axons grow down the distal nerve cable toward the site they previously innervated at a rate of 1-4 mm per day [11].

In cases where a nerve is completely severed, the probability of repair is far lower, especially without surgical intervention. This is because the axons may never find their way to the distal end of the severed nerve cable. Without finding the distal end, the axons will not have the desirable substrate or neurotrophic and neurotropic factors to guide them. Surgeons are able to repair many severed nerves with a process known as primary neurorrhaphy, (i.e., suturing the ends of the severed nerve back together). However, in many cases, the surgeons are unable to perform this operation because a segment of the nerve is destroyed and primary neurorrhaphy would place the nerve under tension. Nerves do not regenerate well under tension [12]; therefore, in situations where a nerve defect is present, a physical support (e.g., graft or conduit) must be used to bridge the gap between the nerve ends and to guide the regenerating axons.

2.3 HISTORY OF SURGICAL APPROACHES

As early as 1608 surgeons attempted to reconnect severed nerve ends. In addition to direct reconnection of nerve ends, surgeons also employed the use of Cargile gauze [13], bone [14], metal tubes [15], and fat sheaths [16] in their attempts to repair nerve defects. Unfortunately, such surgeries resulted in poor functional recovery [17, 18], and most severed nerves resulted in amputation of the limb because function could never be restored. During World War II, surgeons made a concerted attempt to repair nerves. While their success was limited, the sheer number of surgeries performed and the medical data and observations gathered by the surgeons thrust the field of peripheral nerve repair forward [19].

In the 1960s, Dr. Millesi made tremendous advancements in the area of nerve repair by championing microsurgical techniques. He also established that regenerating nerves must not be placed under tension. Thus, Dr. Millesi stressed the importance of using an autologous nerve graft (i.e., autograft) when the defect in a severed nerve was too large for primary neurorrhaphy [20].

2.4 STATE OF THE ART

One of the most significant hurdles remaining in the field of peripheral nerve repair is treating cases of severed nerves in which there is a large gap between the proximal and distal nerve ends. Previous obstacles have been overcome with technological advancements. For example, microsurgery has improved regeneration because it enables surgeons to handle the nerve with more caution, isolate sutures to the connective tissue surrounding the fascicles, and visually align the fascicles. Other technological advancements include smaller needles and biodegradable sutures that allow surgeons to minimize damage caused by the surgical process. Finally, new techniques that use electrical impulses are being developed to identify and align fascicles [19]. While these advancements have significantly improved functional recovery after primary neurorrhaphy, the problems associated with treating larger defects (i.e., nerve gaps of more than 2 cm) still exist [21].

In clinical cases where a significant defect exists in a peripheral nerve, the gap between the proximal and distal nerve ends is typically bridged with an autograft. The graft is removed from another site in the patient that is deemed to be of lesser importance, e.g. sural nerve in the leg [19]. Attempts are made to use a donor nerve with a diameter similar to that of the injured nerve, but there are a limited number of donor sites in a patient from which a surgeon is willing to

harvest because the patient will permanently lose function at the donor site. In addition, the requirement of two surgical procedures associated with an autograft involves longer time under anesthesia and additional opportunity for surgical complications or infection for the patient. Because of these limitations, it is important to find a replacement for the autograft.

2.5 NERVE GUIDANCE CHANNELS

Potential alternatives to the autograft include nerve guidance channels, allografts, and xenografts. The nerve guidance channel (NGC) appears to be the most commonly researched alternative. Made of synthetic materials, natural materials, or both, NGCs are used to bridge the gap between severed nerve ends while avoiding tension on the nerve. The NGC physically directs regenerating axons from the proximal nerve end to the distal end, inhibits the invasion of fibroblasts and the creation of fibrous tissue, and provides a contained environment for neurotrophic and neurotropic factors, endogenous proteins, and support cells that are secreted or migrate from the nerve ends. Many NGCs are designed with a specific porosity to selectively permit molecules below a desired size to diffuse between the lumen of the channel and the surrounding environment, while preventing diffusion of larger molecules and cells [22].

2.6 PHYSICAL PROPERTIES OF NERVE GUIDANCE CHANNELS

Certain physical properties of NGCs are generally accepted as desirable. The most desirable properties of NGCs include: (1) ease of manipulation, (2) resistance to permanent deformation, tearing, or kinking, (3) sterilizability, and (4) consistency in manufacturing. Although originally used for many NGC experiments, non-degradable synthetic materials are less desirable than

biodegradable or resorbable materials because they increase the chance of future complications and compression of the regenerated nerve [23]. For biodegradable conduits, the degradation time varies from one design to another, based on wall thickness and composition. A sample of the various materials used to create nerve conduits to date can be found in **Table 2.1**. Benefits of synthetic biodegradable materials include consistency in manufacturing, tight control over design specifications, and ease of sterilization during or after fabrication. Thus, a well-designed synthetic conduit can meet the four previously mentioned criteria. There are, however, limitations associated with synthetic conduits, including an inherent lack of bioactivity and the potential for an undesirable reaction to the conduit material or its degradation products.

Natural materials can also be used to fabricate an NGC, and they also have both benefits and limitations. A significant benefit to natural materials is that they are often resorbed by the body. Thus, potential complications like nerve compression can be avoided. Such materials include vein tissue, laminin, collagen, and fibronectin (Table 2.1). With naturally derived materials, there is little concern about the toxicity of the conduit material or its degradation products. Additionally, these materials often possess an inherent bioactivity that stimulates regeneration. However, natural materials are typically more difficult to fabricate consistently, especially without denaturing the proteins that provide the bioactivity. Because the materials are derived from cadavers or animals, there is intrinsic variation from one conduit to the next. Also, there are concerns about immunological rejection and the passing of disease from donor to patient.

Currently, there exist a few NGCs fabricated from synthetic and natural materials that are FDA approved and commercially available. Salumedica

produces a conduit made with a synthetic hydrogel. The conduit is not biodegradable, but its elastic properties help avoid compression of the regenerated nerve. Integra Life Sciences produces an absorbable collagen conduit. Possibly the most significant limitation associated with these NGCs is that they do not possess the numerous stimulatory factors present in an autograft; the NGCs have the advantage of not requiring the loss of function at a donor site. Thus, they provide an excellent clinical alternative for repairing short defect nerve injuries (e.g., less than 2 cm). However, until additional bioactivity is incorporated into an NGC, the autograft will continue to be the clinical choice for longer nerve defects [24]. For a more thorough discussion of the clinical trials with nerve conduits, see the 2002 review by Meek et al. [25].

2.7 NEXT GENERATION NERVE GUIDANCE CHANNELS

To improve regeneration rates in NGCs, researchers are fabricating more advanced channels. The advancements are typically associated with one of three facets of the NGC: (1) the nerve conduit material, (2) the incorporation of cells or growth factors, or (3) an internal matrix. Researchers are attempting to shift from an inert physical guide to a bioactive material (e.g., biologically or electrically stimulatory). Because regeneration through an autograft is more rapid and complete than in an inert NGC, it can be concluded that the biological composition and/or the internal structure of the autograft provides additional stimulation to the regenerating axons. Thus, many researchers are focusing on the contents of the NGC lumen rather than the composition of the channel wall.

By incorporating growth factors, cells, or some combination of these into the channel, researchers are attempting to mimic the natural environment of nerve tissue and coax the regenerating nerves into moving through the channel more

rapidly. Cells and growth factors have been suspended in solutions and added to the NGC at the time of implantation [26-30]. Methods have even been developed to periodically inject cells and growth factors in case they lose their activity or leak out of the lumen [31]. In addition, matrices have been used to trap cells and growth factors. A partial list of the cells and growth factors that have been used with NGCs can be found in **Table 2.2**.

Internal matrices are sometimes used to trap cells or growth factors within the NGCs [32], and they are also used to provide regenerating axons with a structural environment similar to that of native nerve [33]. Thus, natural ECM proteins are commonly used to stimulate axons. In some cases, only the peptide sequences believed to be responsible for stimulation are used. Examples of the matrices that have been researched can be found in **Table 2.3**. As knowledge is gained about the regenerative process and material processing techniques improve, researchers will continue to develop better NGCs.

2.8 ALLOGRAFTS AND XENOGRAFTS

While researchers attempt to create NGCs with similarities to native nerve tissue, other researchers are developing natural tissue grafts as a replacement for the autograft. These grafts may come from a donor of the same species (i.e., allografts) [34] or another species (i.e., xenografts) [35]. By using actual tissue, researchers can provide regenerating nerves with an environment that more closely mimics native nerve. In addition, cadaver tissue can be used instead of tissue from a living donor.

Like transplanted organs, the primary concern when using allografts and xenografts is immunological rejection. For this reason, allografts and xenografts

are not currently used in the clinic. Transplant rejection is the result of antigens present in the graft that elicit a cell-mediated immune response. These histocompatibility antigens are commonly expressed on the cellular components of nerve tissue (e.g., Schwann cells, endothelial cells and resident macrophages)[36-38]. T-helper cells are activated upon binding to the histocompatibility antigens, and they subsequently recruit CD4⁺ and CD8⁺ cytotoxic T-cells (**Figure 2.4**). Cytotoxic T-cells can eventually destroy the graft. Immunosuppressive drugs like cyclosporine A can be administered to the patient to reduce the potential for rejection [39], but the drugs make the patient more susceptible to disease and may also negatively impact regeneration [40].

2.9 ACELLULAR NERVE GRAFTS

To overcome the immunological limitations associated with fresh nerve grafts, researchers have turned to acellular nerve grafts. The guiding principle behind such acellular grafts is that the immunogenic response associated with allografts is sufficiently reduced through the removal of cells [37]. Once implanted, the acellular graft serves as a natural scaffold into which cells from the surrounding tissue readily migrate, forming the foundation for new tissue.

2.9.1 Thermal Decellularization

Theoretically, an acellular graft should be non-immunogenic and possess the extracellular environment of native nerve tissue. A number of different techniques, including thermal [41], radiation [42], and chemical [43] treatments have been used to create acellular nerve grafts. The most common method for creating acellular nerve grafts is thermal decellularization, a process in which the tissue is subjected to repeated freeze-thaw cycles. Although thermal

decellularization does kill the cells and render the graft generally non-immunogenic [44, 45], the repeated shrinking and swelling associated with freezing and thawing of the tissue can fracture the basal lamina tubes. Also, this process was not designed to extract the remnants of the destroyed cells. Schwann cells and macrophages must then invade the basal lamina tubes to clear the cellular debris during the first days following implantation. This cellular invasion is believed to delay the regenerative process and further damage the basal laminae [46-48].

2.9.2 Radiation Decellularization

Radiation has also been used to decellularize nerve tissue, and studies show that radiation does not damage the tissue morphology [49]. Like thermal decellularization, however, radiation alone also fails to extract cellular debris from inside the basal lamina tubes. Thus, the same cellular invasion occurs after implantation, potentially damaging the basal laminae. Of the three treatment methods, radiation is the least common in the literature.

2.9.3 Chemical Decellularization

Several chemical treatments have been designed to render nerve grafts non-immunogenic while also removing much of the cellular debris. Although successful at removing cellular material from the tissue, the chemical treatments reported to date do not yield a well-preserved ECM structure, as compared to native or even thermally decellularized nerve tissue [34, 43, 50]. The predominant chemical decellularization treatments used in the literature entail washing the nerve tissue with detergents. By the identity of the detergents used (e.g., sodium deoxycholate and Triton X-100), it can be assumed that researchers selected the

detergents based on their previous use in the isolation of membrane-bound proteins, but probably not on their ability to preserve the ECM of nerve tissue.

2.9.3.1 Detergents

Detergents are ideal chemicals for removing membrane-bound antigens from peripheral nerve tissue because cellular membranes and myelin are primarily composed of lipids. Detergents and lipids are similar in structure; both have hydrophilic head groups and hydrophobic tails. Thus, detergents can penetrate and break apart lipid membranes, entrap the lipids in detergent micelles, and remove them from the nerve tissue. However, detergents also interact with other proteins and can therefore damage the extracellular matrix of the tissue. The charge carried by the hydrophilic head group of a detergent partially determines how strongly that detergent interacts with lipids and proteins. Detergents are divided into four categories based on the charge of their head group. Anionic detergents carry a negative charge, and cationic detergents have a positive charge. Non-ionic detergents have no charge associated with their hydrophilic head. Amphoteric (i.e., zwitterionic) detergents have regions of positive and negative charge within their hydrophilic head, but under a neutral pH, the charges usually cancel one another. Detergent selection plays a crucial role in determining how well a chemical decellularization procedure removes cellular material and how it affects the structural proteins in the tissue.

2.9.3.2 The Sondell Protocol

In a study to assess the composition of nerve ECM, Johnson et al. developed a method for chemically decellularizing nerve tissue using sodium deoxycholate, Triton X-100, and distilled water [51]. The protocol was later

modified to create an implantable acellular nerve graft [34]. In 1998, Sondell et al. developed the most recent version of this graft [43]. By removing cellular material from nerve tissue, they demonstrated that chemically-derived acellular grafts permit regeneration without immunological rejection. The regeneration rate in the acellular grafts was reported to be 1.2 mm/day after an initial delay of 4.9 days. However, they did not report the regeneration rate in the autografts, despite using the autograft as a control. To date, no decellularized nerve graft has demonstrated a regenerative capacity equivalent to that of the autograft [52].

2.10 KEY FACTORS IN REGENERATION THROUGH ACELLULAR GRAFTS

Several factors are potentially responsible for the inability of acellular grafts to match the regenerative potential of the autograft. The process of chemical decellularization affects cellular components [53, 54], growth factors [55], and the ECM [56, 57]. Since each of these factors plays a vital role during peripheral nerve regeneration, removing or damaging them through the decellularization process likely reduces the ability of a graft to support regeneration.

2.10.1 The Role of Cellular Components

Schwann cells play an active role in stimulating and guiding regenerating axons in nerve grafts. Immediately after implantation, both cell types invade the graft and begin clearing myelin and axonal debris in preparation for regeneration [9]. Schwann cells provide a supportive environment for regenerating axons and produce ECM, cell adhesion molecules, integrins and neurotrophins [58-61]. Schwann cells are also critically involved in guiding axons to form synapses at the neuromuscular junction [62].

Macrophages also actively participate in nerve regeneration, but their role has not been as extensively studied. It is known that in addition to clearing debris, macrophages produce neurotrophins that stimulate regeneration, especially in the presence of Schwann cells [63, 64].

2.10.2 The Role of Growth Factors in Regeneration

Examples of neurotrophins released during the regenerative process include nerve growth factor (NGF) and vascular endothelial growth factor (VEGF). Their ability to stimulate nerve regeneration has been established through numerous in vitro and in vivo experiments [65-68]. Other growth factors have also been documented, and many others may still be identified through future research. For more information about growth factors that are known to play a role in peripheral nerve regeneration, see reviews by Schmidt et al. [69] and Lundborg [70].

2.10.3 The Role of the Extracellular Matrix (ECM) in Regeneration

The ECM of native peripheral nerve provides a favorable substrate through which regenerating axons can grow. Of particular significance are the basal laminae (i.e., basement membranes) that guide regenerating nerves back to their previous innervation site, providing a roadmap for regenerating axons [71]. Basal laminae are composed of fibronectin, type IV and type V collagen, and laminin [72]. Laminin is located on the interior surface of the tubes. It is along the laminin substrate that axons preferentially adhere and migrate [47, 73, 74], potentially guided by calcium signaling [75]. In vivo work has demonstrated that the topography of a laminin-coated surface influences the rate of nerve extension,

and a smooth, uniform surface is best for maximizing regeneration [76]. The biochemical integrity of the laminin is also important, and denaturing the basal lamina proteins significantly reduces the rate of regeneration [77].

While cellular components, growth factors and the ECM are all known to be important, the specific impact of each factor on regeneration is still unclear, especially in a complex environment with numerous variables. For example, there is debate over how well nerves can regenerate in the absence of Schwann cells if the nerves are provided with an otherwise favorable environment [78]. Based on a thorough review of the literature, it was determined that inadequate preservation of the ECM structure in the decellularized grafts represents a significant but understudied factor in the regenerative process. It was hypothesized that damage to the ECM during decellularization is a contributing factor to the failure of previous acellular grafts to adequately support regeneration.

2.10.4 The Importance of Removing Cellular Material

Altering the process of decellularization to better preserve the nerve ECM would constitute an important step in the development of an optimal acellular graft, but the process also needs to remove the cellular remnants from the graft. The importance of removing cellular material was demonstrated by research with predegenerated nerve grafts. To predegenerate a nerve graft, an initial surgery is performed on the donor animal in which the donor nerve is severed, but not removed from the animal. After a period of days or weeks of Wallerian degeneration, the donor nerve is removed and used as a nerve graft. Compared to fresh grafts, predegenerated grafts demonstrated an improved regenerative capacity during the first few days after implantation. The macrophages and Schwann cells that migrate into the tissue during predegeneration contributed to

this improvement because they supported regeneration and released cytokines. However, axons extended into decellularized predegenerated grafts more quickly than they extended into acellular allografts that were not predegenerated [46]. This suggested that the removal of myelin and cellular debris by invading cells might have improved the ability of the graft to support regeneration immediately after implantation. Thus, in these studies we developed an optimized nerve decellularization procedure that preserved the ECM and reduced the amount of cellular material in the graft.

2.11 RESEARCH GOALS

It was proposed that a new chemical decellularization treatment could be developed that would preserve the native ECM structure of nerve tissue while also removing the cellular components from nerve tissue. Cell membranes and myelin are composed primarily of lipids, so detergents were identified as chemicals that could selectively remove cellular material. By studying the impact of a variety of detergents on nerve tissue, an optimized chemical decellularization process could be created. With such a process, an acellular graft could be produced that would be immunologically tolerated and would guide regenerating axons across the graft and into the distal nerve cable. The optimized acellular graft would also serve as a model system for studying the regenerative process because of its simplicity, natural structure, and biological composition.

Table 2.1: Nerve Conduit Materials

Material	Reference
<i>Nonbiodegradable synthetic materials</i>	
Silicone	[79, 80]
Gore-Tex or ePTFE	[81-84]
<i>Biodegradable synthetic materials</i>	
Poly(lactic acid) (PLA)	[85, 86]
Poly(lactic-co-glycolic acid) (PLGA)	[87]
Poly(urethane)	[88-90]
Poly(organo)phosphazenes	[91]
Poly(3-hydroxybutyrate)	[92]
Biodegradable glass	[93, 94]
<i>Electrically active materials</i>	
Piezoelectric	[95]
Electrically conducting	[96]
<i>Natural Materials</i>	
Vein	[97]
Amnionic membrane	[98]
Collagen, laminin and fibronectin	[99],[100], [101]
Hyaluronic acid	[102, 103]

Table 2.2: Cells and Growth Factors Used in NGCs

Bioactive Agent	Reference
<i>Cells</i>	
Schwann cells	[26, 27]
Olfactory bulb ensheathing cells	[28]
<i>Growth factors</i>	
Nerve growth factor (NGF)	[30, 31]
Vascular endothelial growth factor (VEGF)	[65]
Basic fibroblastic growth factor (bFGF)	[29]
Glial growth factor	[104]

Table 2.3: Nerve Conduit Internal Matrices

Matrix Material	Reference
<i>ECM based materials</i>	
Fibronectin	[105]
Laminin	[106]
Collagen	[107]
Matrigel	[32]
Hyaluronic acid	[108]
<i>Peptides</i>	
Heparin-binding peptide	[109]
Laminin oligopeptide	[110]
<i>Other</i>	
Synthetic filaments	[33, 111]
Alginate gels	[112]
Agarose	[113]

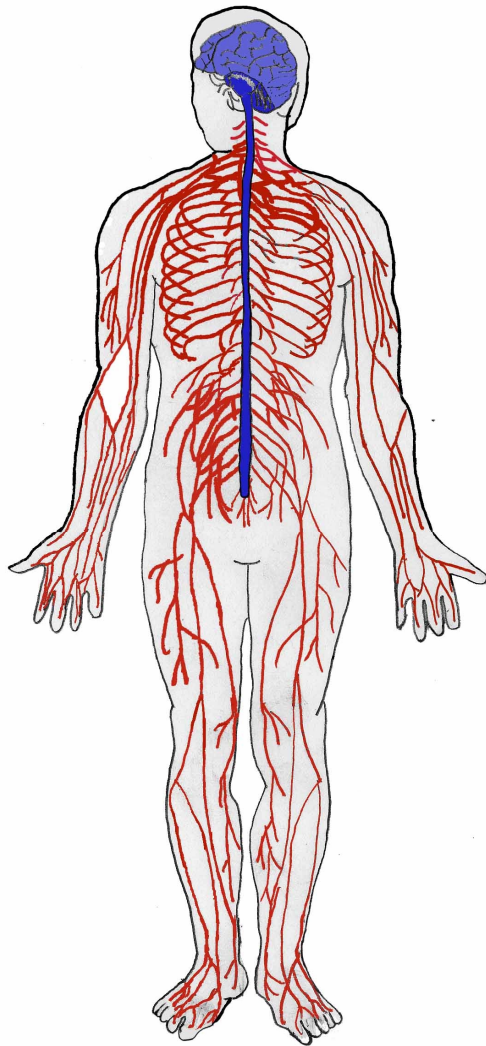


Figure 2.1: Schematic of the nervous system. The brain and spinal cord are the primary components of the central nervous system. The central nervous system (blue) is composed of the brain and spinal cord. The peripheral nervous system (red) is composed of the nerves outside the central nervous system. Cell bodies of peripheral nerves are located within or near the spinal cord, and axons extend from the cell bodies to their site of innervation.

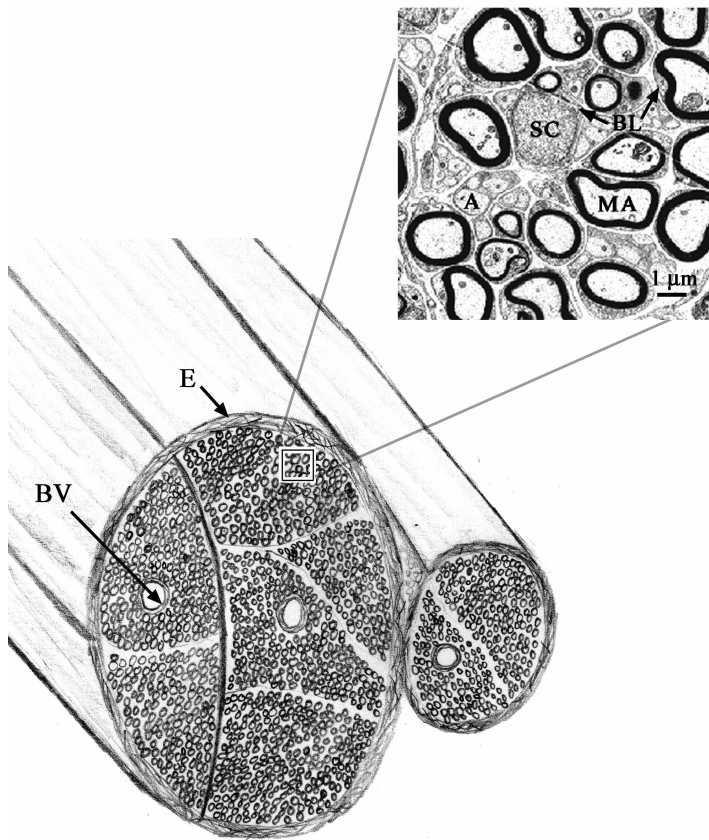


Figure 2.2: Illustration and transmission electron microscopy (TEM) image of a cross section of a typical peripheral nerve. The TEM image is of resin embedded rat sciatic nerve, stained with lead citrate and uranyl acetate. Large peripheral nerve cables originate at or near the spinal cord where the peripheral nerve cell bodies are located. Smaller cables branch off the larger cable and lead to different regions of the body. In this manner, the entire peripheral nervous system is connected to the spinal cord. Axons (A) (i.e., nerve cell extensions), support cells (e.g., Schwann cells (SC)), blood vessels (BV), and an extracellular matrix are held within the cable by a sheath called the epineurium (E). Schwann cells wrap around individual axons to form myelinated axons (MA). The extracellular matrix includes the epineurium, basal laminae (BL), and the interstitial endoneurium. Basal laminae surround myelinated axons, and the interstitial endoneurium provides structural support between the basal laminae.

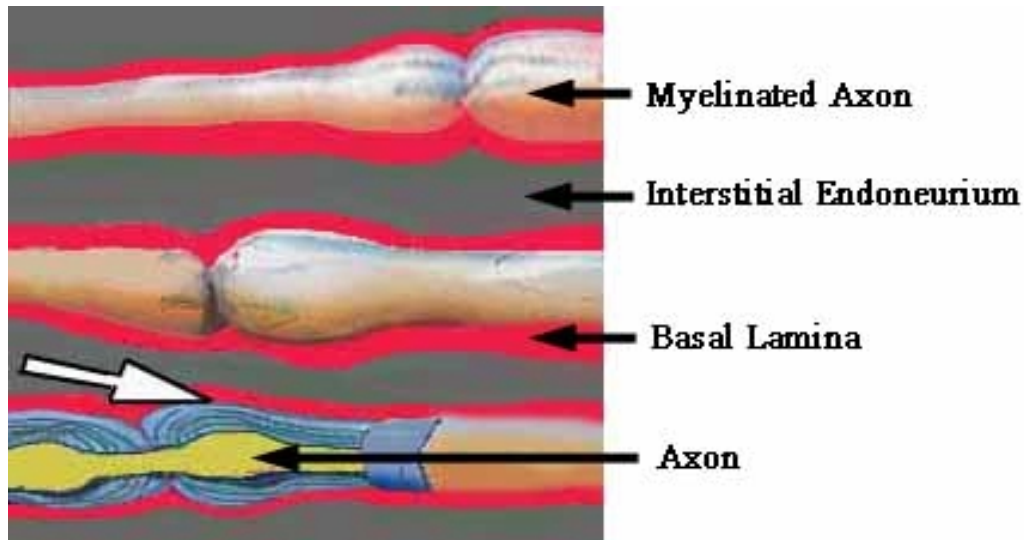


Figure 2.3: Schematic of myelinated axons and the surrounding ECM. Myelinated axons in the peripheral nervous system are found within basal lamina tubes. A cut away of a basal lamina tube shows an axon (yellow) and Schwann cells (blue) wrapped around the axon forming myelin. The basal laminae (red) surround the myelinated axons and are composed of extracellular matrix proteins. Laminin protein is found on the inner surface (white arrow), at the interface with the myelin. The interstitial endoneurium (gray) provides structural support between the basal laminae and also contains extracellular matrix proteins, including collagen I.

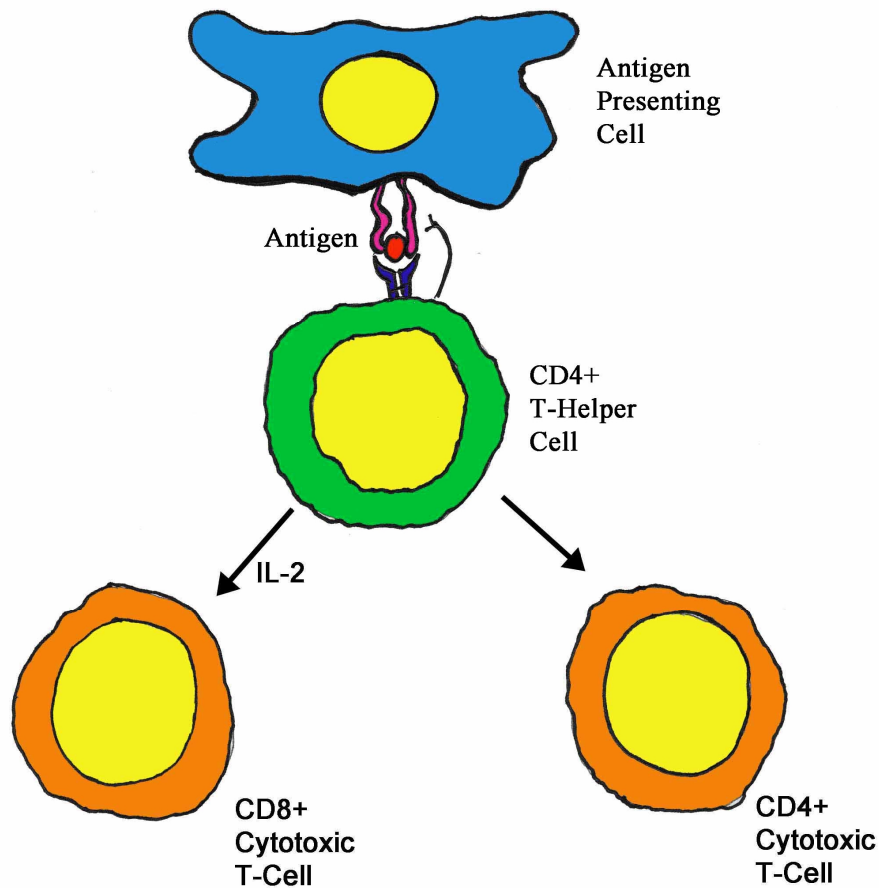


Figure 2.4: Schematic of cell-mediated immune rejection of an allograft. CD4+ T-helper cells bind to transplanted antigens and become activated. The activated T-helper cells proliferate and release interleukin-2 (IL-2), which promotes proliferation of CD8+ cytotoxic T-cells. The activated T-cells also promote proliferation of CD4+ cytotoxic T-cells. Cytotoxic T-cells then attack and kill the transplanted tissue.

Chapter 3: Materials and Methods

3.1 INTRODUCTION

To create an optimized acellular nerve graft, a new chemical process needed to be developed for decellularizing nerve tissue. The first step in developing that process was to study the effect of detergents on peripheral nerve tissue. Cellular removal and structural preservation was evaluated in the tissue segments treated with the detergents. Based on knowledge gained from those evaluations, three detergents were selected for further experiments. With those three detergents, an optimized chemical decellularization procedure was developed. Subsequently, grafts decellularized with the optimal decellularization treatment were compared to grafts decellularized with the protocol developed by Sondell et al. (i.e., the most common chemical decellularization approach reported in the literature). The optimized acellular grafts were also tested in vivo to determine their immunogenicity and regenerative capacity. The levels of CD8+ cells and macrophages in the grafts were examined to determine whether or not the grafts elicited cell-mediated immune rejection or a severe inflammatory response. The density of axons at the midpoint of the grafts was used to evaluate the regenerative capacity of the grafts.

3.2 SELECTION OF DETERGENTS FOR CHEMICAL TREATMENT

To understand the impact of detergents on peripheral nerve, a sampling of detergents from each of the four major detergent categories, based on the charge of the hydrophilic head group, was used to treat nerve tissue. All chemicals were purchased from Sigma unless otherwise noted. From the four categories, the following chemicals were tested: (1) the non-ionic detergents Tween 20 (Bio-Rad,

Hercules, CA), Tween 80, and Triton X-100; (2) the amphoteric detergents Empigen BB, sulfobetaine-10 (SB-10), sulfobetaine-16 (SB-16), and 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS); (3) the anionic detergents Triton X-200, dodecylbenzene sulfonate, sodium caprylate, and sodium deoxycholate; and (4) the cationic detergent hexadecyltrimethylammonium bromide (CTAB). Only one representative from the cationic detergent category was selected because the entire class of cationic detergents was predicted to be damaging to the ECM; cationic detergents commonly cause structural proteins to denature. In addition, cationic detergents are generally the most cytotoxic of the detergents [114], so there was concern about the negative effects of possible residual detergent in the grafts after processing. CTAB is a standard cationic detergent, and thus served as a representative sample from this detergent category. The anionic detergent sodium deoxycholate is a common component in published chemical decellularization protocols [34, 43, 51]. Other detergents were selected based on commercial availability, reports of mild toxicity in low concentrations relative to similarly classified detergents, and predicted effectiveness for disrupting cell membranes [114-117].

3.3 CHEMICAL TREATMENT OF NERVE TISSUE

The impact of the various detergents on peripheral nerve tissue was evaluated by washing sciatic nerve segments in solutions of the detergents. Segments of sciatic nerve (20 mm long) were harvested from 350 g Harlan Sprague Dawley rats. Peripheral fat and connective tissue was trimmed from the tissue, and then washed in either "high" or "low" concentrations of each detergent (see **Table 3.1** for specific values of detergent concentrations). When possible, the detergent concentration was chosen based on the critical micelle concentration

(CMC) of the detergent, with "low" concentrations equal to the CMC and "high" concentrations equal to 10 times the CMC. In a few cases, however, solubility limits forced the selection of a "high" concentration less than 10 times the CMC (e.g., SB-10, sodium caprylate). The detergents were prepared in a 10 mM phosphate/50 mM sodium buffer. The pH of all detergent solutions was adjusted to between 7.2 and 7.6 except sodium caprylate and sodium deoxycholate (pH 7.8-8.2); the higher pH was necessary to maintain solubility of these anionic detergents. The tissue and detergent solution were agitated on a vertical rotator (10 rpm) for 24 hours at room temperature. The effects of each detergent were examined in nerves from 2 rats, and multiple sets of data were collected from each nerve.

3.4 HISTOLOGICAL ANALYSIS OF NERVE GRAFTS

Histology was used to examine the effects of the detergent treatments on the nerve grafts. From the center of each graft, a 5 mm segment was used for histology. Two concerns motivated the use of a tissue segment from the middle of the graft. First, handling of the nerve during processing could damage the structure. Thus, the tissue was only handled on the ends so that any damage to the extracellular structure at the midpoint was a direct result of the detergent treatment. Second, diffusion of the detergent micelles could be limited, resulting in removal of cellular components from the nerve ends, but not the middle of the tissue. The end goal of this work was a 10 mm nerve graft, so histological analysis of the midpoint of a tissue segment at least 10 mm long ensured that mass transport limitations were taken into account.

The harvested tissue segment was dehydrated with graded alcohol solutions and xylene, then embedded in paraffin. The tissue was not fixed prior to

embedding because the auto-fluorescence that results from glutaraldehyde or paraformaldehyde fixation would have hindered the use of fluorescence microscopy as an evaluation technique. Cross-sections of tissue 7 μm thick were cut with a microtome and mounted on glass slides. Tissue from each nerve segment was stained with a Harris Hematoxylin and Eosin (H&E) stain for observation of general morphology. A more detailed explanation of the H&E staining protocol can be found in **Appendix A**.

Additionally, immunostains were employed to visualize the basal laminae (anti-laminin antibody, Developmental Studies Hybridoma Bank, Iowa City, IO), interstitial regions of the endoneurium [7, 118] (anti-collagen I antibody, Abcam, Cambridge, UK), axons (anti-neurofilament 200 antibody, Sigma, St. Louis, MO), and Schwann cells (anti-S-100 antibody, Dako, Carpinteria, CA). The anti-collagen I and anti-S-100 primary antibodies were labeled with FITC-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA), while the anti-laminin and anti-neurofilament 200 antibodies were labeled with TRITC-conjugated secondary antibodies (Jackson ImmunoResearch). A more detailed explanation of the immunostaining protocols is found in **Appendix B**. The stained sections were visualized on an Olympus IX70 (Melville, NY) inverted microscope, and the images were captured with an Optronics MagnaFire (Goleta, CA) digital color camera. For each stain, a fixed exposure time was used (e.g., 3 s for S-100, 5 s for laminin). To eliminate non-specific staining from the images, the exposure time was selected such that sections of fresh nerve tissue incubated only in secondary antibody (negative control) appeared black.

3.5 ANALYSIS OF CELLULAR REMOVAL AND STRUCTURAL PRESERVATION IN THE GRAFTS

A scoring system was devised to evaluate the histological images, thus providing a means to quantify the effectiveness of the detergent treatments at cellular removal and structural preservation. In a blind evaluation, four individuals independently rated each of the images on a scale of 1 to 5. Under this scoring system, sections exhibiting desirable characteristics received a higher score. The desirable characteristics that we evaluated were: (1) general morphological preservation, (2) basal laminae preservation, (3) interstitial endoneurium preservation, (4) axonal removal, and (5) Schwann cell removal. The analysis can be grouped into two main categories: structural preservation (characteristics 1-3) and cellular removal (characteristics 4-5).

For evaluation of structural preservation, the stained sections (i.e., H&E, laminin and collagen I-stained sections) were rated by comparing the morphology of the ECM and the intensity of the laminin and collagen I staining to that observed in fresh nerve (positive control). A higher score is correlated with better ECM preservation. Tissue sections that appeared structurally indistinguishable from fresh nerve received a score of 5. Denatured proteins (identified by faint staining) or poor structural integrity (e.g., collapsed or fragmented basal lamina tubes) lowered the score. Sections that exhibited staining intensity similar to that of the negative control (non-specific staining) or severe structural damage received a score of 1. In summary, a desirable nerve graft should exhibit a preserved extracellular structure and would therefore receive a higher score.

The second category of analysis focused on the removal of cellular components by the detergent treatments, evaluated by the removal of Schwann

cells and axons. The previously described scoring system was modified in this analysis to account for the shift from preservation to removal as the desirable effect. A score of 5 was still the top (i.e., desirable) score, but it was correlated with removal of cellular components (i.e., the absence of staining). Sections stained for S-100 and neurofilament that exhibited a fluorescence intensity equivalent to the non-specific staining seen in the negative control (sections of untreated nerve tissue only incubated in secondary antibody) received a score of 5, the desired outcome. However, a tissue section that demonstrated S-100 or neurofilament staining as bright as that of fresh nerve (positive control) received a score of 1, an undesired outcome.

3.6 GEL ELECTROPHORESIS AND WESTERN ANALYSIS

After the middle segment was removed from the detergent-treated tissue for histological analysis, the remaining tissue was used to quantify the level of Schwann cell debris in the graft. Fresh nerve served as a control. Myelin basic protein (MBP) was selected as a marker for Schwann cells. Unlike S-100, which is a soluble protein that may be easily removed by the detergent treatment, MBP is a membrane bound protein that should be less easily removed. MBP is abundant in peripheral nerve myelin (18% of the total protein [119]), and an antibody against the protein is commercially available. Each detergent-treated section was rinsed for 30 minutes in distilled water to remove residual detergent and placed in a 1.5 ml microfuge tube. All subsequent steps were carried out in the same tube to minimize the loss of protein. The tissue was dehydrated and weighed. Then, 100 μ l of 3 M sucrose and protease inhibitor cocktail ((0.05 ml/l, Sigma) was added, and the tissue was minced for 5 minutes with microsurgical scissors. Next, 10 μ l of 1.67 M of acetic acid was added to dissociate the basic proteins (e.g., MBP) from complexes with charged and neutral lipids in the

myelin sheath [120]. The tissue was sonicated for 5 minutes and crushed for 5 minutes with a pestle. In preparation for electrophoresis, sample buffer solution (11% glycerol, 2% sodium dodecyl sulfate (BioRad), 1.7% DL-dithiothreitol, 0.8 M Tris buffer, pH of 8.8) was added to yield a final concentration of 9.5 mg/ml (tissue mass per diluent). Finally, 16 μ l of 1 M sodium hydroxide was added to the tube to neutralize the acetic acid and to raise the pH to 8.8, and the mixture was again homogenized with a pestle for 30 seconds.

Post-treatment weight was used as the basis for calculating protein concentration in the homogenized nerve solutions to reduce the number of animals sacrificed. By performing both histological and Western analysis on each detergent-treated nerve segment, only 24 animals were required in the detergent treatment experiment. If one nerve segment had been used exclusively for histological analysis and another for Western analysis, an additional 24 animals would have been required. To perform both histological and Western analysis on each piece of tissue, the post-treatment weight of tissue being homogenized for Western analysis had to serve as the basis for concentration. Pre-treatment weight could not be used because part of the tissue was removed for histological examination after detergent treatment. Using the post-treatment weight was slightly less accurate than using pre-treatment weight because the final concentration did not take into account the weight of the proteins removed by the treatment, which varied by detergent. However, it was decided that the reduction in accuracy was not significant enough to warrant sacrificing 24 additional animals.

After homogenizing the detergent-treated tissues, the proteins in each solution were separated on 15% acrylamide gels using electrophoresis. The

solutions of homogenized tissue, standards (MBP purified from bovine brain), and a negative control (sample buffer) were loaded into separate lanes and separated for 50 minutes at 200 V. The proteins were then transferred from the acrylamide gels onto Immuno-Blot PVDF membranes (BioRad) for 900 minutes at 30 V. Western analysis was performed using anti-MBP primary antibodies (Calbiochem, San Diego, CA), HRP-conjugated secondary antibodies (Jackson ImmunoResearch), SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL), and Hyperfilm ECL (Amersham Biosciences, Piscataway, NJ). More details about the gel electrophoresis and Western protocols are found in **Appendix C**. Developed films were digitized with a Perfection 1200s scanner (Epson, Long Beach, CA). Scion Image software (Scion Corporation, Frederick, MA) was used to measure the optical density of the protein bands, and the MBP concentration of each band was calculated based on comparison to standard solutions containing 0, 250, 500 and 750 ng of purified MBP run in each gel. For each Western, the density measurements for the standard bands were plotted and fit with a trendline. If the trendline for the standards did not exhibit a fit of $r^2 > 0.9$, then the data for all experimental cases on the same Western were not used, and the Western was repeated. This requirement ensured a linear correlation between protein concentration and optical density. The concentration of MBP in each sample was quantified from at least four independent Westerns.

3.7 OPTIMIZED GRAFT DECELLULARIZATION PROTOCOL

Based on the results from the first detergent treatment experiments, a combination of detergents was selected for further optimization. These detergents were Triton X-200, SB-10, and SB-16. A decellularization protocol was conducted as follows. The tissue was cut into 15 mm segments and placed in a 15 ml conical tube filled with deionized distilled water. All washing steps were

carried out at 25°C with agitation. After 7 hours, the water was aspirated and replaced by a solution containing 125 mM SB-10, 10 mM phosphate, and 50 mM sodium. The nerves were agitated for 15 hours. The tissue was then rinsed once for 15 minutes in a washing solution of 50 mM phosphate and 100 mM sodium. Next, the washing solution was replaced by a solution containing 0.14% Triton X-200, 0.6 mM SB-16, 10 mM phosphate, and 50 mM sodium. After agitating for 24 hours, the tissue was rinsed with the washing solution 3 times at 5 minutes per rinse. The nerve segments were again agitated in the SB-10 solution (7 hours), washed once, and agitated in the SB-16/Triton X-200 solution (15 hours). Finally, the tissue segments were washed 3 times for 15 minutes in a solution containing 10 mM phosphate and 50 mM sodium, then stored in the same solution at 4°C.

To evaluate the ECM preservation after this decellularization treatment, tissue segments created by the protocol were compared to tissue treated with a published chemical decellularization protocol by Sondell et al.[43]. Briefly, the nerve tissue was agitated in distilled water for 7 hours, 46 mM Triton X-100 in distilled water overnight, and then 96 mM sodium deoxycholate in distilled water for 24 hours. These steps were repeated before performing a final wash in distilled water. All treatment steps were performed at room temperature, and the tissue was subsequently stored in a 10 mM phosphate buffered saline (PBS) solution at 4°C.

Structural preservation in grafts created by thermal decellularization was also examined. The freeze/thaw grafts were created by dipping nerve tissue in liquid nitrogen for 20 seconds, thawing it in PBS at room temperature for 60 seconds, and then repeating the process four additional times [121].

Tissue segments decellularized by the optimized protocol were compared with segments decellularized with the Sondell protocol and fresh nerve tissue. Histology, Western analysis, and gel electrophoresis were employed in this comparison. The structure of freeze/thaw nerve grafts was also evaluated histologically, but no other analysis was performed on that model. In addition to the previously described histological stains, a fluorescent stain for lipids (DiI, Molecular Probes, Eugene, OR) was used to visualize myelin and cell membranes in the optimized, Sondell, and fresh nerve grafts.

The concentrations of MBP and myelin protein zero (P0), the most abundant structural protein in peripheral nerve myelin [122], in homogenized tissue solutions were analyzed. Unlike MBP, however, the P0 concentration could not be quantified using Western analysis because an anti-P0 antibody was not commercially available. Instead, equally concentrated volumes of homogenized tissue, based on the pre-treatment mass of the nerve segments, were separated on an acrylamide gel. The proteins bands were then non-specifically stained with Colloidal Blue (Novex, Toronto, Canada). By comparing the protein bands to molecular weight standards (BioRad) that were also separated on the gel, a 28-30 kDa band [123] believed to be P0 was identified.

3.8 CREATION OF GRAFTS FOR IN VIVO EXPERIMENTS

Three different decellularized nerve grafts were created for in vivo experiments: optimized acellular grafts, grafts created with the Sondell protocol, and freeze/thaw grafts. Fresh isografts were also used in the in vivo experiments as a positive control. Fresh isografts are a mimic of the clinical autograft. All steps in the preparation of the grafts were carried out under aseptic conditions, and all solutions used were sterile. The tissue was only handled on the ends to

limit structural damage to the grafts. Tissue that was to be decellularized with the optimized or Sondell treatment was placed in RPMI 1640 (a common cell culture medium) solution at 4°C immediately after harvest. Fatty and connective tissue was removed from the nerve epineurium. The tissue was trimmed to 15 mm and decellularized according to the optimized or Sondell protocol as previously described (**Figure 3.1**). To create freeze/thaw grafts, tissue was harvested, immediately treatment, and then placed in PBS until implantation. Like the freeze/thaw grafts, fresh grafts were placed in PBS until implantation. The time between harvest and implantation was never longer than 30 minutes for the freeze/thaw or the fresh grafts (**Table 3.2**). For all four types of graft, the ends of the tissue were trimmed immediately prior to implantation to attain a clean-cut, 10 mm graft (**Figure 3.2**).

3.9 IN VIVO EVALUATION OF GRAFT IMMUNOGENICITY AND REGENERATIVE CAPACITY

Isografts and allografts were used to test the immunogenicity of the optimized grafts. Isografts are harvested from a donor animal of the same strain as the host animal (e.g., Lewis rats); they mimic the autograft. Allografts are harvested from a donor animal of a different strain (e.g., a Harlan Sprague-Dawley rat) than the host animal (e.g., a Lewis rat). The differences between these rat strains are addressed in Section 5.5. Four experimental conditions were tested: (a) fresh isografts (n=6) (b) fresh allografts (n=5); (c) optimized acellular isografts (n=5); and (d) optimized acellular allografts (n=5) (**Table 3.3**).

The ability of the optimized grafts to support nerve regeneration was also evaluated in vivo. Regeneration through the optimized grafts was compared to regeneration in fresh grafts, grafts created with the Sondell protocol, and

freeze/thaw grafts (n=6 for each type of graft). Donor and host animals were HSD rats.

The rats were anesthetized with an intra-peritoneal injection of 120 mg/kg body weight ketamine and 15 mg/kg body weight xylazine. The sciatic nerve on the right side was exposed, transected, 5 mm of nerve was removed, and a 10 mm graft was sutured to both the proximal and distal nerve ends using 10-0 vicryl sutures (Ethicon, Somerville, NJ) (**Figure 3.3**). The muscle was drawn back together with 5-0 chromic gut sutures (Ethicon), and the skin was closed with wound clips (Becton Dickinson, Sparks, MD). Surgical methods were in accordance with regulations established by the National Research Council in the Guide for the Care and Use of Laboratory Animals [124].

All of the grafts were harvested after 28 days. The animals were re-anesthetized, and the nerve graft was exposed. Prior to harvesting, the graft and surrounding tissue was submerged in 3% glutaraldehyde/4% paraformaldehyde in PBS for 1 minute to initiate fixation. Then, the sciatic nerve was transected 5 mm above and below the graft, the distal end was marked with a stitch, and the graft was placed in a microfuge tube containing the glutaraldehyde/paraformaldehyde fixative and stored at 4°C. After 30 minutes, the graft was transferred to PBS and stored at 4°C until it was embedded in paraffin.

3.9.1 Immune Response to Grafts Analyzed Histologically

Histology was used to inspect the nerve grafts for signs of immunological rejection. Longitudinal sections of tissue 7 µm thick were immunostained with anti-macrophage (Chemicon, Temecula, CA) and anti-CD8a+ (PharMingen, San Diego, CA) primary antibodies. HRP-conjugated secondary antibodies (Jackson

ImmunoResearch), 3,3'-diaminobenzidine (DAB) substrate (Vector Labs, Burlingame, CA), and an eosin counterstain were used to visualize the invading cells and axons. After capturing images of the sections with a digital camera, the images were combined in Adobe Photoshop to create a composite of the entire graft. The percentage of area of the graft covered with positively stained CD8a+ and macrophage cells was quantified using Scion Image software.

3.9.2 Regeneration in Grafts Analyzed Histologically

To evaluate the regenerative potential of the acellular grafts, longitudinal tissue sections were stained for regenerated axons using an anti-neurofilament primary antibody called RT97 (Developmental Studies Hybridoma Bank), an HRP-conjugated secondary antibody, and DAB. Subsequently, cross sections were cut from the midpoint of the grafts and stained for neurofilaments. The stained sections were visualized with a 20X objective and images were captured with the digital camera. A 20 cm X 16 cm image was printed for each sample. The number of nerve fibers in each image was counted, and the area of nerve cable in the image was measured. Because a portion of the nerve cable had been removed by sectioning the tissue longitudinally prior to taking cross-sections, the total number of axons in each nerve cable could not be determined. Instead, axon density was calculated by dividing the number of nerve fibers by the area of the cable from which the count was taken. Regions of connective tissue at the periphery of the graft, based on morphological evaluation, were excluded from the analysis.

3.10 STATISTICAL ANALYSIS

The Student's t-Test was performed to determine the statistical significance of the differences between results for all quantitative experiments

(e.g., Western analysis and axon density). A significance level of $p < 0.05$ was used as the cutoff (i.e., p values are reported only for cases in which $p < 0.05$).

Table 3.1: Detergents and Concentrations Used in Initial Experiment

Detergent	Literature CMC Value (mM)	Low Concentration (mM)	High Concentration (mM)
<i>Amphoteric</i>			
CHAPS	6-10	10	100
Empigen BB	1.8	1.8	18
SB-10	25-40	40	160
SB-16	0.01-0.06	0.06	0.6
<i>Anionic</i>			
Dodecylbenzene Sulfonate	0.15-0.17	0.17	1.7
Sodium Caprylate	351-360	138	360
Sodium Deoxycholate	1.5-70	15	70
Triton X-200	Unknown *	0.07 % w/w *	0.7% w/w *
<i>Cationic</i>			
CTAB	1.0	1.0	10
<i>Non-Ionic</i>			
Triton X-100	0.25	0.25	2.5
Tween 20	0.059	0.059	0.59
Tween 80	0.012	0.012	0.12

CMC = critical micelle concentration, CMC value given is in water at a neutral pH

* CMC and molecular weight are proprietary to the manufacturer; Triton X-200 is known to be an alkylaryl polyether sulfonate.

Table 3.2: Creation of Nerve Grafts

Graft Type	Decellularization Protocol	Time Harvested
Fresh	None	< 30 minutes before implantation
Optimized Acellular	Chemical (Triton X-200, SB-16, and SB-10)	Days before implantation
Sondell Acellular	Chemical (sodium deoxycholate and Triton X-100)	Days before implantation
Freeze/Thaw Acellular	Thermal (liquid nitrogen and PBS)	< 30 minutes before implantation

Table 3.3: Implants to Examine Immunological Tolerance of Optimized Grafts

Graft Type	Donor Strain	Host Strain	Number of Implants
Fresh Isograft	Lewis*	Lewis	3
	HSD [†]	HSD	3
Fresh Allograft	Lewis	HSD	5
Acellular Isograft	HSD	HSD	5
Acellular Allograft	Lewis	HSD	5

* Lewis rats are an inbred strain.

[†] HSD rats are an outbred strain, but the animals used were from a closed colony.

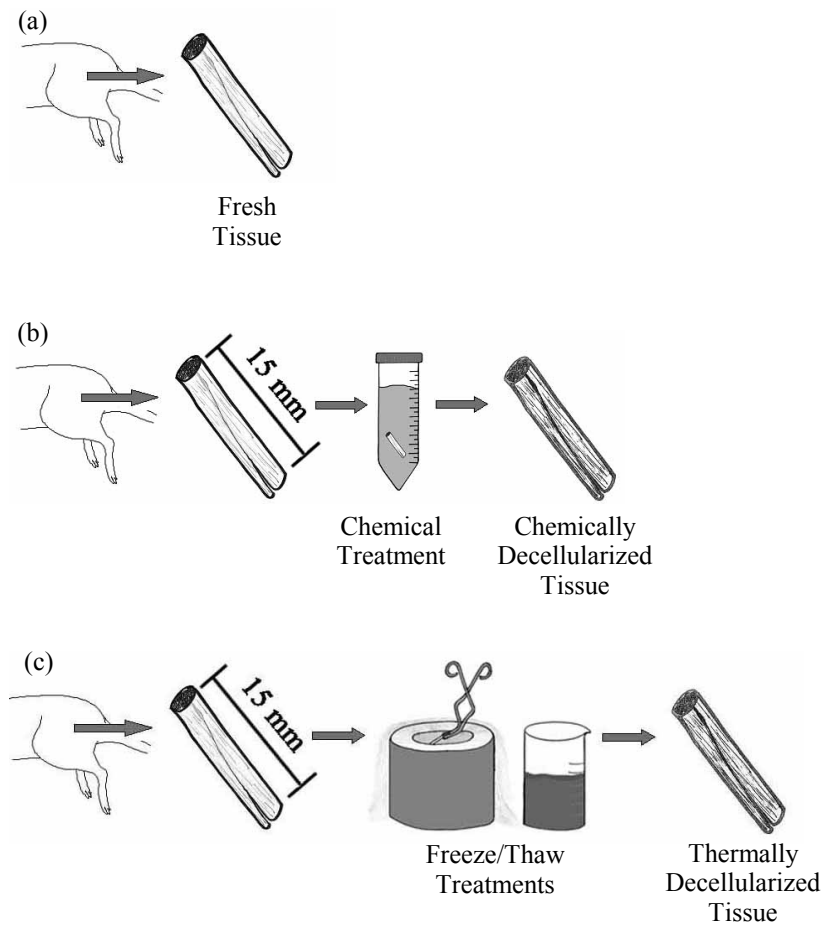


Figure 3.1: The grafts used for in vivo experiments were (a) fresh, (b) chemically decellularized, or (c) thermally decellularized. The fresh grafts were harvest from the sciatic nerve of a donor animal immediately prior to implantation. Chemically decellularized grafts were created from 15 mm segments of rat sciatic nerve tissue. The tissues were decellularized by washing them in detergent solutions as outlined in the treatment protocols. The optimized acellular grafts and the Sondell grafts were chemically decellularized. Freeze/thaw grafts were thermally decellularized by cycling the tissue segments through liquid nitrogen and PBS.

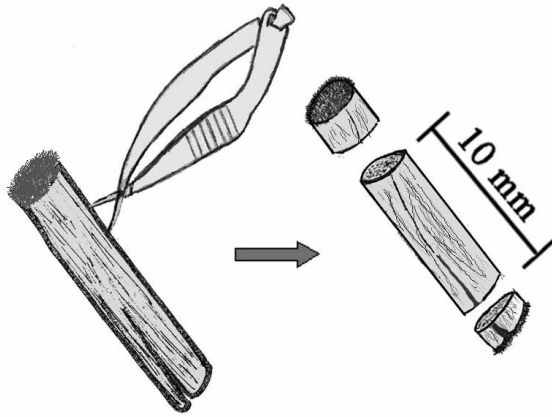


Figure 3.2: The ends of the grafts were often frayed or compressed by washing and handling the tissue. By trimming the damaged ends immediately prior to implantation, the grafts were always 10 mm long with clean-cut ends.

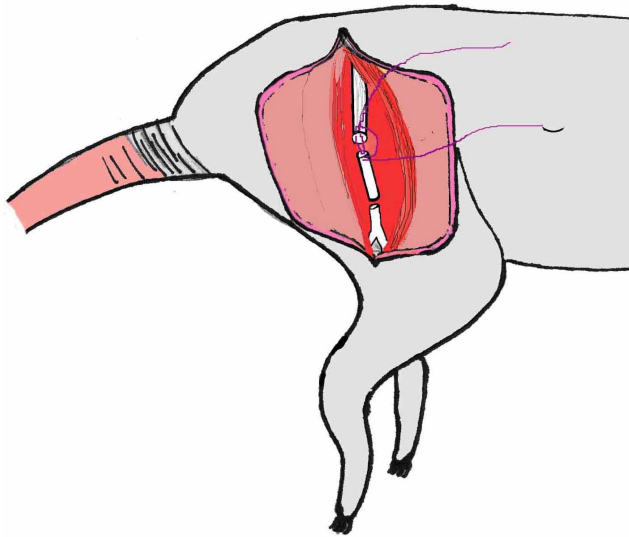


Figure 3.3: The sciatic nerve on the right side of the rat was exposed. The nerve was transected, and 5 mm of nerve was removed. A 10 mm nerve graft was sutured to both the proximal and distal nerve ends using 10-0 vicryl sutures. After implantation, the muscle was drawn back together with 5-0 chromic gut sutures, and the skin was closed with wound clips.

Chapter 4: Results

4.1 INTRODUCTION

Evaluation of detergent-treated nerve tissue was performed with histological and Western analysis. This analysis elucidated general trends in how detergents affect peripheral nerve tissue. Based on those general trends and data for the specific chemicals, three detergents were selected for use in a single decellularization protocol. The decellularization protocol was optimized for the removal of cellular material and preservation of the extracellular structure in the grafts. Histological and Western analysis demonstrate the accomplishment of these goals. After optimization, the grafts were examined *in vivo*. Using two different strains of rats, optimized acellular allografts were shown to be immunologically benign. In addition, the regenerative capacity of the grafts was compared to that of the isograft (a mimic of the autograft) and two published acellular grafts. The axon density in the optimized acellular grafts surpassed that in the Sondell and freeze/thaw acellular grafts by 42% and 96%, respectively.

4.2 GENERAL TRENDS IN DECELLULARIZATION

In an effort to engineer an optimized acellular nerve graft, two primary goals were established: preservation of the extracellular matrix and removal of cellular components. To better understand the impact of a variety of chemicals on nerve tissue, a detailed analysis was performed by examining five specific indicators of ECM preservation and cellular removal: (1) general morphological preservation, (2) basal laminae preservation, (3) interstitial endoneurium preservation, (4) axonal removal, and (5) Schwann cell removal. By averaging the data for each detergent type, a general trend is evident. The amphoteric and non-

ionic detergents scored higher at preserving the extracellular matrix (**Figure 4.1**), but the anionic and cationic detergents were more effective at removing cellular components (**Figure 4.2**). These results were expected because amphoteric and non-ionic detergents are relatively mild detergents, while anionic detergents and cationic detergents are typically stronger detergents [115].

4.3 MOST EFFECTIVE DETERGENTS IDENTIFIED

Of all the detergents tested, those with the five highest total effectiveness scores are reported, along with their individual scores for each category (**Table 4.1**). Three of these detergents were anionic: dodecylbenzene sulfonate, sodium deoxycholate, and Triton X-200. The other two detergents were amphoteric: SB-10 and SB-16. Neither of the treatments with Triton X-100, a non-ionic detergent used in the Sondell protocol, scored in the top five for total effectiveness. The total effectiveness scores for the low and high concentrations for Triton X-100 were 15.4 and 16.3, respectively. For basal laminae preservation, the Triton X-100 treatments received scores of 2.8 ± 1.0 and 2.6 ± 0.7 , the lowest scores out of all the non-ionic and amphoteric detergents (**Appendix D**). Examining the individual scores for both axon and Schwann cell removal by the five most effective detergents, it was evident that an anionic detergent was needed in order to remove the majority of cellular material from nerve tissue. Of the three anionic detergents, sodium deoxycholate scored the highest in the cellular removal categories, but it also scored the lowest in each of the structural preservation categories. Of particular importance was the sodium deoxycholate score of 2.9 for basal laminae preservation. The basal laminae are the structures through which regenerating nerves preferentially grow, and sodium deoxycholate was more destructive to these structures than the other top performing detergents.

Western analysis was also performed as a complementary means of assessing cellular removal. The concentration of MBP remaining in each tissue segment after detergent treatment was measured. The MBP concentration in native nerve tissue was also measured. From these data, the percentage of MBP removed by each detergent was calculated. Average MBP removal by each detergent category (**Figure 4.3**) demonstrates the same general trend as that observed with histological analysis (**Figure 4.2**): cationic and anionic detergents possess a greater ability to remove cellular material than non-ionic and amphoteric detergents. Looking at the anionic detergent solutions that scored the highest under histological examination: sodium deoxycholate removed 97.9% of MBP, Triton X-200 removed 84.6%, and dodecylbenzene sulfonate removed only 37.2% (**Table 4.2**).

4.4 OPTIMIZED PROCESS PRESERVES THE ECM AND REMOVES CELLULAR MATERIAL

Based on the histological and Western analysis data, an optimized decellularization protocol was developed using Triton X-200, SB-10 and SB-16 (**Figure 4.4**). The rationale for selecting these detergents is provided in Chapter 5. Tissue treated with this protocol was analyzed and compared to fresh nerve tissue and tissue treated with the protocol by Sondell et al. [43, 125]. Comparing the H&E stained tissue sections, the morphology of the fresh nerve tissue appears to more closely resemble tissue created with the optimized protocol and the freeze/thaw protocol than with the Sondell protocol (**Figure 4.5**). In addition to a greater loss in structural integrity, the Sondell protocol treated tissue is also characterized by a reduction in graft diameter. Higher magnification images of tissue sections stained for laminin allow for the comparison of basal laminae preservation by the treatment protocols (**Figure 4.6**). The ring-like structures in

native nerve tissue are open columns of basal laminae, and similar structures are apparent in the tissue treated with the optimized decellularization protocol and the freeze/thaw protocol. The Sondell treatment appears to fragment the basal laminae.

Tissue sections were stained for S-100 protein and lipids to evaluate the removal of cellular components by the optimized decellularization protocol. Schwann cell remnants in the tissue after decellularization were visualized by staining for S-100 (**Figure 4.7**). There was no visible difference in the ability of the decellularization protocols to remove Schwann cells. Similarly, the reduction in lipid content in the decellularized tissues was comparable (**Figure 4.8**).

In addition to examining tissue at the midpoint of the optimized acellular graft, tissue 2.5 mm from the end of the graft was also examined to determine if the structural preservation and cellular removal were consistent throughout the graft. No difference could be detected in the sections taken from near the end of the graft when compared to sections from the midpoint (data not shown). Sections were not taken from the final 2.5 mm of the graft because that portion of the graft would be trimmed off prior to implantation.

4.5 GEL ELECTROPHORESIS AND WESTERN ANALYSIS CONFIRM CELLULAR REMOVAL

Cellular removal was further evaluated by examining the concentrations of the Schwann cell proteins P0 and MBP in homogenized tissue solutions (**Figure 4.9**). In Colloidal Blue stained acrylamide gels, the reduction of P0 by the optimized treatment (lane 2) was apparent when compared to fresh nerve tissue (lane 3). As expected, the Sondell protocol (lane 4) appears to completely remove

the P0 band. Western analysis provided quantitative values for the removal of the cellular protein MBP. Fresh nerve was found to contain 1.6 ± 0.51 μg of MBP per mg of tissue, while tissue decellularized with the optimized protocol and the Sondell contained 0.054 ± 0.047 and 0.036 ± 0.048 μg of MBP per mg of tissue, respectively. The data were normalized, based on fresh nerve tissue, and presented in **Figure 4.10**. Both decellularization protocols significantly reduced the MBP concentration from that measured in fresh nerve ($p < 0.001$), and the MBP levels in the decellularized tissues could not be statistically distinguished from the negative control (sample buffer). In summary, the optimized protocol is comparable to the Sondell protocol in its ability to remove cellular components and better at retaining the natural ECM, especially the intricate structure of the basal laminae.

4.6 GRAFTS ARE IMMUNOLOGICALLY TOLERATED

To evaluate the immunological response by a host to the optimized acellular grafts, four experimental conditions were tested with sciatic nerve graft implants (**Table 4.3**). A fresh isograft was used to establish the inflammatory and immune response that results from surgery alone. This served to mimic the current clinical approach (i.e., autograft) for nerve repair (positive control). The fresh allograft established the cellular response associated with cell-mediated rejection (negative control). The acellular allografts were used to determine if there was a reduction or avoidance of immunological rejection by decellularizing the grafts. Finally, acellular isografts were used to determine the immune response that resulted from the processing technique and any residual chemicals remaining in the graft after decellularization.

By immunostaining longitudinal sections of the grafts for cytotoxic T-cells and macrophages, the level of cell-mediated immune response was determined. CD8+ cytotoxic T-cells were visualized by staining for CD8a cell surface markers. Elevated levels of cytotoxic T-cells are expected in tissues undergoing cell-mediated rejection. Increased levels of macrophage cells are also expected in rejected allografts. However, macrophages are also recruited during Wallerian degeneration to clear debris and release neurotrophic factors for regenerating nerves. At 28 days, both cell types could be seen throughout the full length of all the grafts (**Figures 4.11, 4.13**). The infiltration of CD8a+ cells into the fresh allografts was higher than into the fresh isografts ($p<0.01$) and the acellular grafts ($p<0.005$) (**Figure 4.12**). Meanwhile, the levels of CD8a+ cells in the acellular isografts and allografts were lower than those observed in the fresh isografts ($p<0.05$). Macrophage invasion into the fresh isografts was lower than into the fresh allografts ($p<0.05$), but the differences between other grafts were not statistically significant (**Figure 4.14**).

4.7 OPTIMIZED GRAFTS SUPPORT REGENERATING AXONS

The capacity of the optimized acellular graft to support nerve regeneration was tested by examining the growth of axons through the various nerve grafts after 4 weeks. The grafts tested included (1) fresh isografts, (2) optimized acellular grafts, (3) grafts created with the Sondell protocol, and (4) freeze/thaw grafts. This was accomplished by staining longitudinal sections and cross-sections of the grafts for neurofilaments. At 28 days, new axons had grown completely across the grafts (**Figure 4.15**). The axons appeared to meet some resistance in crossing from the proximal nerve end into the graft and from the graft into the distal nerve end, as demonstrated by the non-linearity in neurofilaments around the suture points on both ends of the graft (**Figure 4.15a, 4.15c**). However, once

the axons extended into graft, they appeared to grow linearly, as demonstrated by the parallel neurofilaments at the midpoint of the graft (Figure 4.15b). Similarly, once the axons found their way into the distal end of the graft, they appeared to grow straight (data not shown). Thus, the optimized acellular nerve grafts supported axonal regeneration and guided the axons toward the distal nerve end.

4.8 REGENERATIVE CAPACITY OF OPTIMIZED GRAFTS SURPASSES OTHER ACELLULAR MODELS

In addition to visually examining the growth of axons through the grafts, axon density at the midpoint of the grafts was determined. The fresh grafts and optimized nerve grafts were nearly identical with axon densities of 9.9 and 9.8 axons/100 μm^2 , respectively. The freeze/thaw grafts had 5.0 axons/100 μm^2 , and the Sondell grafts had 6.9 axons/100 μm^2 . The axon density in the freeze/thaw grafts was statistically lower than in the fresh grafts and the optimized acellular grafts ($p < 0.01$). The axon density in the Sondell grafts was also statistically lower than in the fresh grafts ($p < 0.01$) and the optimized acellular grafts ($p < 0.05$). Since cellular debris is not removed by the freeze/thaw decellularization process and the ECM is damaged by the Sondell decellularization process, the data suggest that removing cellular debris and preserving the ECM in acellular nerve grafts improves their regenerative capacity.

4.9 CONCLUSIONS

In conclusion, the optimized decellularization protocol removes cellular material from peripheral nerve tissue while preserving the extracellular structure, as demonstrated by histological and Western analysis. Optimized acellular allografts did not elicit cell-mediated immunological rejection after 28 days in vivo. Additionally, in initial 4-week studies, the axon density at the midpoint of

the optimized acellular grafts was higher than that in the Sondell and freeze/thaw acellular grafts and was statistically indistinguishable from the axon density in the fresh isograft. This would suggest that the regenerative capacity of the optimized acellular graft might surpass that of previous acellular graft models.

Table 4.1: Most Effective Detergent Treatments, Based on Histological Analysis of Treated Nerve Tissue

Detergent	Conc.	Morphology Preservation	Basal Laminae Preservation	Interstitial Endoneurium Preservation	Axon Removal	Schwann Cell Removal	Total Score
<i>Amphoteric</i>							
SB-10	High	3.9 ± 0.6	4.0 ± 0.5	4.1 ± 0.6	1.9 ± 0.8	3.9 ± 0.6	17.8
SB-16	High	4.8 ± 0.5	4.8 ± 0.5	4.9 ± 0.4	2.1 ± 1.0	1.3 ± 0.5	17.8
<i>Anionic</i>							
Dodecylbenzene Sulfonate	Low	3.3 ± 0.7	4.3 ± 0.7	4.5 ± 0.5	3.4 ± 0.7	3.8 ± 1.0	19.1
Sodium Deoxycholate	High	2.6 ± 1.7	2.9 ± 1.1	3.7 ± 1.0	3.8 ± 1.0	4.7 ± 0.6	17.6
Triton X-200	Low	3.8 ± 0.5	3.9 ± 1.1	3.8 ± 1.4	3.4 ± 0.7	3.9 ± 0.6	18.6

Conc. = Concentration

5 = superior (e.g., optimal preservation of morphology, basal laminae and interstitial endoneurium; optimal removal of axons and Schwann cells)

1 = poor (e.g., destruction of morphology, basal laminae and endoneurium; no removal of axons and Schwann cells)

Individual evaluations presented as "score" ± "standard deviation"

Total Score = combination of score in 5 individual categories (25 = best possible score)

Table 4.2: Cellular Removal by Anionic Detergent Treatments with Highest Total Effectiveness Scores, Based on Western Analysis of Myelin Basic Protein Concentration

Detergent	Detergent Concentration	MBP Removal (Relative to Untreated Nerve)
Untreated Nerve Tissue	-	0.0 %
Dodecylbenzene Sulfonate	Low	37.2 %
Sodium Deoxycholate	High	97.9 %
Triton X-200	Low	84.6 %

MBP = Myelin basic protein

Table 4.3: Implants to Examine Immunological Tolerance of Optimized Graft

Graft Type	Strain of Donor & Host Animal	Analyzing Response to:
Fresh Isograft	Same	Surgical procedure (negative control)
Fresh Allograft	Different	Natural antigens (positive control)
Acellular Isograft	Same	Treatment protocol
Acellular Allograft	Different	Residual antigens

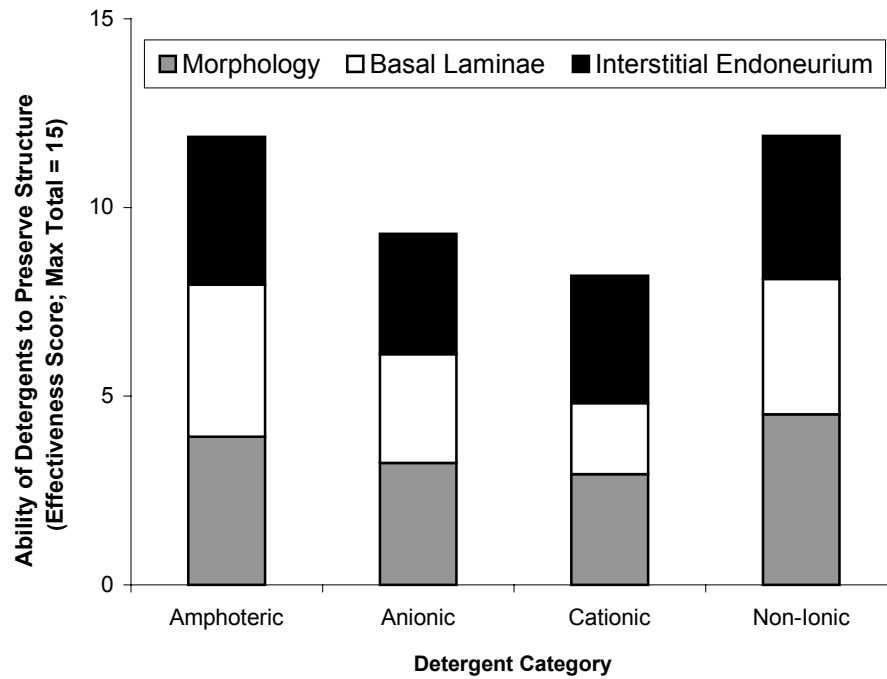


Figure 4.1: Effectiveness of detergents at structural preservation, based on histological evaluation. The average ability of each detergent category to preserve the natural structure of peripheral nerve tissue is illustrated. Overall structural preservation was measured as a composite of the individual scores for preservation of the general morphology, basal laminae, and the interstitial region of the endoneurium. Amphoteric and non-ionic detergents were less damaging to tissue structure than anionic and cationic detergents.

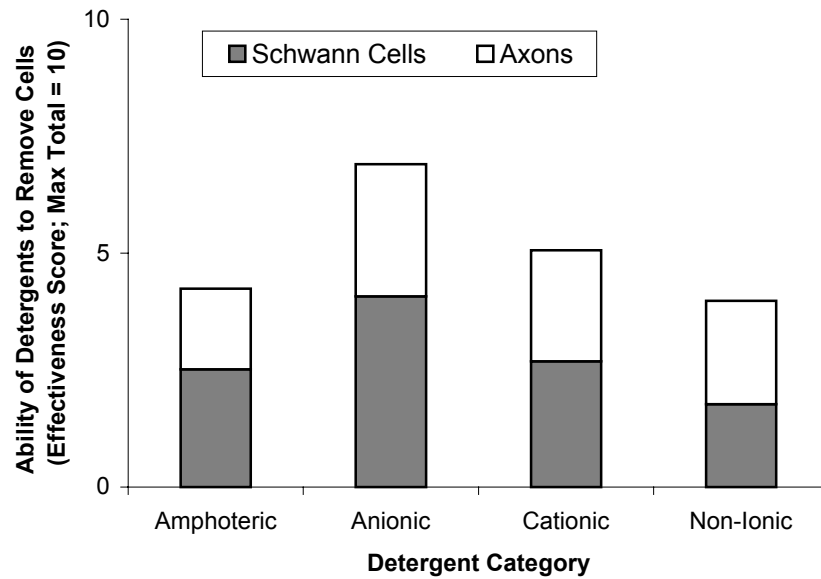


Figure 4.2: Effectiveness of detergents at cellular removal, based on histological evaluation. The average ability of each detergent category to remove cellular material from peripheral nerve tissue is illustrated. Cellular removal was measured as a composite of the scores for removal of Schwann cells and axons. Anionic and cationic detergents removed more of the cellular components than amphoteric and non-ionic detergents.

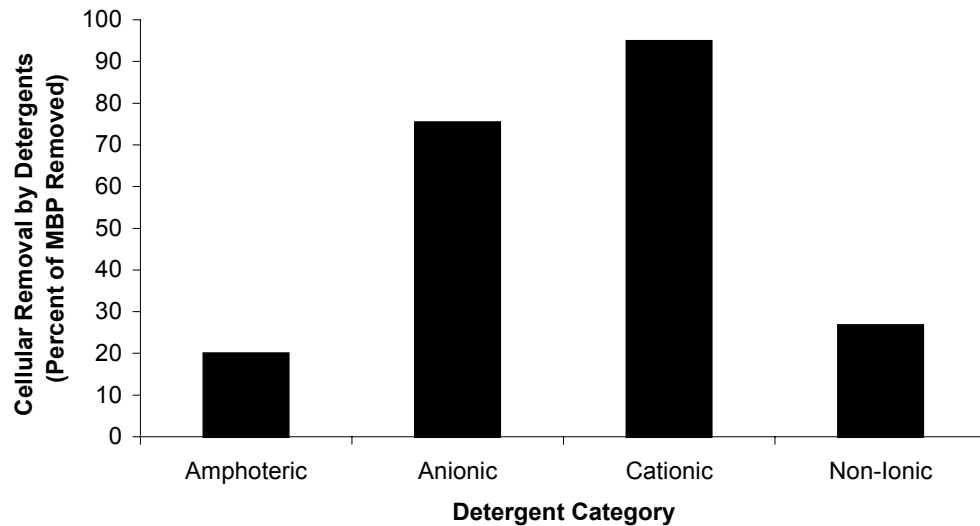
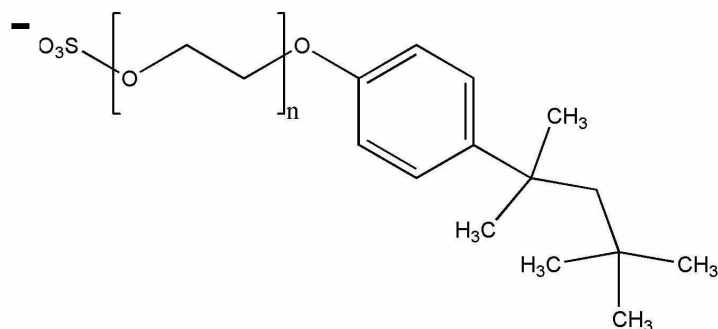
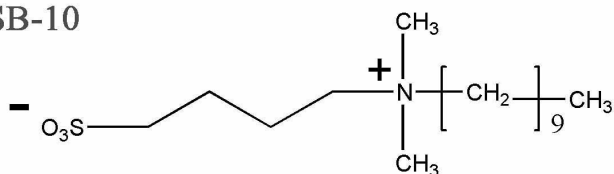


Figure 4.3: Removal of myelin basic protein (MBP) by each category of detergent, based on a Western analysis of homogenized tissues. After measuring the optical density of MBP bands in the Westerns, the concentration of MBP remaining in each detergent treated nerve segment was determined and compared to the concentration of MBP in fresh nerve tissue. On average, anionic and cationic detergents remove more cellular material than amphoteric and non-ionic detergents.

Triton X-200



SB-10



SB-16

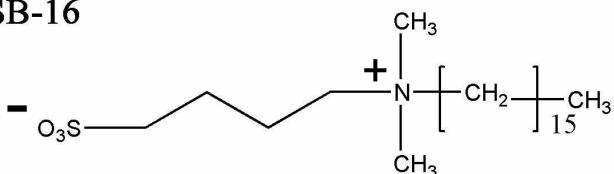


Figure 4.4: The chemical structures of the detergents selected for the optimized decellularization protocol are illustrated. The negatively charged hydrophilic head group on Triton X-200 makes it an anionic detergent. SB-10 and SB-16 have both negative and positive charges within their hydrophilic head groups, making them amphoteric. All three detergents have hydrophobic tails that allow them to penetrate and disrupt cell membranes and lipid layers (e.g., myelin).

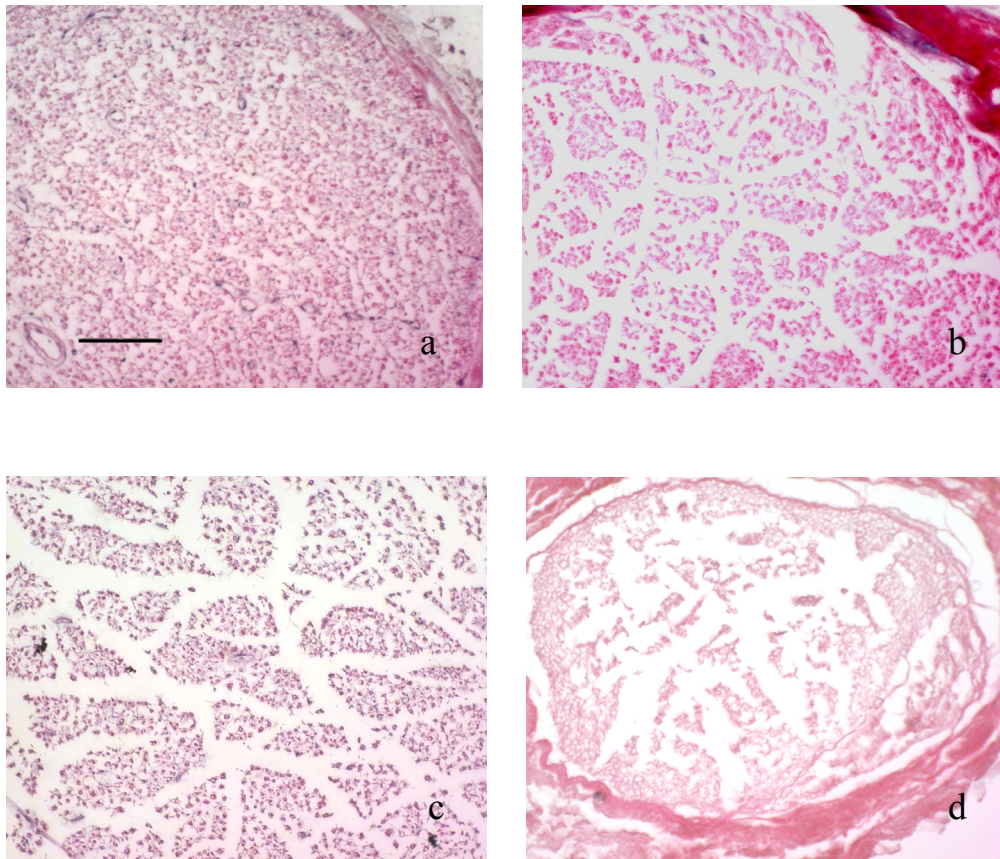


Figure 4.5: Harris Hematoxylin and Eosin stained cross-sections of rat sciatic nerve tissue were used to compare the general morphology of (a) fresh peripheral nerve to (b) tissue treated with the optimized decellularization protocol, (c) tissue treated with the freeze/thaw protocol, and (d) tissue treated with the Sondell protocol. The reduced tissue diameter and ECM fragmentation of the Sondell protocol treated tissue are evidence of structural damage. Scale bar = 100 μm .

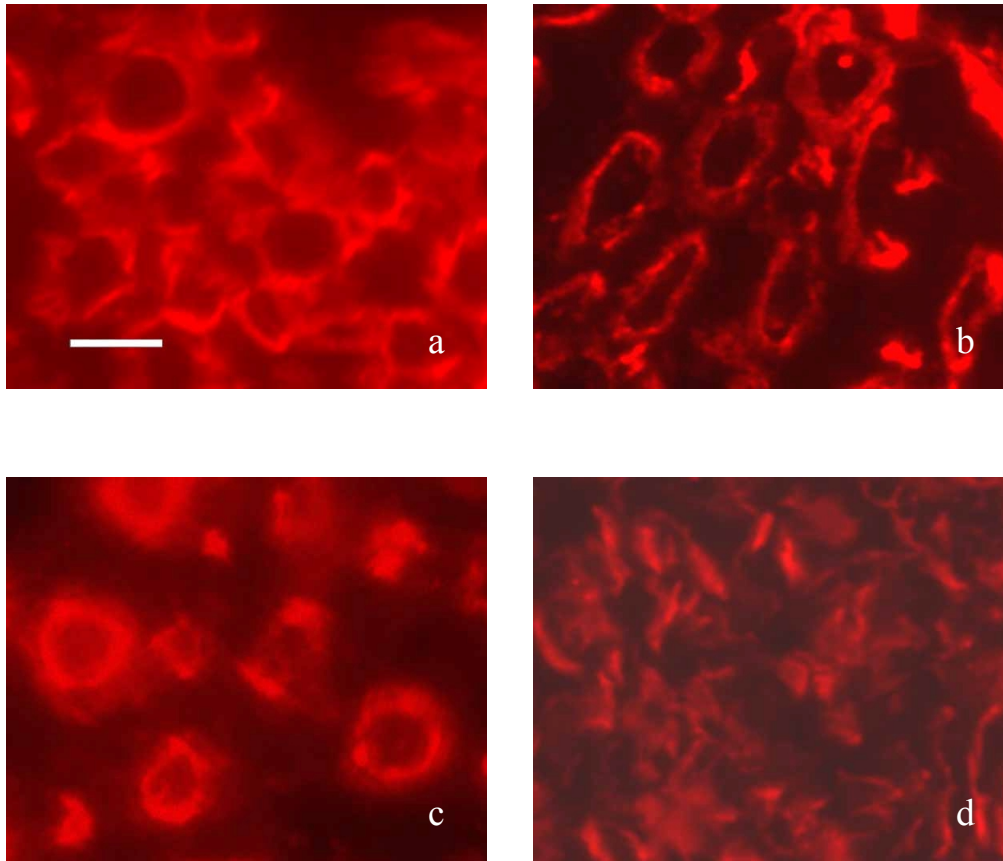


Figure 4.6: Cross-sections of basal laminae were visualized by the staining of laminin protein. The stained tissue sections were visualized under higher magnification to examine the structure of the basal laminae. The ring-like appearance of the open tubes in (a) fresh nerve tissue, (b) tissue decellularized with the optimized protocol, and (c) tissue decellularized with the freeze/thaw protocol suggest the preservation of the basal laminae. Rings are difficult to distinguish in (d) the Sondell treated tissue, suggesting that the basal laminae were damaged during the decellularization treatment. Scale bar = 10 μm .



Figure 4.7: The removal of Schwann cells was evaluated by staining tissue cross-sections for S-100 protein. The fluorescence in (a) fresh nerve tissue was considerably more intense than in nerve treated with (b) the optimized decellularization protocol and (c) the Sondell protocol. Image exposure times were constant for all three images, suggesting that both decellularization treatments removed Schwann cells. Scale bar = 100 μm .

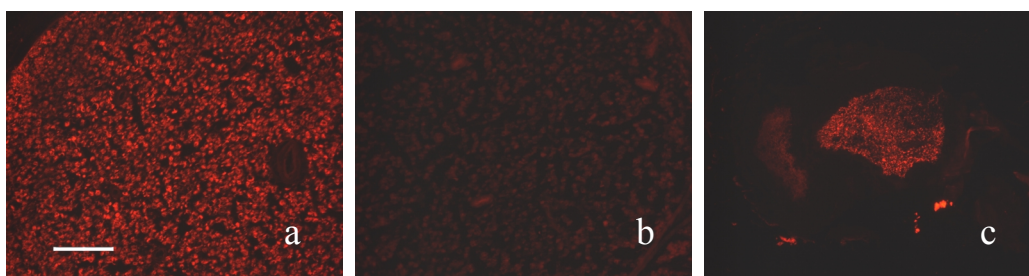


Figure 4.8: The removal of myelin and cell membranes was evaluated by staining tissue cross-sections for lipids. The fluorescence in (a) fresh nerve tissue was again considerably more intense than in nerve treated with (b) the optimized protocol and (c) the Sondell protocol. Image exposure times were constant for all three images. Scale bar = 100 μm .

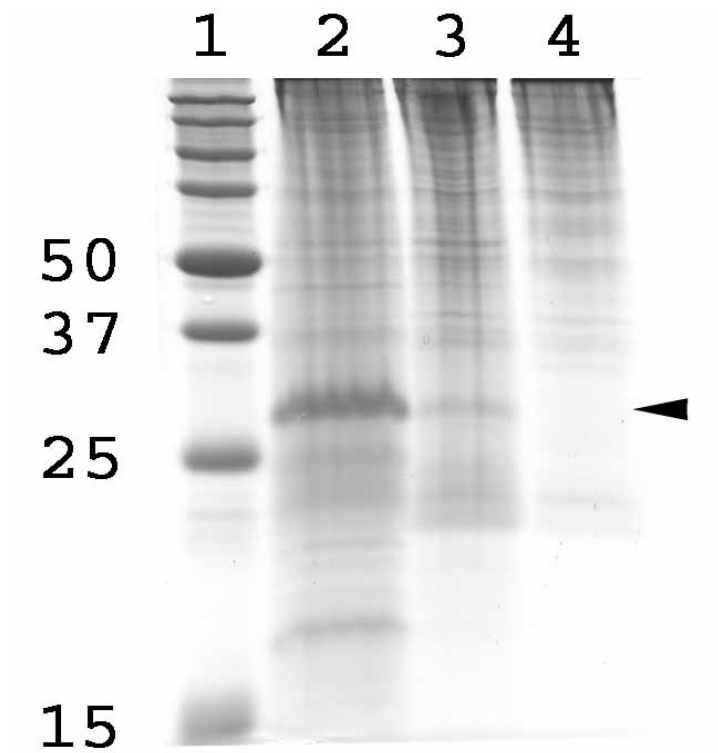


Figure 4.9: Proteins from homogenized tissue samples were separated by electrophoresis on acrylamide gels and visualized with a Colloidal Blue stain. Lane 1 contains molecular weight markers (units are in kDa). Lane 2 contains fresh nerve tissue. Lanes 3 and 4 contain tissue treated with the optimized decellularization protocol and the Sondell decellularization protocol, respectively. An arrowhead denotes the band that is presumed to be myelin protein zero (P0), a 28-30 kDa glycoprotein. Both decellularization treatments visibly reduce the P0 concentration.

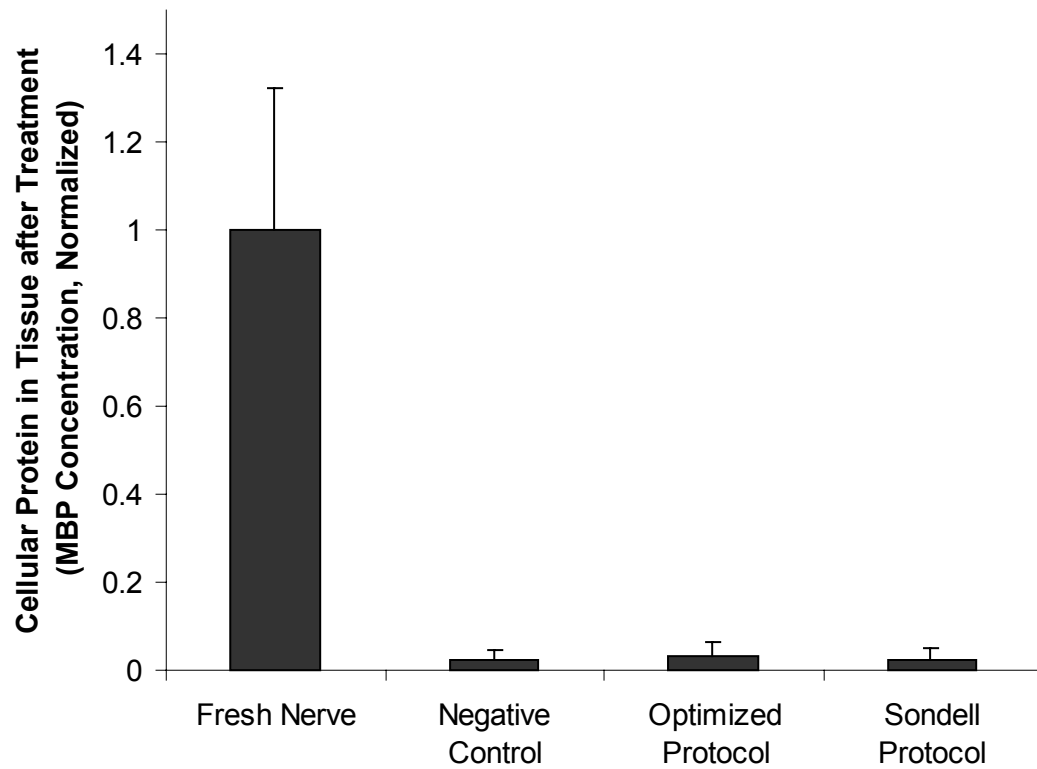


Figure 4.10: Western analysis was used to calculate the MBP concentration in tissue samples before and after decellularization. The data were normalized based on the concentration in fresh nerve tissue. Both the optimized decellularization treatment and Sondell treatment yield MBP concentrations statistically indistinguishable from the negative control (sample buffer).

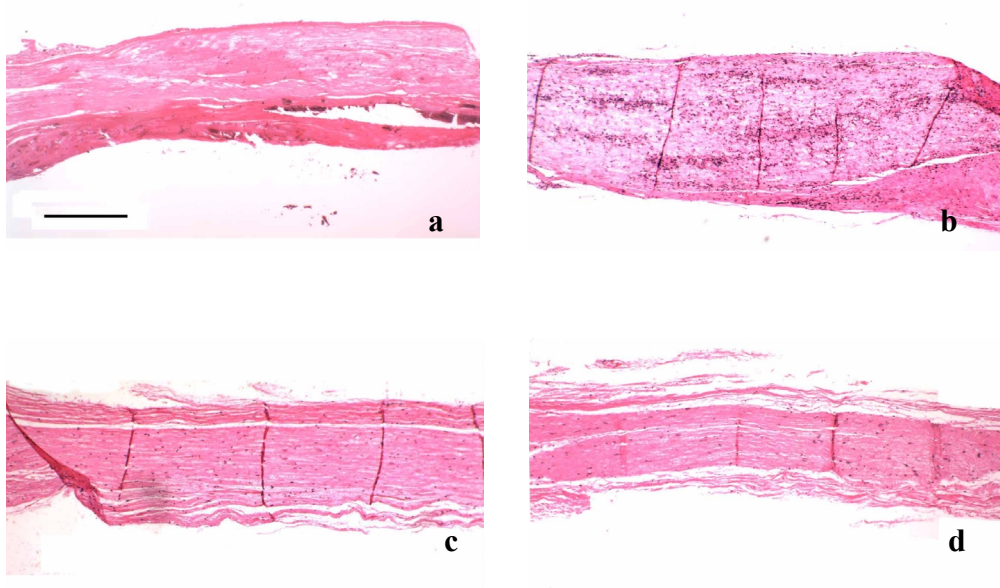


Figure 4.11: Longitudinal sections of tissue were cut from (a) fresh isografts, (b) fresh allografts, (c) optimized acellular isografts, and (d) optimized acellular allografts that were harvested 28 days after implantation. The tissue sections were stained for CD8a, a surface marker on cytotoxic T-cells. The level of staining in the fresh allografts was visibly higher, but the optimized acellular grafts appeared indistinguishable from the fresh isografts.

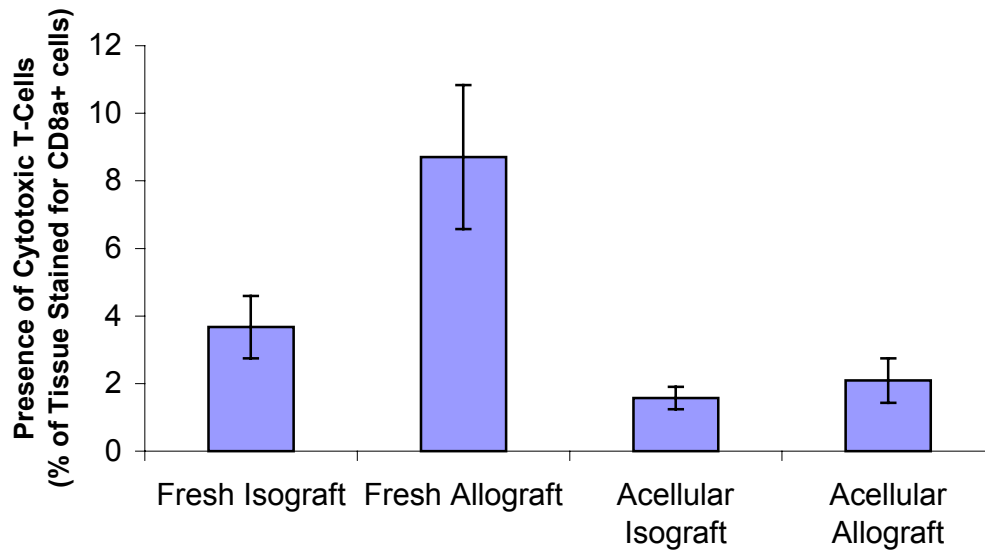


Figure 4.12: The cell-mediated immune response in the fresh and acellular nerve grafts was evaluated by determining the percentage of tissue covered by CD8a+ cells. Fresh allografts demonstrated a statistically significant elevation in CD8a+ cells. The optimized acellular isografts and allografts were statistically indistinguishable from the fresh isografts, indicating that after 28 days, cell-mediated immune rejection was only occurring in the fresh allografts.

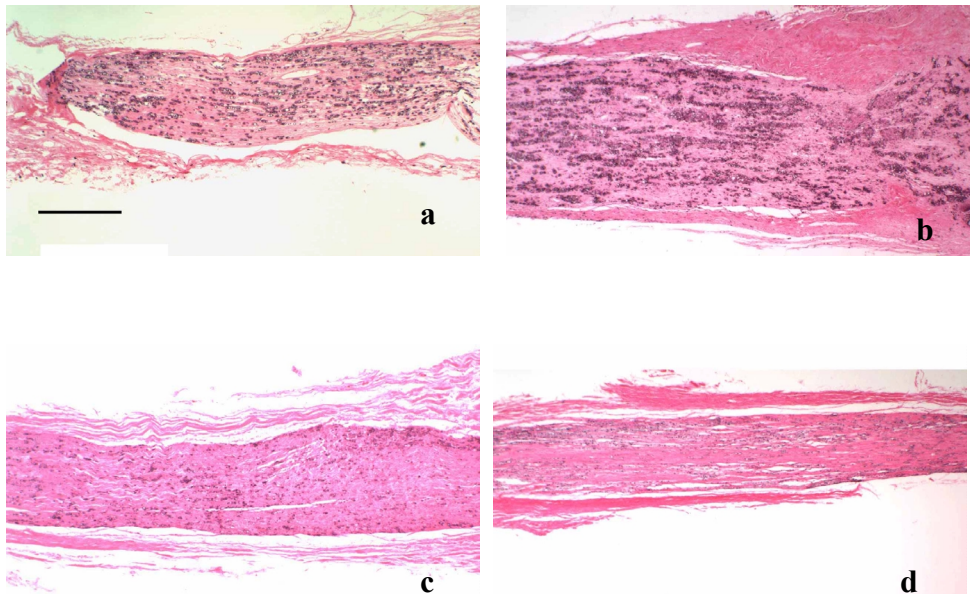


Figure 4.13: Longitudinal sections of tissue were cut from (a) fresh isografts, (b) fresh allografts, (c) optimized acellular isografts, and (d) optimized acellular allografts that were harvested 28 days after implantation. The tissue sections were stained for macrophages, immune cells involved in Wallerian degeneration, nerve regeneration, and tissue inflammation.

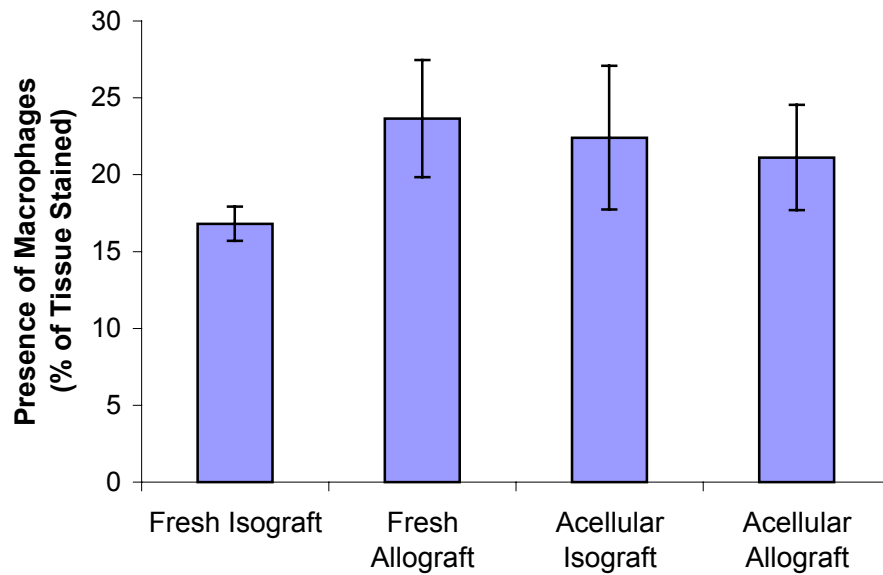


Figure 4.14: The level of macrophages present in fresh and acellular nerve grafts after 28 days was evaluated by determining the percentage of area stained in longitudinal tissue sections. Fresh allografts demonstrated a statistically significant elevation in macrophages compared to fresh isografts. The optimized acellular isografts and allografts were statistically indistinguishable from the fresh isografts, the fresh allografts, and each other, suggesting that any residual chemicals in the graft did not cause a significant inflammatory response.

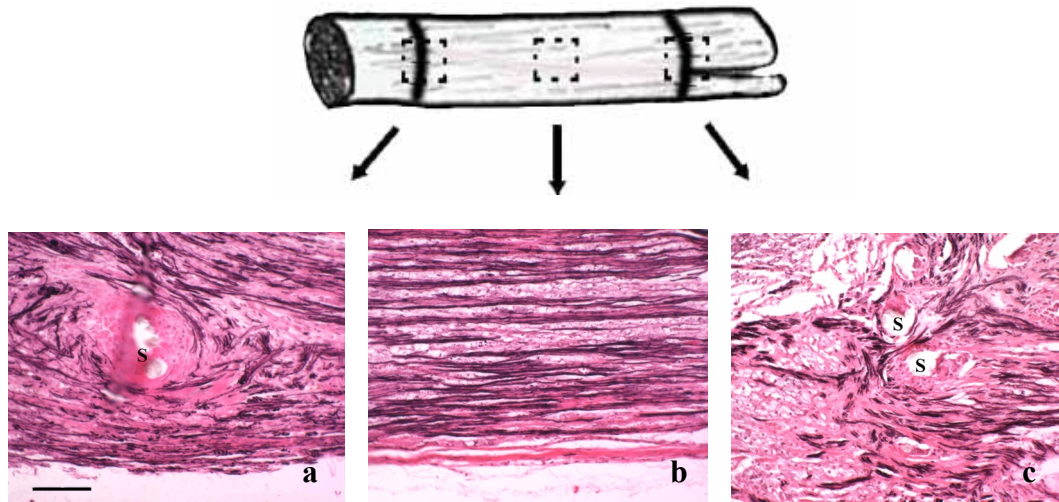


Figure 4.15: Axonal regeneration through acellular nerve grafts was demonstrated by staining longitudinal tissue segments for neurofilaments. The random patterns in the axons at the junctions of the proximal nerve and graft (a) and the graft and distal nerve (c) suggest a lack of guidance as the axons crossed into and out of the graft. However, at the midpoint of the graft (b) the axons were highly aligned, suggesting they were guided by the extracellular structure of the graft. Suture marks (S) at the nerve/graft junction can be seen in images A and C. Scale bar = 100 μm .

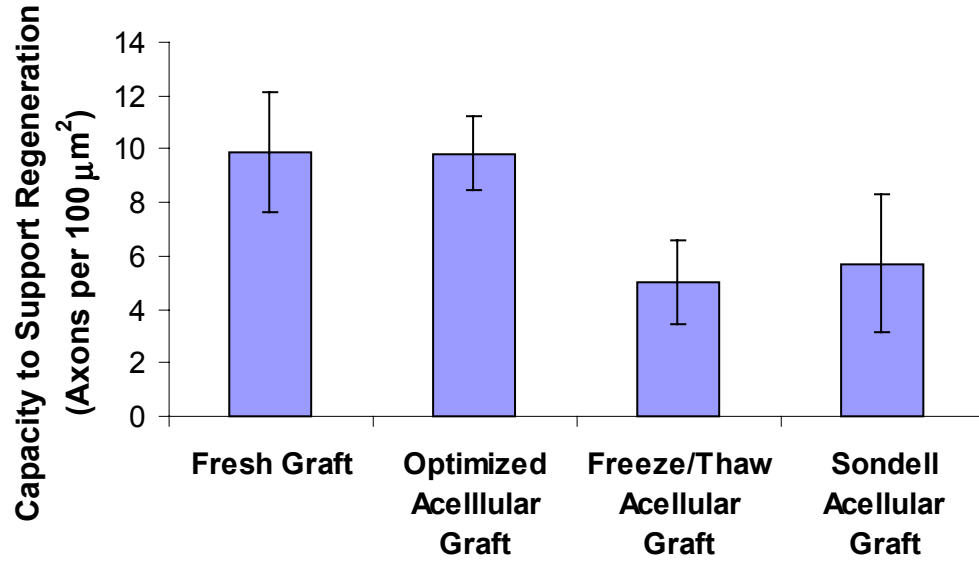


Figure 4.16: The regenerative capacity of four nerve graft models was evaluated by measuring axon density in the grafts 28 days after implantation. Fresh isografts served as a model for the autograft (positive control). Axon density in the fresh grafts and the optimized acellular grafts was statistically indistinguishable. Freeze/thaw grafts had the lowest axon density, implying that the presence of cellular debris may reduce the regenerative capacity of an acellular graft. The Sondell grafts also demonstrated a statistically lower axon density than the optimal acellular grafts, suggesting that preservation of the ECM increased the regenerative capacity of acellular grafts.

Chapter 5: Discussion

5.1 INTRODUCTION

An alternative method for treating severed peripheral nerves is needed to avoid the requirement of multiple surgeries, donor site morbidity, and other drawbacks associated with the autograft. Acellular nerve grafts are derived from donor nerve tissue (e.g., cadaver tissue), so they are composed of proteins endogenous to nerve tissue. Because of their natural composition and the fact that axons preferentially grow through the basal lamina tubes found in nerve tissue, acellular nerve grafts exhibit potential for use as a next generation nerve graft. Before acellular grafts can be used clinically, however, they must be immunologically tolerated and support regeneration as well as the autograft. Based on a thorough examination of the impact of detergents on peripheral nerve tissue, we have developed a method of removing the cellular material responsible for immunological rejection while also preserving the ECM of nerve tissue. Nerve grafts created with this decellularization treatment were tested in vivo and determined to be immunologically tolerated and capable of supporting regeneration better than previously published acellular graft models and similar to the fresh nerve graft.

5.2 IMPACT OF DETERGENTS ON NERVE TISSUE

The first step in creating an optimized acellular nerve graft was a thorough examination of the effects of different detergents on cellular removal and structural preservation in peripheral nerve tissue. Results from that experiment were used to develop an optimized acellular nerve graft with a well-preserved ECM. Based on histological and Western analysis of detergent treated tissues, it

was determined that detergent selection plays a key role in creating an optimized natural acellular graft. The importance of selecting proper chemicals for the decellularization treatment was demonstrated by the varied effects of detergents from different classifications (e.g., anionic or amphoteric) on nerve tissue (Figures 4.1 and 4.2).

5.3 SELECTION OF DETERGENTS FOR DECELLULARIZATION TREATMENT

Of the detergents tested in this study, Triton X-200 was identified as the detergent most capable of chemically decellularizing nerve tissue while also preserving the structure of the ECM. In the initial histological analysis for overall detergent effectiveness, it was determined that Triton X-200 and dodecylbenzene sulfonate were the best detergents for accomplishing our goals (Table 4.1). In a subsequent experiment based on Western analysis, Triton X-200 removed 47.4% more of the Schwann cell protein MBP than dodecylbenzene sulfonate (Table 4.2). Thus, for the purposes of creating an acellular nerve graft with a well-preserved ECM, Triton X-200 appeared to be an optimal detergent with which to create a decellularization protocol. However, the cellular removal achieved with Triton X-200 treatment under the original experimental conditions needed to be improved while maintaining structural preservation.

Literature suggested that combining an amphoteric detergent with an anionic detergent would lead to tighter packing of the detergent micelles [117]. In a micelle of anionic detergents, the packing density is limited by the charge repulsion of the head groups. Packing density is related to detergency because it affects the size and the durability of the micelles that penetrate into the tissue. In a solution containing an amphoteric and an anionic detergent, the neutrally charged amphoteric molecules will be interspersed between the anionic molecules,

lowering the repulsion between head groups and increasing the packing density of the micelles. It was hypothesized that this tighter packing would result in smaller and more robust micelles, better penetration into the tissue, and better removal of hydrophobic materials (e.g., myelin, cell membranes). Thus, an optimized decellularization protocol was developed that used Triton X-200 in combination with the two most effective amphoteric detergents in this study, SB-10 and SB-16 (Table 4.1).

5.4 OPTIMIZED PROTOCOL ACHIEVES GOALS OF CELLULAR REMOVAL AND ECM PRESERVATION

The acellular grafts created with this optimized decellularization protocol were compared to acellular grafts created with the Sondell protocol, a published chemical decellularization treatment [125, 126]. The optimized decellularization protocol and the Sondell protocol both decellularize nerve tissue, removing the majority of lipids and cellular proteins. Decellularization by the protocols was demonstrated with histology (Figures 4.7 and 4.8), Colloidal Blue staining of proteins separated in acrylamide gels (Figure 4.9), and Western analysis (Figure 4.10). However, the decellularization protocols affect the ECM of the tissue differently. The Sondell protocol uses Triton X-100 and sodium deoxycholate, while the optimized treatment uses Triton X-200, SB-10 and SB-16. Comparing these detergents individually, Triton X-100 and sodium deoxycholate were both found to cause more structural damage to nerve tissue than Triton X-200, SB-10 or SB-16 (Table 4.1, Appendix A). Comparing the decellularization protocols, the Sondell protocol was found to cause more damage to the general morphology (Figure 4.5) and the basal laminae (Figure 4.6) than the optimized protocol. In the Sondell protocol, the tissue contracted during treatment with sodium deoxycholate. Subsequently, a 30% increase in tissue diameter and weight was

observed upon transferring the tissue to distilled water (data not shown). It is possible that this swelling is partially responsible for the structural damage caused by the Sondell protocol. The optimized protocol was designed to avoid rapid transitions from detergent solutions to distilled water so that the tissue is not damaged by such osmotic stresses. As a result, the natural structure of the ECM is better preserved in the optimized grafts, especially the basal lamina tubes that help to guide regenerating axons [36-38].

5.5 IMMUNOLOGICAL TOLERANCE OF OPTIMIZED GRAFTS WAS CONFIRMED

Cellular antigens are predominantly responsible for the immunological rejection associated with nerve allografts, particularly the antigens associated with Schwann cells, endothelial cells, and macrophages [36-38]. The removal of cellular components from the graft by the optimized protocol was correlated with the immunological response of a host animal of a different strain than the donor animal. The major histocompatibility complex (MHC) of the rat is called RT1 and is highly polymorphic [127]. Rat strains can be characterized by their RT1 haplotype, the combination of several alleles in the gene cluster. Matching of haplotypes plays a predominant role in allograft survival. Gulati et al. demonstrated that in allografts involving strains of different RT1 haplotypes, the increased presence of immune cells associated with rejection was readily detectable at 28 days [37]. Thus, fresh nerve tissue from an HSD rat (RT1 b) implanted into a Lewis rat (RT1 l) (i.e., a fresh allograft) should display signs of immunological rejection after 28 days. Similarly, an acellular allograft should be rejected if the graft contains membrane bound antigens associated with the RT1 haplotype.

Rat cytotoxic T-cells carry a CD8a cell surface marker (i.e., they are CD8a⁺ cells), and the presence of cytotoxic t-cells is an important indicator of cell-mediated graft rejection. However, a moderate number of CD8a⁺ cells that are not cytotoxic should be present in any nerve graft after 28 days, whether or not it is being rejected. The non-cytotoxic CD8⁺ cells are a subset of macrophages that are known to invade after sciatic nerve injuries, even in the absence of immunological rejection [128]. Macrophages are immune cells that respond to nerve injury [129], clear cellular debris during nerve degeneration [130], and support regeneration by inducing and producing growth factors and adhesion molecules [131]. In the case of a rejected allograft, higher numbers of macrophages should be present [132]. However, macrophages also respond to other cues in the regenerating nerve, so an increase in macrophages without a concomitant increase in CD8a⁺ cells does not indicate rejection. Thus, the presence of CD8a⁺ and macrophage cells was anticipated in all four grafts being tested. However, a statistically significant increase in both CD8a⁺ and macrophage cells in a graft, when compared to a fresh isograft, would indicate cell-mediated graft rejection.

As anticipated, the fresh allografts did exhibit a statistical increase in both CD8a⁺ cells and macrophages, compared to fresh isografts (Figures 4.12 and 4.14). The acellular grafts did not show any increase in CD8a⁺ cells, compared to fresh isografts, so they did not elicit cell-mediated immune rejection. Macrophage invasion into the acellular grafts did appear to be slightly higher than in the fresh isografts, though not significantly. We do not believe this was the result of immunological rejection because the acellular isografts and allografts demonstrated similar levels of macrophage cells, as well as CD8a⁺ cells. One possible cause for the elevation in the level of macrophages is that the open basal

lamina tubes and the absence of myelin allowed a higher number of macrophages to invade and remain inside the acellular grafts. This may be beneficial since macrophages produce growth factors and adhesion molecules. By removing cellular components from nerve tissue, the antigens that would normally initiate cell-mediated immunological rejection of an allograft were removed, and the acellular grafts were immunologically tolerated after 28 days in vivo.

5.6 OPTIMIZED ACELLULAR GRAFTS SUPPORT REGENERATION

When engineering the optimized acellular grafts, the two major goals were to remove cellular material and to provide structural support for regenerating nerves. It was believed that accomplishing those two goals would lead to improved levels of regeneration in comparison to the levels seen with other acellular grafts. The importance of structural support was evident with histological examination of longitudinal tissue sections. Axons grew linearly in regions of defined structure (e.g., in the nerve graft and distal nerve cable), but their path was irregular in the regions where the graft was attached to the nerve ends (Figure 4.15). The irregular patterns were potentially caused by the misalignment of basal laminae at the junctions between the nerve ends and the graft. As the axons crossed into and out of the graft, they had to find new basal laminae to provide them with guidance.

5.7 REGENERATIVE CAPACITY CORRELATED TO GRAFT STRUCTURE AND CONTENT

In addition to providing guidance, the optimized acellular grafts also supported higher axon densities than the other published acellular graft models (Figure 4.16). The lowest axon densities were found in the freeze/thaw grafts. While the structural preservation in the freeze/thaw grafts was similar to that in

the optimized grafts (Figures 4.5 and 4.6), the freeze/thaw procedure was the only decellularization procedure that did not wash dead cells out of the grafts. Thus, a correlation is suggested between the presence of cell debris and a reduction in the level of nerve regeneration. The primary difference between the Sondell protocol and the optimized decellularization protocol was preservation of the ECM. Consequently, the higher axon density in the optimized acellular grafts suggests that providing regenerating axons with an ECM structure that mimics that of native nerve is important for maximizing regeneration in an acellular graft.

Since fresh isografts were the only grafts that contained living cells (e.g., Schwann cells) that aid regeneration, they were expected to support higher axon densities than any of the acellular grafts. However, the data suggest that in 10 mm nerve grafts, the combination of proper structure and the removal of cellular debris were sufficient to attain axon densities statistically indistinguishable from those in fresh isografts.

5.8 CONCLUSIONS

In summary, this work presents a detailed study of the effect of twelve different detergents, representing the four major categories of detergents, on the general morphology, ECM, and cellular components in peripheral nerve tissue. These results led to an optimized decellularization treatment for peripheral nerve tissue that selectively removes cellular components while preserving the ECM. Acellular grafts created with this optimized decellularization treatment were demonstrated to be immunologically benign after 28 days *in vivo* and they demonstrated a higher regenerative capacity than previously published acellular graft models. This work suggests that when engineering a synthetic or natural peripheral nerve graft to replace the autograft, removing cellular debris and

providing an ECM structure that closely mimics native nerve are critical factors for attaining optimal nerve regeneration.

Chapter 6: Conclusions and Recommendations

This dissertation describes the rational design and testing of an optimized acellular peripheral nerve graft. Based on a thorough examination of the effects of various detergents on nerve tissue, a chemical treatment using Triton X-200, SB-10, and SB-16 was developed to remove cellular material from nerve tissue while preserving the natural ECM structure. Nerve grafts created with this decellularization protocol demonstrated the ability to support and guide regenerating nerves without eliciting the cell-mediated immune response typically associated with transplanted tissues.

Regeneration through the optimized acellular grafts was compared to regeneration through three other published nerve graft models. One of these models was the Sondell graft, a chemically decellularized nerve graft. The second model was the freeze/thaw graft, the most common acellular nerve graft model in the literature. In initial experiments, the regenerative capacity of the optimized acellular graft appears to surpass both of these acellular models. The third model was the fresh isograft, an experimental model that mimics the autograft. The regenerative capacity of the optimized acellular graft was statistically indistinguishable from the regenerative capacity of the fresh isograft. These findings suggest that the absence of cellular debris and the presence of a natural extracellular structure are advantageous for supporting nerve regeneration in an acellular nerve graft. The similarity in axon densities observed in the optimized acellular grafts and the fresh isografts suggest that regeneration levels superior to those seen in the autograft could potentially be achieved by incorporating additional stimulatory factors (e.g., NGF) into the optimized acellular graft.

To more fully evaluate the regenerative capacity of the optimized acellular grafts in comparison to these other models, further studies need to be conducted. Longer time points need to be used, functional recovery must be evaluated, and the studies should utilize more animals (e.g., $n=30$ per experimental condition) for improved statistical significance. These long-term studies should be performed with allografts to ensure that the grafts are not rejected even after several months. Additionally, control groups should be included to examine the impact of graft rejection and inflammation on regeneration. The correlation between rejection and regeneration can be examined by allowing fresh allografts to endure complete rejection. Because of the pain the animals may endure during the later stages of cell-mediated graft rejection, this experiment was postponed until the potential of the optimized grafts was assessed. The correlation between inflammation and regenerative capacity can be examined by implanting grafts that contain a high concentration of residual detergent (i.e., the final washing step in the decellularization process is omitted). Again, this experiment was postponed until the viability of the optimized graft was established. Based on the promising results detailed in this work, such additional experiments should now be performed.

This work demonstrates the importance of a natural ECM structure in nerve regeneration, but other questions about the role of the ECM still persist. For example, why are axons preferentially attracted to the laminin surface on the interior side of the basal laminae? Is the bioactivity of the laminin protein more important than the physical structure of the basal laminae? Furthermore, as micro-fabrication techniques improve, it will become possible to engineer a synthetic nerve graft that closely mimics the structure of native nerve, complete with artificial basal laminae. Laminin protein or adhesive peptide sequences found in

laminin could be incorporated into the graft to impart bioactivity. In the future, such a graft may potentially become an off-the-shelf treatment for severed peripheral nerves.

Because the optimized acellular graft presented in this work is a simpler model than the autograft, it can also serve as a tool with which to study individual facets of the regenerative process. Cells, bioactive factors, or both, can be selectively incorporated into the acellular graft to study their impact. For example, hyaluronic acid (HA) could be incorporated into the graft. HA is a glycosaminoglycan found in the extracellular spaces of the body, and HA fragments that result from its degradation are known to promote angiogenesis [133]. Thus, incorporation of HA into the optimized acellular graft could be used to study the role of a vascular network in nerve regeneration.

In conclusion, it appears that a crucial design criterion for the next generation of nerve grafts is an ECM that mimics the structure of native nerve tissue. Using the optimized acellular graft described in this work, more information can be gained about the role of the ECM, support cells, growth factors, and biological processes that influence nerve regeneration. With this knowledge, grafts superior to the autograft can be engineered for treating severed peripheral nerves. Those grafts could be assembled on a completely synthetic scaffold, or they may be built on a foundation of the optimized acellular graft presented here.

Appendices

APPENDIX A: HEMATOXYLIN AND EOSIN STAINING OF TISSUE SECTIONS

Materials

Glass containers
Plastic containers
Dipping rack
Glass coverslips
Ethanol
Mayer's Hematoxylin
Eosin Y (Electron Microscopy Sciences)
Magnesium sulfate
Sodium bicarbonate
Distilled de-ionized water (ddH₂O)
Acetic acid, glacial
Xylene, histological grade
Permout mounting medium

Preparation of Solutions

Eosin

2 g Eosin Y
230 ml ddH₂O
570 ml Ethanol
Immediately prior to use, add 4 ml glacial acetic acid

Scott Solution

10 g Magnesium sulfate
2 g Sodium bicarbonate
Add ddH₂O to 1000 ml

Methods

Glass containers should be used for xylene. Plastic containers can be used for the other solutions. Place slides with tissue sections on them in the dipping rack. Then, dip the slides in the following solutions:

Xylene, 3 changes	2 min each
Absolute alcohol	10 dips
Alcohol, 95%, 2 changes	10 dips each
ddH ₂ O water	rinse until water runs off evenly
Hematoxylin	1 to 3 min
ddH ₂ O water, 2 changes	5 dips each
Scott solution	dip until blue

ddH ₂ O water, 2 changes	10 dips each
Eosin	10 dips
Alcohol, 95%, 2 changes	10 dips each
Absolute alcohol, 3 changes	10 dips each
Xylene, 3 changes	10 dips each

Apply 10-15 µl of Permount mounting medium to the slides. Slowly lower a coverslip onto each slide. Lightly press on the coverslips to remove any air bubbles.

APPENDIX B: IMMUNOSTAINING OF TISSUE SECTIONS

Materials

Pepsin
Phosphate buffered saline (PBS)
Glass containers
Plastic containers
Dipping rack
Glass coverslips
Ethanol
Eosin Y
Distilled de-ionized water (ddH₂O)
Xylene, histological grade
Permout mounting medium
Vectashield fluorescent mounting medium (Vector Labs)
3,3'-diaminobenzidine (DAB) substrate staining kit (Vector Labs)
Rubber cement (Elmer's)
Syringe with an 18 gauge needle
2 M Hydrochloric acid
Bovine serum albumin (BSA) (Jackson ImmunoResearch)
Fingernail polish

Preparation of Solutions

Pepsin digestion solution

9.5 ml of ddH₂O
0.5 ml of 2 M HCl
20 mg of pepsin
Store at 4°C for up to 1 week or aliquot and freeze. Avoid repeated freeze and thawing.

5% BSA

Described in the gel electrophoresis and Western analysis protocol (Appendix D)

Eosin

Described in the hematoxylin and eosin staining protocol (Appendix A)

Methods

Partially fill 3 glass containers with xylene. Plastic containers can be used for the ethanol solutions. Place slides with tissue sections on them in the dipping rack. Then, dip the slides in the following solutions:

Xylene, 3 changes	2 min each
100% Ethanol, 2 changes	10 dips each
95% Ethanol	5 dips
90% Ethanol	5 dips

70% Ethanol	5 dips
50% Ethanol	5 dips
30% Ethanol	5 dips
ddH ₂ O	2 min

Fill a syringe with rubber cement. Remove the slides from the water and dry off the glass surrounding the tissue sections. With the rubber cement, make a well around the tissue sections on each slide and allow the rubber cement to dry.

Depending on the protocol for each antibody, the tissue sections may or may not be incubated in the pepsin digestion solution. If the protocol calls for pepsin digestion, cover the sections with the pepsin digestion solution and incubate the slides for 30 min at 37°C. Then, wash the slides 3 times for 5 min in PBS. If the protocol does not call for pepsin digestion, skip to the next step.

Cover the tissue sections with a 5% BSA solution and incubate them for 30 min at room temperature. Then, dip the slides in PBS and wick off excess solution with a Chemwipe.

Cover the tissue sections with the primary antibody solution. Approximately 200 µl is usually required to cover the sections. Incubate the sections in the primary solution as outlined for that specific antibody. After incubation, wash the slides 5 times for 5 min in PBS and then once in ddH₂O.

Wick off the excess solution with a Chemwipe, and then cover the sections with secondary antibody solution. Incubate as outlined for that specific antibody. After incubation, wash the slides 5 times for 5 min in PBS and then once in ddH₂O.

If the secondary antibody was tagged with a fluorescent molecule (e.g., TRITC), remove the rubber cement well. Then, 20 µm of Vectashield should be placed on the slide, followed by a coverslip. Ensure that the sections are completely covered by the mounting medium, and then seal the coverslip to the slide with fingernail polish.

If, however, the secondary antibody was tagged with horseradish peroxidase, incubate the sections in DAB solution for 10 min at room temperature. Next, wash off the DAB and remove the rubber cement well. Place the slides in the dipping rack and dip the slides in the following solutions:

Eosin (if counterstaining desired)	10 dips
95% Ethanol, 2 changes	10 dips each
100% Ethanol, 3 changes	10 dips each
Xylene, 3 changes	10 dips each

Apply 10-15 µl of Permout mounting medium to the slides. Slowly lower a coverslip onto each slide. Lightly press on the coverslips to remove any air bubbles.

Antibodies and Dilutions

Primary	Dilution	Incubation	Secondary	Dilution	Incubation
S-100	1:400	Ovnt at 4°C	GAR FITC	1:200	1 hr at RT
Neurofilament 200	1:200	Ovnt at 4°C	GAM TRITC	1:400	1 hr at RT
Coll I	1:100	Ovnt at 4°C	GAR FITC	1:200	1 hr at RT
Laminin	1:25	Ovnt at 4°C	GAM TRITC	1:400	1 hr at RT
RT97	1:25	Ovnt at 4°C	GAM HRP	1:100	1 hr at RT
Macrophage	1:75	30 min at 37°C	GAM HRP	1:100	1 hr at RT
CD8a	1:50	Ovnt at 4°C	GAM HRP	1:100	1 hr at RT

Abbreviations: Ovnt = overnight, GAR = goat anti-rabbit IgG, GAM = goat anti-mouse IgG, FITC = fluorescein isothiocyanate, TRITC = tetramethyl rhodamine isothiocyanate, HRP = horseradish peroxidase, RT = room temperature

APPENDIX C: GEL ELECTROPHORESIS AND WESTERN ANALYSIS PROTOCOL

Materials

Vacuum flask
Vacuum Pump
Powerpac 200 power supply with tanks (BioRad)
Mini PROTEAN 3 Electrophoresis System (1.0 mm glass spacer plates) (BioRad)
Multi-gel caster (BioRad)
Prot/Elec Protein Gel Loading Pipet Tips (BioRad)
Mini Trans-Blot Electrophoretic Transfer Cell (BioRad)
PVDF membranes (BioRad)
60 cc Syringe with 21-gauge needle
20 cc Syringe with 21-gauge needle
Lazy-susan
Medium Tupperware container (~1000 ml)
Small Tupperware container with Methanol (~250 ml)
Stir plate
Rocker
Magnetic stir bar
Roller
De-ionized distilled water (ddH₂O)
Sodium dodecyl sulfate (SDS)
1,4-Dithio-DL-threitol (DTT)
Bromophenol blue
30% Acrylamide solution (BioRad)
Ammonium persulfate (APS)
1,2-Bis(dimethylamino)ethane (TEMED)
N-Butanol
Tris (Fisher Scientific)
10X Tris-glycine solution (BioRad)
Concentrated hydrochloric acid
Precision Plus Protein Standards (BioRad)
Bovine Serum Albumin (BSA) (Jackson ImmunoResearch)
Igepal CA-630 (NP40)
SuperSignal ECL kit (Pierce)

Preparation of Buffers and Solutions

4X Separating buffer

18.15 g Tris (1.5 M)
Add ddH₂O to 75 ml
Adjust pH to 8.8 with conc. HCl (~3 ml)
Add ddH₂O to 100 ml
Store up to 3 months in dark at 4°C

4X Stacking buffer

3.0 g Tris base (0.5 M)

Add ddH₂O to 40 ml

Adjust pH to 6.8 using conc. HCl (~2 ml)

Add ddH₂O to 50 ml

Store up to 3 months in the dark at 4°C

Water-saturated n-butanol

50 ml n-butanol

5 ml ddH₂O

Combine and shake – use top phase to overlay gels

Store at RT indefinitely

10% SDS

10 g SDS

Add ddH₂O to 100 ml

Store up to 6 months at room temperature

10% APS

0.1 g Ammonium persulfate

Add ddH₂O to 1 ml (~700 µl)

Use fresh, no more than 1 week old

Transfer Buffer

100 ml 10X Tris-glycine stock solution

900 ml ddH₂O

Store at 4°C

Tank Buffer

100 ml 10X Tris-glycine stock solution

10 ml 10% SDS

890 ml ddH₂O

Store up to 1 month at RT

TBS (Tris buffered saline)

12.11 g Tris (100 mM)

900 ml ddH₂O

Adjust pH to 7.5 with conc. HCl (~7ml)

9 g NaCl

Add ddH₂O to 1 L

Store up to 3 years (if sterile) at 4°C

5% BSA

50 g BSA

1000 ml TBS

Filter-sterilize and store at 40°C

10X NP40 buffer

60.6 g Tris (500 mM)

87.7 g NaCl (1.5 M)

18.61 g EDTA (50 mM)

5 ml NP40

Add ddH₂O to 1 L

Final pH should be 7.5

Store at RT

4X Sample Buffer

4g SDS

20mL glycerol

25mL 4X stacking buffer

3.084g DTT

0.4mL of 50mg/mL stock

Store aliquots at -20°C for up to 6 months

Methods

Casting Separating Gels

In vacuum flask, combine:

ddH ₂ O	11.64 ml
4X separating Buffer	12.36 ml
10% SDS	495 µl
30% acrylamide	24.84ml

De-aerate the solution with a vacuum pump for at least 15 minutes. Meanwhile, arrange the 1.0 mm glass plates and short plates in the multi-caster. Make sure all the equipment is completely dry, otherwise the plates will not seal well. Be sure to place plastic separating sheets between each pair of glass plates, and ensure that the short plates are flush with the bottom of the multi-caster. Place the multi-caster on the lazy-susan. Remove the plunger from the 60 cc syringe.

Remove the flask from vacuum pump carefully so as not to create bubbles.

To solution in flask, (without creating bubbles) add:

10% APS	206 µl
TEMED	20.6 µl

Gently swirl the solution to mix in the APS and TEMED. Carefully pour the solution down the side of the syringe, and then add the plunger. Fill the multi-caster to 3 cm below the top of the short plates. With the cap on the needle, stand the syringe up to polymerize the remainder of solution in the syringe.

Line the top of each gel with 300 µl of water-saturated n-butanol. Allow the solution to polymerize for an hour. The solution in the syringe can serve as an indicator of polymerization.

After the acrylamide gels are formed between the glass plates, wick off the water-saturated n-butanol. Then, remove the gel/plate units from the multi-caster and discard any excess polyacrylamide. Rinse the gel/plate units with ddH₂O. Finally, store them in 1X separating buffer at 4°C or immediately cast stacking gels on top of the separating gels.

Casting the Stacking Gels

In a vacuum flask, combine:

ddH ₂ O	5 ml
4X stacking Buffer	2.1 ml
10% SDS	83.3 µl
30% acrylamide	1.1 ml

De-aerate the solution with a vacuum pump for at least 15 minutes. Meanwhile, rinse the gel/plate units with distilled H₂O and arrange them in the casting apparatus. Remove all excess H₂O from gel/plate units with Chemwipes. Place the casting apparatus on the lazy-susan. Remove the plunger from the 20 cc syringe.

Remove the flask from the vacuum pump carefully so as not to create bubbles.

To the solution in flask, add:

10% APS solution	41.7 µl
TEMED	8.3 µl

Gently swirl the solution to mix in the APS and TEMED. Carefully pour the solution down the side of the syringe, and then add plunger. Fill the gel/plate units to 1 mm below the top of short plate. Stand the syringe up to polymerize any remaining solution. Put 100 µl of TEMED on a Chemwipe and lightly brush the TEMED onto the well combs. Insert the well combs into the gel/plate units.

Allow the solution to polymerize for an hour. The solution in the syringe can serve as an indicator of polymerization. Discard any excess acrylamide on the bottom of the plates. Rinse the gel/plate units with ddH₂O and store them in 1X separating buffer at 4°C.

Loading the Gels with Protein Solutions

Centrifuge the homogenized tissue samples for 15 s at 10,000 rpm. Remove the well combs from the gel/plate units and rinse them with ddH₂O. Place the gel/plate units in the electrophoresis apparatus. Fill the space between the gel/plate units with tank buffer. Carefully position the apparatus in the tank, using the electrodes to lift and move the apparatus. Pour tank buffer into tank in the area surrounding the apparatus until the liquid level is one half inch above wire in the bottom of the apparatus.

Place the loading guide between the gel/plate units and add 25 µl of sample (e.g., homogenized tissue solution or molecular weight standard) to each lane, using

electrophoresis pipette tips. Use caution so as not to mix solution from one lane to another. When all lanes are properly loaded, remove the loading guide and place the lid on the tank, aligning the proper leads (i.e., red on red, black on black). Turn on the power source, and enter the following settings:

200 V, 2.0 A, 50 min, constant voltage

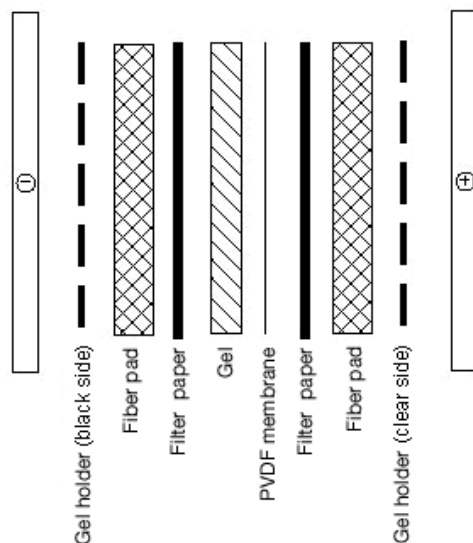
Start the power, and ensure that the gel is running; bubbling will occur and the solutions will start to move downward. If the solutions appear to be moving up instead of down, the leads are reversed. When the running time is complete, look to see where the dye front is. If it has not reached the bottom of the gel, run the gel a little longer to get the dye front to the bottom of the gel.

Transferring Proteins to the PVDF Membrane

While the gel is running, prepare the PVDF membranes as follows. Do not touch the membranes with anything except flat-headed forceps. Proteins from your hands will contaminate them. Cut a notch in top right corner of each membrane. Wet the membranes in methanol, and then place them in transfer buffer. Agitate the solution by placing it on a rocker. The membranes must equilibrate for at least 45 minutes.

When gel is finished running (i.e., the blue line is at the bottom of the gel or gone), remove the gel/plate units and rinse them with ddH₂O. Separate the glass plates with a razor blade. Use caution because the short plates are easily cracked. Cut between stacking and separating gel and down the sides of gel to loosen the gel from the sidewalls. Remove and discard the stacking gel. Cut a notch in top right corner of the gel to mark lane number 1.

Place all the fiber pads and pieces of filter paper to be used in the medium Tupperware container. Cover them with transfer buffer. Prepare the gel transfer unit in the following order:



Use care when handling the membranes, as they are easily torn. Close the holder and place it in the transfer apparatus with the black side of holder closest to the black side of the apparatus (i.e., the negative side). Place a stir bar, the transfer apparatus, and an ice pack in tank. Fill the tank with cold transfer buffer until all holes in holder are covered. Put the tank in the large Tupperware container and place it on a stir plate. Ensure that the stir bar is spinning. Place the lid on the tank with the leads properly aligned. Connect it to the power source. Fill the Tupperware container with ice and water. Set power source to the proper settings:

30 V, 2.0 A, 900 min, constant voltage

Start the power source. When the transfer is complete, the membrane is ready for Western analysis.

Incubating the Membranes for Western Analysis

Place the membrane in a container slightly larger than the gel and add enough 5% BSA solution to cover the membrane. Make sure that the side of the membrane that was in contact with the gel faces up. Ensure that the container has a tight lid so that the membrane does not dry out. Place the container on a rocker for at least 5 hours at room temperature. All subsequent incubation steps also take place on the rocker.

Make antibody solutions (primary and secondary) by diluting the stock antibody solution to the desired concentration using a diluent made of 1 part 5% BSA solution and 3 parts PBS solution. As a general rule, polyclonal antibodies work best with Westerns, but monoclonal antibodies can also be used.

Primary Antibody:

After blocking the membrane, remove the blocking solution and add the primary antibody solution. Only enough to cover membrane is needed. This incubation step is usually performed on a rocker at 4°C overnight. Myelin basic protein (MBP) antibody was diluted 1:1000 and incubated at 4°C overnight.

Example Dilution:

2.5 ml 5% BSA solution

7.5 ml PBS

10 µl MBP antibody

10 ml total

Remove the primary antibody solution. Recapture it because it can be stored at 4°C and reused for up to 1 week. Wash the membrane 5 X 5min with 1X NP40 buffer solution. Then, rinse the membrane for 5 min in ddH₂O.

Secondary Antibody:

Remove the ddH₂O and add the secondary antibody solution. Incubate the membrane for 1 hour at room temperature. A horseradish peroxidase (HRP)

conjugated, goat anti-mouse secondary antibody at a 1:100,000 dilution was used in conjunction with the MBP primary antibody.

Example Dilution:

2.5 ml BSA solution

7.5 ml PBS

0.2 µl HRP conjugated GAM antibody (aliquots were pre-diluted 1:2)

10 ml total

Remove the secondary antibody and wash the membrane 5 X 5min in 1X NP40 buffer solution. Then, rinse the membrane for 5 min in ddH₂O.

In a separate container, add 2.5 ml of each of the 2 reagents in the ECL kit. These reagents should not be combined until immediately before the membrane is incubated in the ECL. Incubate the membrane on a rocker at room temperature for exactly 5 minutes. After incubation, pick up the membrane with forceps and touch one corner to a ChemWipe for about 15 seconds to wick off excess ECL solution. Do not dry out the membrane. Place the membrane on the inside of a plastic cover sheet. This works best if the cover sheet has been cut on all sides except one. Cover the membrane with the top sheet and use a roller to remove any air bubbles. Wipe the edges with a paper towel to soak up any ECL solution that spills out of the cover sheet.

After ECL incubation, wait at least 15 minutes, but no longer than 1 hour to get the best signal. Take the membranes in the cover sheet and a developing cassette to the darkroom. With all lights off except a red light, open the film container. Place a sheet of film and the cover sheet with membranes in the developing cassette. It works well to tape the cover sheet to the inside lid of cassette and place the sheet of film in the bottom of the cassette. Close the cassette lid for the desired exposure time (10 seconds was typically used with the MBP antibody). Open the cassette and quickly extract the film. Develop the film.

When development is complete and the unexposed film sheets are safely stored in the film box, turn on the light to inspect film. If the image is under or over exposed, expose the film again with a modified exposure time.

APPENDIX D: DETERGENT EFFECTIVENESS BASED ON HISTOLOGICAL ANALYSIS OF POST-TREATED NERVE TISSUE

Detergent	Conc.	Morphology Preservation	Basal Laminae Preservation	Interstitial Endoneurium Preservation	Axon Removal	Schwann Cell Removal	Total Score
CHAPS	Low	4.4 ± 0.7	4.5 ± 0.5	4.5 ± 0.5	1.5 ± 0.5	2.3 ± 0.4	17.1
	High	4.0 ± 0.5	4.3 ± 0.5	4.4 ± 0.5	1.1 ± 0.4	3.1 ± 1.6	16.9
Empigen BB	Low	3.3 ± 0.7	3.25 ± 1.2	2.4 ± 0.9	1.4 ± 0.5	1.9 ± 1.0	12.1
	High	3.8 ± 0.5	3.5 ± 0.5	2.9 ± 0.8	2.0 ± 1.1	2.6 ± 1.5	14.8
SB-10	Low	2.9 ± 2.0	3.4 ± 1.8	3.5 ± 1.4	2.1 ± 1.2	4.1 ± 1.0	16.0
	High	3.9 ± 0.6	4.0 ± 0.5	4.1 ± 0.6	1.9 ± 0.8	3.9 ± 0.6	17.8
SB-16	Low	4.5 ± 0.8	4.6 ± 0.5	4.8 ± 0.5	1.6 ± 0.7	1.0 ± 0	16.5
	High	4.8 ± 0.5	4.8 ± 0.5	4.9 ± 0.4	2.1 ± 1.0	1.3 ± 0.5	17.8
Dodecylbenzene Sulfonate	Low	3.3 ± 0.7	4.3 ± 0.7	4.5 ± 0.5	3.4 ± 0.7	3.8 ± 1.0	19.1
	High	3.3 ± 0.7	1.8 ± 0.7	2.1 ± 1.1	2.6 ± 0.7	3.5 ± 0.5	13.3
Sodium Caprylate	Low	3.6 ± 0.7	2.7 ± 1.3	3.7 ± 1.5	3.0 ± 0.8	3.5 ± 1.1	16.4
	High	3.4 ± 0.5	2.5 ± 1.4	2.8 ± 1.7	1.5 ± 0.5	4.1 ± 1.0	14.3
Sodium Deoxycholate	Low	3.2 ± 1.1	2.9 ± 1.5	2.6 ± 1.5	1.8 ± 0.7	4.3 ± 0.8	14.8
	High	2.6 ± 1.7	2.9 ± 1.1	3.7 ± 1.0	3.8 ± 1.0	4.7 ± 0.6	17.6
Triton X-200	Low	3.8 ± 0.5	3.9 ± 1.1	3.8 ± 1.4	3.4 ± 0.7	3.9 ± 0.6	18.6
	High	2.9 ± 0.6	2.4 ± 1.1	2.4 ± 1.1	3.1 ± 1.4	4.9 ± 0.4	15.6
CTAB	Low	3.4 ± 0.5	2.1 ± 0.8	3.6 ± 1.3	1.6 ± 0.7	1.9 ± 0.4	12.6
	High	2.5 ± 0.9	1.6 ± 0.7	3.1 ± 1.0	3.1 ± 2.0	3.5 ± 1.7	13.9
Triton X-100	Low	4.4 ± 0.7	2.8 ± 1.0	3.5 ± 1.4	1.8 ± 0.9	3.0 ± 1.7	15.4
	High	4.3 ± 0.7	2.6 ± 0.7	3.3 ± 1.9	3.6 ± 0.7	2.5 ± 1.6	16.3
Tween 20	Low	4.8 ± 0.5	4.4 ± 0.5	4.0 ± 0.8	1.6 ± 0.5	1.5 ± 0.5	16.3
	High	4.9 ± 0.4	3.0 ± 0.8	4.1 ± 0.6	2.9 ± 1.0	1.3 ± 0.5	16.1
Tween 80	Low	3.9 ± 1.1	4.4 ± 0.8	4.0 ± 0.8	1.8 ± 0.5	1.4 ± 0.5	15.4
	High	5.0 ± 0.0	4.4 ± 0.5	3.9 ± 1.0	1.6 ± 0.5	1.0 ± 0	15.9

5 = superior (e.g., optimal preservation of morphology, basal laminae and interstitial endoneurium; optimal removal of axons and Schwann cells)

1 = poor (e.g., destruction of morphology, basal laminae and endoneurium; no removal of axons and Schwann cells)

Total Score = combination of score in 5 individual categories (25 = best possible score)

Glossary

Allograft: Tissue graft taken from a genetically dissimilar donor of the same species as the host.

Antigen: A protein or oligosaccharide marker on the surface of cells that identifies the cell as *self* or *non-self*, identifies the cell type (e.g., dermal, glial), stimulates the production of antibodies against the cell; and stimulates a cytotoxic response against the cell.

Autograft: Tissue graft taken from one location in the host and implanted at another site in the host (e.g., an autologous nerve graft taken from the sural nerve).

Axon: A process of a nerve cell that conducts impulses away from the cell body. Sensory nerve cell processes that conduct impulses from the site of innervation to the nerve cell body are also called axons, even though they are technically dendrites.

Basal lamina (plural: basal laminae): Tube of extracellular matrix that surrounds a myelinated axon. Proteins in the basal lamina include laminin, collagen IV, and fibronectin. Laminin is found primarily on the interior surface of the basal lamina.

Central nervous system (CNS): The brain and spinal cord.

Critical micelle concentration (CMC): Concentration at which detergents in solution transition from forming only a surface layer to forming micelles that are suspended in solution.

Decellularization: The process of destroying the cells in a tissue. The process may or may not include removing of the cellular material after the cells are destroyed.

Distal: Farthest from the point of attachment. In reference to severed nerves, the end that is closer to the site of innervation.

Endoneurium: Connective tissue and extracellular matrix that surrounds all axons. In the case of myelinated axons, the endoneurium is outside the basal laminae.

Freeze/thaw graft: Decellularization treatment that uses repeated submersion in liquid nitrogen and room temperature PBS to destroy cells in tissue.

Immunogenic: Capable of inducing an immune response.

Innervation: The stimulation of a part through the action of nerves.

Integrin: The receptor on cell surfaces that links with proteins and chemical mediators to enhance cell-to-cell communication.

Isograft: Tissue graft taken from a donor that is genetically identical to the host.

Laminin: Extracellular matrix protein that is found in basement membranes. Laminin protein lines the inner surface of the basal laminae that surrounds myelinated axons.

Myelin: The phospholipid-protein of the cell membranes of Schwann cells (peripheral nervous system) that form the myelin sheath around axons. Myelin acts as an insulator, increasing the conduction velocity of impulse transmission down the axons.

Myelinated axon: A nerve process around which Schwann cells have wrapped, forming a myelin layer.

Myelination: The process of Schwann cells forming myelin around axons.

Neurotrophic: Stimulates nerve cells to proliferate or extend processes.

Neurotrophin: Biological agent that stimulates nerve cells to proliferate or extend processes.

Neurotropic: Attracts neurons to migrate or grow towards a stimulus.

Peripheral nervous system (PNS): The portion of the nervous system outside the central nervous system. These nerves contain sensory and somatic motor neurons, as well as the motor neurons of the autonomic nervous system.

Predegenerated: Nerve segment that is allowed to undergo Wallerian degeneration prior to being harvested and used as a nerve graft.

Primary neurorrhaphy: Surgical procedure in which severed nerve ends are sutured together.

Proximal: Nearest the point of attachment. In reference to severed nerves, the end attached to the spinal column.

Schwann cell: A support cell of the peripheral nervous system that forms myelin sheaths around axons. Schwann cells also produce neurotrophic factors.

Sondell protocol: Decellularization treatment that uses distilled water and solutions of sodium deoxycholate and Triton X-100 to destroy and remove cells from tissue.

Xenograft: Tissue graft taken from a donor of a different same species than the host.

References

1. National Center for Health Statistics Based on Classification of Diseases, 9th Revision, Clinical Modifications for the Following Categories: ICD-9 CM Codes 04.3, 04.5, 04.6, 04.7.
2. de Medinaceli, L., Prayon, M., and Merle, M. (1993) Percentage of nerve injuries in which primary repair can be achieved by end-to-end approximation: review of 2,181 nerve lesions. *Microsurgery* **14**, 244.
3. Evans, G.R. (2000) Challenges to nerve regeneration. *Semin Surg Oncol* **19**, 312.
4. David, S., and Aguayo, A.J. (1981) Axonal elongation into peripheral nervous system "bridges" after central nervous system injury in adult rats. *Science* **214**, 931.
5. Lorimier, P., Mezin, P., Labat Moleur, F., Pinel, N., Peyrol, S., and Stoeber, P. (1992) Ultrastructural localization of the major components of the extracellular matrix in normal rat nerve. *J Histochem Cytochem* **40**, 859.
6. Tohyama, K., and Ide, C. (1984) The localization of laminin and fibronectin on the Schwann cell basal lamina. *Arch Histol Jpn* **47**, 519.
7. Shellswell, G.B., Restall, D.J., Duance, V.C., and Bailey, A.J. (1979) Identification and differential distribution of collagen types in the central and peripheral nervous systems. *FEBS Lett* **106**, 305.
8. Fournier, A.E., and Strittmatter, S.M. (2002) Regenerating nerves follow the road more traveled. *Nat Neurosci* **5**, 821.
9. Stoll, G., Griffin, J.W., Li, C.Y., and Trapp, B.D. (1989) Wallerian degeneration in the peripheral nervous system: participation of both

- Schwann cells and macrophages in myelin degradation. *J Neurocytol* **18**, 671.
10. Chaudhry, V., Glass, J.D., and Griffin, J.W. (1992) Wallerian degeneration in peripheral nerve disease. *Neurol Clin* **10**, 613.
 11. MacKinnon, S.E., Dellon, A.L. (1988) Anatomy and physiology of the peripheral nerve, in: S.E. MacKinnon, Dellon, A.L., ed. *Surgery of the peripheral nerve*. Thieme, New York, pp. 1.
 12. Millesi, H. (1990) Peripheral nerve surgery today: turning point or continuous development? *J Hand Surg [Br]* **15**, 281.
 13. Huber, G.C. (1895) A study of the operative treatment for loss of nerve substance in peripheral nerves. *J Morphol* **11**, 629.
 14. Gluck, T. (1880) Ueber neuroplastik auf dem wege der transplantation. *Arch Klin Chir* **25**, 606.
 15. Payr, E. (1900) Beitrage zur technik der blutgefass und nervennaht nebst mittheilungen uber die verevendung eines resorbibaren metalles in der chirurgie. *Arch Klin Chir* **62**, 67.
 16. Kirk, E.G., Lewis, D. (1915) Fascial tubulization in the repair of nerve defects. *JAMA* **65**, 486.
 17. Letievant, J.J.E. (1873) *Traite des sections nerveuses*, J.B. Bailliereetfils, Paris.
 18. Mackenzie, J.A.J. (1909) Resection of the sciatic nerve, neuroplasty, end results. *Surg Gynecol Obstet* **9**, 30.
 19. Evans, G.R. (2001) Peripheral nerve injury: a review and approach to tissue engineered constructs. *Anat Rec* **263**, 396.
 20. Millesi, H., Meissl, G., and Berger, A. (1976) Further experience with interfascicular grafting of the median, ulnar, and radial nerves. *J Bone Joint Surg Am* **58**, 209.

21. Moneim, M.S. (1982) Interfascicular nerve grafting. *Clin Orthop* , 65.
22. Aebischer, P., Guenard, V., and Brace, S. (1989) Peripheral nerve regeneration through blind-ended semipermeable guidance channels: effect of the molecular weight cutoff. *J Neurosci* **9**, 3590.
23. Heath, C.A., and Rutkowski, G.E. (1998) The development of bioartificial nerve grafts for peripheral-nerve regeneration. *Trends Biotechnol* **16**, 163.
24. Strauch, B. (2000) Use of nerve conduits in peripheral nerve repair. *Hand Clin* **16**, 123.
25. Meek, M.F., and Coert, J.H. (2002) Clinical use of nerve conduits in peripheral-nerve repair: review of the literature. *J Reconstr Microsurg* **18**, 97.
26. Rutkowski, G.E., and Heath, C.A. (2002) Development of a bioartificial nerve graft. I. Design based on a reaction-diffusion model. *Biotechnol Prog* **18**, 362.
27. Guenard, V., Kleitman, N., Morrissey, T.K., Bunge, R.P., and Aebischer, P. (1992) Syngeneic Schwann cells derived from adult nerves seeded in semipermeable guidance channels enhance peripheral nerve regeneration. *J Neurosci* **12**, 3310.
28. Verdu, E., Navarro, X., Gudino-Cabrera, G., Rodriguez, F.J., Ceballos, D., Valero, A., and Nieto-Sampedro, M. (1999) Olfactory bulb ensheathing cells enhance peripheral nerve regeneration. *Neuroreport* **10**, 1097.
29. Laquerriere, A., Peulve, P., Jin, O., Tiollier, J., Tardy, M., Vaudry, H., Hemet, J., and Tadie, M. (1994) Effect of basic fibroblast growth factor and alpha-melanocytic stimulating hormone on nerve regeneration through a collagen channel. *Microsurgery* **15**, 203.
30. Mohammad, J.A., Warnke, P.H., Pan, Y.C., and Shenaq, S. (2000) Increased axonal regeneration through a biodegradable amnionic tube

nerve conduit: effect of local delivery and incorporation of nerve growth factor/hyaluronic acid media. *Ann Plast Surg* **44**, 59.

31. Santos, X., Rodrigo, J., Hontanilla, B., and Bilbao, G. (1998) Evaluation of peripheral nerve regeneration by nerve growth factor locally administered with a novel system. *J Neurosci Methods* **85**, 119.
32. Rodriguez, F.J., Verdu, E., Ceballos, D., and Navarro, X. (2000) Nerve guides seeded with autologous schwann cells improve nerve regeneration. *Exp Neurol* **161**, 571.
33. Arai, T., Lundborg, G., and Dahlin, L.B. (2000) Bioartificial nerve graft for bridging extended nerve defects in rat sciatic nerve based on resorbable guiding filaments. *Scand J Plast Reconstr Surg Hand Surg* **34**, 101.
34. Hall, S.M. (1986) Regeneration in cellular and acellular autografts in the peripheral nervous system. *Neuropathol Appl Neurobiol* **12**, 27.
35. Hebebrand, D., Zohman, G., and Jones, N.F. (1997) Nerve xenograft transplantation. Immunosuppression with FK-506 and RS-61443. *J Hand Surg [Br]* **22**, 304.
36. Pollard, J.D., and McLeod, J.G. (1981) Fresh and predegenerate nerve allografts and isografts in trembler mice. *Muscle Nerve* **4**, 274.
37. Gulati, A.K., and Cole, G.P. (1990) Nerve graft immunogenicity as a factor determining axonal regeneration in the rat. *J Neurosurg* **72**, 114.
38. Gulati, A.K. (1998) Immune response and neurotrophic factor interactions in peripheral nerve transplants. *Acta Haematol* **99**, 171.
39. Midha, R., Mackinnon, S.E., Evans, P.J., Best, T.J., Hare, G.M., Hunter, D.A., and Falk-Wade, J.A. (1993) Comparison of regeneration across nerve allografts with temporary or continuous cyclosporin A immunosuppression. *J Neurosurg* **78**, 90.

40. Meirer, R., Babuccu, O., Unsal, M., Nair, D.R., Gurunluoglu, R., Skugor, B., Meirer, B., and Siemionow, M. (2002) Effect of chronic cyclosporine administration on peripheral nerve regeneration: a dose-response study. *Ann Plast Surg* **49**, 96.
41. Ide, C., et al. (1998) Long acellular nerve transplants for allogeneic grafting and the effects of basic fibroblast growth factor on the growth of regenerating axons in dogs: a preliminary report. *Exp Neurol* **154**, 99.
42. Hiles, R.W. (1972) Freeze dried irradiated nerve homograft: a preliminary report. *Hand* **4**, 79.
43. Sondell, M., Lundborg, G., and Kanje, M. (1998) Regeneration of the rat sciatic nerve into allografts made acellular through chemical extraction. *Brain Res* **795**, 44.
44. Zalewski, A.A., and Gulati, A.K. (1982) Evaluation of histocompatibility as a factor in the repair of nerve with a frozen nerve allograft. *J Neurosurg* **56**, 550.
45. Gulati, A.K., and Cole, G.P. (1994) Immunogenicity and regenerative potential of acellular nerve allografts to repair peripheral nerve in rats and rabbits. *Acta Neurochir (Wien)* **126**, 158.
46. Danielsen, N., Kerns, J.M., Holmquist, B., Zhao, Q., Lundborg, G., and Kanje, M. (1995) Predegeneration enhances regeneration into acellular nerve grafts. *Brain Res* **681**, 105.
47. Osawa, T., Tohyama, K., and Ide, C. (1990) Allogeneic nerve grafts in the rat, with special reference to the role of Schwann cell basal laminae in nerve regeneration. *J Neurocytol* **19**, 833.
48. Pollard, J.D., and Fitzpatrick, L. (1973) An ultrastructural comparison of peripheral nerve allografts and autografts. *Acta Neuropathol (Berl)* **23**, 152.

49. Marmor, L. (1964) The repair of peripheral nerves by irradiated homografts. *Clin Orthop* **34**, 161.
50. Krekoski, C.A., Neubauer, D., Zuo, J., and Muir, D. (2001) Axonal regeneration into acellular nerve grafts is enhanced by degradation of chondroitin sulfate proteoglycan. *J Neurosci* **21**, 6206.
51. Johnson, P.C., Duhamel, R.C., Meezan, E., and Brendel, K. (1982) Preparation of cell-free extracellular matrix from human peripheral nerve. *Muscle Nerve* **5**, 335.
52. Frerichs, O., Fansa, H., Schicht, C., Wolf, G., Schneider, W., and Keilhoff, G. (2002) Reconstruction of peripheral nerves using acellular nerve grafts with implanted cultured Schwann cells. *Microsurgery* **22**, 311.
53. Gulati, A.K., Rai, D.R., and Ali, A.M. (1995) The influence of cultured Schwann cells on regeneration through acellular basal lamina grafts. *Brain Res* **705**, 118.
54. Sorensen, J., Fugleholm, K., Moldovan, M., Schmalbruch, H., and Krarup, C. (2001) Axonal elongation through long acellular nerve segments depends on recruitment of phagocytic cells from the near-nerve environment. Electrophysiological and morphological studies in the cat. *Brain Res* **903**, 185.
55. Frostick, S.P., Yin, Q., and Kemp, G.J. (1998) Schwann cells, neurotrophic factors, and peripheral nerve regeneration. *Microsurgery* **18**, 397.
56. Ide, C., Osawa, T., and Tohyama, K. (1990) Nerve regeneration through allogeneic nerve grafts, with special reference to the role of the Schwann cell basal lamina. *Prog Neurobiol* **34**, 1.

57. Martini, R. (1994) Expression and functional roles of neural cell surface molecules and extracellular matrix components during development and regeneration of peripheral nerves. *J Neurocytol* **23**, 1.
58. Geller, H.M., and Fawcett, J.W. (2002) Building a bridge: engineering spinal cord repair. *Exp Neurol* **174**, 125.
59. Thanos, P.K., Okajima, S., and Terzis, J.K. (1998) Ultrastructure and cellular biology of nerve regeneration. *J Reconstr Microsurg* **14**, 423.
60. Bunge, R.P. (1994) The role of the Schwann cell in trophic support and regeneration. *J Neurol* **242**, S19.
61. Bunge, M.B. (2001) Bridging areas of injury in the spinal cord. *Neuroscientist* **7**, 325.
62. Son, Y.J., Trachtenberg, J.T., and Thompson, W.J. (1996) Schwann cells induce and guide sprouting and reinnervation of neuromuscular junctions. *Trends Neurosci* **19**, 280.
63. Miyauchi, A., Kanje, M., Danielsen, N., and Dahlin, L.B. (1997) Role of macrophages in the stimulation and regeneration of sensory nerves by transposed granulation tissue and temporal aspects of the response. *Scand J Plast Reconstr Surg Hand Surg* **31**, 17.
64. Hikawa, N., and Takenaka, T. (1996) Myelin-stimulated macrophages release neurotrophic factors for adult dorsal root ganglion neurons in culture. *Cell Mol Neurobiol* **16**, 517.
65. Hobson, M.I. (2002) Increased vascularisation enhances axonal regeneration within an acellular nerve conduit. *Ann R Coll Surg Engl* **84**, 47.
66. Sondell, M., Sundler, F., and Kanje, M. (2000) Vascular endothelial growth factor is a neurotrophic factor which stimulates axonal outgrowth through the flk-1 receptor. *Eur J Neurosci* **12**, 4243.

67. Fine, E.G., Decosterd, I., Papaloizos, M., Zurn, A.D., and Aebischer, P. (2002) GDNF and NGF released by synthetic guidance channels support sciatic nerve regeneration across a long gap. *Eur J Neurosci* **15**, 589.
68. Kimpinski, K., Campenot, R.B., and Mearow, K. (1997) Effects of the neurotrophins nerve growth factor, neurotrophin-3, and brain-derived neurotrophic factor (BDNF) on neurite growth from adult sensory neurons in compartmented cultures. *J Neurobiol* **33**, 395.
69. Schmidt, C.E., Baier Leach J. (2003) Neural tissue engineering: strategies for repair and regeneration. *Annual Review in Biomedical Engineering* **5**, in press.
70. Lundborg, G. (2000) A 25-year perspective of peripheral nerve surgery: evolving neuroscientific concepts and clinical significance. *J Hand Surg [Am]* **25**, 391.
71. Nguyen, Q.T., Sanes, J.R., and Lichtman, J.W. (2002) Pre-existing pathways promote precise projection patterns. *Nat Neurosci* **5**, 861.
72. Carbonetto, S. (1984) The extracellular matrix of the nervous system. *Trend Neuro Sci* **7**, 382.
73. Ide, C., Tohyama, K., Yokota, R., Nitatori, T., and Onodera, S. (1983) Schwann cell basal lamina and nerve regeneration. *Brain Res* **288**, 61.
74. Wang, G.Y., Hirai, K., and Shimada, H. (1992) The role of laminin, a component of Schwann cell basal lamina, in rat sciatic nerve regeneration within antiserum-treated nerve grafts. *Brain Res* **570**, 116.
75. Kuhn, T.B., Williams, C.V., Dou, P., and Kater, S.B. (1998) Laminin directs growth cone navigation via two temporally and functionally distinct calcium signals. *J Neurosci* **18**, 184.
76. Tai, H.C., and Buettner, H.M. (1998) Neurite outgrowth and growth cone morphology on micropatterned surfaces. *Biotechnol Prog* **14**, 364.

77. Sketelj, J., Bresjanac, M., and Popovic, M. (1989) Rapid growth of regenerating axons across the segments of sciatic nerve devoid of Schwann cells. *J Neurosci Res* **24**, 153.
78. Bajrovic, F., Srpacic, M., and Sketelj, J. (2001) Schwann cell dependence of regenerating rat sensory neurons is inversely related to the quality of axon growth substratum. *J Neuropathol Exp Neurol* **60**, 65.
79. Midgley, R.D., and Woolhouse, F.M. (1968) Silicone rubber sheathing as an adjunct to neural anastomosis. *Surg Clin North Am* **48**, 1149.
80. Dahlin, L., and Lundborg, G. (2001) The use of silicone tubing in the late repair of the median and ulnar nerves in the forearm. *J Hand Surg [Br]* **26**, 393.
81. Danielsen, N., Williams, L.R., Dahlin, L.B., Varon, S., and Lundborg, G. (1988) Peripheral nerve regeneration in Gore-tex chambers. *Scand J Plast Reconstr Surg Hand Surg* **22**, 207.
82. Vasconcelos, B.C., and Gay-Escoda, C. (2000) Facial nerve repair with expanded polytetrafluoroethylene and collagen conduits: an experimental study in the rabbit. *J Oral Maxillofac Surg* **58**, 1257.
83. Pitta, M.C., Wolford, L.M., Mehra, P., and Hopkin, J. (2001) Use of Gore-Tex tubing as a conduit for inferior alveolar and lingual nerve repair: experience with 6 cases. *J Oral Maxillofac Surg* **59**, 493.
84. Miloro, M., Halkias, L.E., Mallery, S., Travers, S., and Rashid, R.G. (2002) Low-level laser effect on neural regeneration in Gore-Tex tubes. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **93**, 27.
85. Evans, G.R., Brandt, K., Niederbichler, A.D., Chauvin, P., Herrman, S., Bogle, M., Otta, L., Wang, B., and Patrick, C.W., Jr. (2000) Clinical long-term in vivo evaluation of poly(L-lactic acid) porous conduits for peripheral nerve regeneration. *J Biomater Sci Polym Ed* **11**, 869.

86. Evans, G.R., et al. (2002) Bioactive poly(L-lactic acid) conduits seeded with Schwann cells for peripheral nerve regeneration. *Biomaterials* **23**, 841.
87. Molander, H., Olsson, Y., Engkvist, O., Bowald, S., and Eriksson, I. (1982) Regeneration of peripheral nerve through a polyglactin tube. *Muscle Nerve* **5**, 54.
88. Borkenhagen, M., Stoll, R.C., Neuenschwander, P., Suter, U.W., and Aebischer, P. (1998) In vivo performance of a new biodegradable polyester urethane system used as a nerve guidance channel. *Biomaterials* **19**, 2155.
89. Soldani, G., Varelli, G., Minnocci, A., and Dario, P. (1998) Manufacturing and microscopical characterisation of polyurethane nerve guidance channel featuring a highly smooth internal surface. *Biomaterials* **19**, 1919.
90. Robinson, P.H., van der Lei, B., Hoppen, H.J., Leenslag, J.W., Pennings, A.J., and Nieuwenhuis, P. (1991) Nerve regeneration through a two-ply biodegradable nerve guide in the rat and the influence of ACTH4-9 nerve growth factor. *Microsurgery* **12**, 412.
91. Langone, F., Lora, S., Veronese, F.M., Caliceti, P., Parnigotto, P.P., Valenti, F., and Palma, G. (1995) Peripheral nerve repair using a poly(organo)phosphazene tubular prosthesis. *Biomaterials* **16**, 347.
92. Young, R.C., Wiberg, M., and Terenghi, G. (2002) Poly-3-hydroxybutyrate (PHB): a resorbable conduit for long-gap repair in peripheral nerves. *Br J Plast Surg* **55**, 235.
93. Lenihan, D.V., Carter, A.J., Gilchrist, T., Healy, D.M., Miller, I.A., Myles, L.M., and Glasby, M.A. (1998) Biodegradable controlled release glass in the repair of peripheral nerve injuries. *J Hand Surg [Br]* **23**, 588.

94. Gilchrist, T., Glasby, M.A., Healy, D.M., Kelly, G., Lenihan, D.V., McDowall, K.L., Miller, I.A., and Myles, L.M. (1998) In vitro nerve repair--in vivo. The reconstruction of peripheral nerves by entubulation with biodegradable glass tubes--a preliminary report. *Br J Plast Surg* **51**, 231.
95. Valentini, R.F., Vargo, T.G., Gardella, J.A., Jr., and Aebischer, P. (1993) Patterned neuronal attachment and outgrowth on surface modified, electrically charged fluoropolymer substrates. *J Biomater Sci Polym Ed* **5**, 13.
96. Schmidt, C.E., Shastri, V.R., Vacanti, J.P., and Langer, R. (1997) Stimulation of neurite outgrowth using an electrically conducting polymer. *Proc Natl Acad Sci U S A* **94**, 8948.
97. Wang, K.K., Costas, P.D., Bryan, D.J., Eby, P.L., and Seckel, B.R. (1995) Inside-out vein graft repair compared with nerve grafting for nerve regeneration in rats. *Microsurgery* **16**, 65.
98. Mohammad, J., Shenaq, J., Rabinovsky, E., and Shenaq, S. (2000) Modulation of peripheral nerve regeneration: a tissue-engineering approach. The role of amnion tube nerve conduit across a 1-centimeter nerve gap. *Plast Reconstr Surg* **105**, 660.
99. Tong, X.J., Hirai, K., Shimada, H., Mizutani, Y., Izumi, T., Toda, N., and Yu, P. (1994) Sciatic nerve regeneration navigated by laminin-fibronectin double coated biodegradable collagen grafts in rats. *Brain Res* **663**, 155.
100. Kauppila, T., Jyvasjarvi, E., Huopaniemi, T., Hujanen, E., and Liesi, P. (1993) A laminin graft replaces neurorrhaphy in the restorative surgery of the rat sciatic nerve. *Exp Neurol* **123**, 181.

101. Whitworth, I.H., Brown, R.A., Dore, C., Green, C.J., and Terenghi, G. (1995) Orientated mats of fibronectin as a conduit material for use in peripheral nerve repair. *J Hand Surg [Br]* **20**, 429.
102. Favaro, G., Bortolami, M.C., Cereser, S., Dona, M., Pastorello, A., Callegaro, L., and Fiori, M.G. (1990) Peripheral nerve regeneration through a novel bioresorbable nerve guide. *ASAIO Trans* **36**, M291.
103. Synthesis and characterization of polypyrrole/hyaluronic acid composites for peripheral nerve regeneration, 1998, Biomedical Engineering.
104. Bryan, D.J., Holway, A.H., Wang, K.K., Silva, A.E., Trantolo, D.J., Wise, D., and Summerhayes, I.C. (2000) Influence of glial growth factor and Schwann cells in a bioresorbable guidance channel on peripheral nerve regeneration. *Tissue Eng* **6**, 129.
105. Chen, Y.S., Hsieh, C.L., Tsai, C.C., Chen, T.H., Cheng, W.C., Hu, C.L., and Yao, C.H. (2000) Peripheral nerve regeneration using silicone rubber chambers filled with collagen, laminin and fibronectin. *Biomaterials* **21**, 1541.
106. Verdu, E., Labrador, R.O., Rodriguez, F.J., Ceballos, D., Fores, J., and Navarro, X. (2002) Alignment of collagen and laminin-containing gels improve nerve regeneration within silicone tubes. *Restor Neurol Neurosci* **20**, 169.
107. Toba, T., et al. (2001) Regeneration of canine peroneal nerve with the use of a polyglycolic acid-collagen tube filled with laminin-soaked collagen sponge: a comparative study of collagen sponge and collagen fibers as filling materials for nerve conduits. *J Biomed Mater Res* **58**, 622.
108. Seckel, B.R., Jones, D., Hekimian, K.J., Wang, K.K., Chakalis, D.P., and Costas, P.D. (1995) Hyaluronic acid through a new injectable nerve guide

- delivery system enhances peripheral nerve regeneration in the rat. *J Neurosci Res* **40**, 318.
109. Sakiyama, S.E., Schense, J.C., and Hubbell, J.A. (1999) Incorporation of heparin-binding peptides into fibrin gels enhances neurite extension: an example of designer matrices in tissue engineering. *Faseb J* **13**, 2214.
 110. Borkenhagen, M., Clemence, J.F., Sigrist, H., and Aebischer, P. (1998) Three-dimensional extracellular matrix engineering in the nervous system. *J Biomed Mater Res* **40**, 392.
 111. Shen, Z.L., Berger, A., Hierner, R., Allmeling, C., Ungewickell, E., and Walter, G.F. (2001) A Schwann cell-seeded intrinsic framework and its satisfactory biocompatibility for a bioartificial nerve graft. *Microsurgery* **21**, 6.
 112. Hashimoto, T., Suzuki, Y., Kitada, M., Kataoka, K., Wu, S., Suzuki, K., Endo, K., Nishimura, Y., and Ide, C. (2002) Peripheral nerve regeneration through alginate gel: analysis of early outgrowth and late increase in diameter of regenerating axons. *Exp Brain Res* **146**, 356.
 113. Yu, X., Dillon, G.P., and Bellamkonda, R.B. (1999) A laminin and nerve growth factor-laden three-dimensional scaffold for enhanced neurite extension. *Tissue Eng* **5**, 291.
 114. Kaestner, W. (1992) Local tolerance (animal tests): mucous membranes and skin, in: C. Gloxhuber and K. Kuenstler, eds. *Anionic Surfactants, Biochemistry, Toxicology, Dermatology*. Marcek Dekker, Inc., New York, pp. 127.
 115. Bartnik, F.G. (1992) Interaction of anionic surfactants with proteins, enzymes, and membranes, in: C. Gloxhuber and K. Kuenstler, eds. *Anioinic Surfactants, Biochemistry, Toxicology, Dermatology*. Marcel Dekker, Inc., New York, pp. 23.

116. Domingo, X. (1996) Betaines, in: E.G. Lomax, ed. *Amphoteric Surfactants*. Marcel Dekker, Inc., New York, pp. 75.
117. Lomax, E.G. (1996) Introduction, in: E.G. Lomax, ed. *Amphoteric Surfactants*. Marcel Dekker, Inc., New York, pp. 1.
118. Fleischmajer, R., Timpl, R., Dziadek, M., and Lebowitz, M. (1985) Basement membrane proteins, interstitial collagens, and fibronectin in neurofibroma. *J Invest Dermatol* **85**, 54.
119. Givogri, M.I., Bongarzone, E.R., and Campagnoni, A.T. (2000) New insights on the biology of myelin basic protein gene: the neural-immune connection. *J Neurosci Res* **59**, 153.
120. Braun, P.E., and Radin, N.S. (1969) Interactions of lipids with a membrane structural protein from myelin. *Biochemistry* **8**, 4310.
121. Gulati, A.K. (1988) Evaluation of acellular and cellular nerve grafts in repair of rat peripheral nerve. *J Neurosurg* **68**, 117.
122. Lemke, G., and Axel, R. (1985) Isolation and sequence of a cDNA encoding the major structural protein of peripheral myelin. *Cell* **40**, 501.
123. D'Urso, D., Ehrhardt, P., and Muller, H.W. (1999) Peripheral myelin protein 22 and protein zero: a novel association in peripheral nervous system myelin. *J Neurosci* **19**, 3396.
124. National Research Council (1996) *Guide for the Care and Use of Laboratory Animals*. Institute of Laboratory Animal Resources Commission on Life Sciences, National Academy Press, Washington, D.C.
125. Sondell, M., Lundborg, G., and Kanje, M. (1999) Vascular endothelial growth factor stimulates Schwann cell invasion and neovascularization of acellular nerve grafts. *Brain Res* **846**, 219.

126. Arai, T., Kanje, M., Lundborg, G., Sondell, M., Liu, X.L., and Dahlin, L.B. (2000) Axonal outgrowth in muscle grafts made acellular by chemical extraction. *Restor Neurol Neurosci* **17**, 165.
127. Guenther, E., Stark, O. (1977) The major histocompatibility system of the rat (Ag-B or H-1 system), in: D. Goetze, ed. The major histocompatibility system in man and animals. Springer-Verlag, New York, pp. 207.
128. Jander, S., Lausberg, F., and Stoll, G. (2001) Differential recruitment of CD8⁺ macrophages during Wallerian degeneration in the peripheral and central nervous system. *Brain Pathol* **11**, 27.
129. Hirata, K., Mitoma, H., Ueno, N., He, J.W., and Kawabuchi, M. (1999) Differential response of macrophage subpopulations to myelin degradation in the injured rat sciatic nerve. *J Neurocytol* **28**, 685.
130. Bruck, W. (1997) The role of macrophages in Wallerian degeneration. *Brain Pathol* **7**, 741.
131. Perry, V.H., and Brown, M.C. (1992) Macrophages and nerve regeneration. *Curr Opin Neurobiol* **2**, 679.
132. Pollard, J.D., and Fitzpatrick, L. (1973) A comparison of the effects of irradiation and immunosuppressive agents on regeneration through peripheral nerve allografts: an ultrastructural study. *Acta Neuropathol (Berl)* **23**, 166.
133. West, D.C., Hampson, I.N., Arnold, F., and Kumar, S. (1985) Angiogenesis induced by degradation products of hyaluronic acid. *Science* **228**, 1324.

Vita

Terry Wayne Hudson was born to Tom and Marijen Hudson on December 28, 1974 in Lake Charles, Louisiana. In 1993, he graduated valedictorian of his high school class in Bay City, Texas. Terry then attended Trinity University in San Antonio, Texas, in pursuit of a Bachelor of Science degree in engineering science. While at Trinity University, Terry received awards including Academic Excellence for a Sophomore Engineering Student and Outstanding Academic Achievement for a Senior Engineering Student. He also performed research under Dr. Mahbub Uddin. For two summers Terry interned at Fluor Daniel, Inc., working as an engineering technician. After graduating in 1997, Terry worked for four months as a process engineer for Flour Daniel, Inc. Then in the fall of 1997, he enrolled in the graduate school of chemical engineering at The University of Texas at Austin where he was the recipient of a NIH Training Fellowship, a College of Engineering Thrust 2000 Graduate Fellowship, and an Engineering Foundation Endowed Graduate Presidential Fellowship. During the summer of 2000, Terry performed an internship as a research scientist for Clear Solutions Biotechnologies, Inc. In January of 2001, Terry married Allison Alexis Hanson. By the completion of his doctorate in 2003, Terry was an author or co-author on five publications concerning tissue engineering and the lead author on a provisional patent.

Permanent address: 37 Sundown Parkway, Austin, TX, 78746

This dissertation was typed by Terry Wayne Hudson.