3-2016

Chitosan Decontamination with Non-Thermal Nitrogen Plasma to Enable Internal Use

Andrew R. Crofton

Follow this and additional works at: http://scholarsrepository.llu.edu/etd

Recommended Citation
Crofton, Andrew R., "Chitosan Decontamination with Non-Thermal Nitrogen Plasma to Enable Internal Use" (2016). Loma Linda University Electronic Theses, Dissertations & Projects. 351.
http://scholarsrepository.llu.edu/etd/351

This Dissertation is brought to you for free and open access by TheScholarsRepository@LLU: Digital Archive of Research, Scholarship & Creative Works. It has been accepted for inclusion in Loma Linda University Electronic Theses, Dissertations & Projects by an authorized administrator of TheScholarsRepository@LLU: Digital Archive of Research, Scholarship & Creative Works. For more information, please contact scholarsrepository@llu.edu.
Chitosan Decontamination with Non-Thermal Nitrogen Plasma
to Enable Internal Use

by

Andrew R. Crofton

A Dissertation submitted in partial satisfaction of
the requirements for the degree of
Doctor of Philosophy in Anatomy

March 2016
Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

______________________________________________________________________________, Chairperson
Wolff M. Kirsch, Professor of Biochemistry and Neurological Surgery

______________________________________________________________________________
Duane Baldwin, Professor of Urologic Surgery

______________________________________________________________________________
Penelope Duerksen-Hughes, Professor of Biochemistry

______________________________________________________________________________
Samuel Hudson, Professor of Polymer Chemistry, College of Textiles, North Carolina State University

______________________________________________________________________________
Michael A. Kirby, Professor of Pathology and Human Anatomy, Pediatrics, and Neurosurgery

______________________________________________________________________________
William Langridge, Professor of Biochemistry
ACKNOWLEDGEMENTS

I would like to thank Dr. Kirsch from the bottom of my heart for his time, mentorship, advice, generosity, and most of all love and friendship over the past 5.5 years. I could not have imagined a better boss or more inspiring scientist to work for during my doctoral training. You have molded me into an outstanding scientist and taught me not only how to do sound scientific research, but also how to navigate bureaucracy and translate my basic science research into medical devices and drugs that may help humanity. Or as you would say, “we are trying to make man whole, by fixing holes in man.” You treated me as a colleague, friend, and equal from day one and imparted on me great responsibility, all of which helped me push myself to be the best I could be. You have also taught me a great deal about being a great husband and family man. The time I have spent with Marie-Claire and Danny is truly cherished. I am certain that I am one of only a select few Ph.D. scientists who can say that I truly loved my time in graduate school and would do it again in a heartbeat. You are the reason I feel that way.

I would also like to express my deepest gratitude to the Anatomy Department for taking a chance on me in the Master’s program and then fighting for my admittance to the Ph.D. program. Drs. Escobar, Kirby, Nava, Oberg, and Wright, you all have been tremendous sources of support and encouragement. I will always be working to make you all proud of my research and teaching and count each of you as friends.
I would like to thank my committee members for their advice and guidance. Each of you has taught me valuable lessons and helped me become a better scientist. All the members of the Kirsch lab, past and present, have made my time in graduate school an enjoyable experience. I especially want to thank Matt Schrag, Matt Zabel, Nathan Oh, Grant McAuley, Jon Baio, Carson Whinnery, Nick Sanchez, and Kristy Howard for their camaraderie and friendship and our numerous summer students for all their hard work and contributions.

To my family and friends, your love, support, and encouragement mean the world to me. I have loved all the visits, phone calls, text messages, and emails that I have received from each and every one of you. I would like to give a special thanks to my wife, Rhea Eva. I cannot thank you enough for loving me so deeply and always pushing me to be the best I can be. Your unwavering support means the world to me. I truly feel I am the luckiest man in the world to be married to you, my soul mate. I love you to the moon and back!

Last but certainly not least, I would like to thank God for providing me the opportunity to use my gifts and talents to study His creation. I have had the privilege of seeing how ingeniously and beautifully the human body is built as well as the insane complexity of its functions through my experience at LLU. I also discovered that at least 90% of science is failure, which makes being a scientist both frustrating and immensely gratifying, at least when things do finally work the way you intend. But it also makes one realize how complex God’s creation is and that we will never fully understand that which we are studying.
CONTENTS

Approval Page ........................................................................................................ iii
Acknowledgements ................................................................................................ iv
List of Figures ........................................................................................................... x
List of Tables .......................................................................................................... xii
List of Abbreviations ............................................................................................ xiii
Abstract ................................................................................................................ xv

Chapter

1. Introduction ........................................................................................................ 1
   Background ........................................................................................................... 1
      Alzheimer's Disease .......................................................................................... 1
         Copper ............................................................................................................ 2
         Dynactin p62 ............................................................................................... 6
         Aβ Immunotherapy ...................................................................................... 8
         Shift from AD and Copper to Chitosan and Nitrogen Plasma....................... 9
      Chitosan .......................................................................................................... 11
         History and Derivation ................................................................................ 11
         Scientific Background .................................................................................. 13
      Plasma ............................................................................................................ 16
         Scientific Background ................................................................................ 16
         Plasma Decontamination ............................................................................ 18
         Plasma Treatment of Chitosan .................................................................. 20
      The Present Research ..................................................................................... 20
         Approach of the Studies ............................................................................. 21
            Study 1 .................................................................................................... 22
            Study 2 .................................................................................................... 22
Study 3 .................................................................................................................. 23
Study 4 .................................................................................................................. 24

Significance of the Studies .................................................................................. 24

2. Formulation and Characterization of a Plasma Sterilized, Pharmaceutical Grade Chitosan Powder .................................................................................. 27

Abstract ............................................................................................................. 28
Introduction ........................................................................................................ 29
Materials and Methods ...................................................................................... 33

Cryomilling ........................................................................................................ 33
Particle Sizing .................................................................................................... 33
Degree of Deacetylation ..................................................................................... 34
Morphologic Characterization ......................................................................... 35
Hygroscopicity ................................................................................................... 35
Molecular Weight ............................................................................................... 36
Fourier-Transform Infrared Spectroscopy ......................................................... 37
Plasma Sterilization ........................................................................................... 37
Endotoxins ......................................................................................................... 39

Results and Discussion ...................................................................................... 39

Particle Size Analysis ........................................................................................ 39
Viscosity and Molecular Weight ....................................................................... 41
Degree of Deacetylation ..................................................................................... 42
Hygroscopicity ................................................................................................... 43
Morphologic Characterization ......................................................................... 44
FTIR ...................................................................................................................... 45
Plasma Decontamination ................................................................................... 46
Solubility ............................................................................................................. 47

Conclusion .......................................................................................................... 47
Acknowledgements ........................................................................................... 48
References .......................................................................................................... 49

3. Intravesical Immunotherapy of Orthotopic Murine Bladder Cancer with Nitrogen Plasma Sterilized Chitosan+Interleukin-12 ............................................. 53

Abstract ............................................................................................................. 54
Introduction ........................................................................................................ 56
Materials and Methods ...................................................................................... 58

Chitosan ............................................................................................................. 58
Plasma Decontamination ................................................................................... 59
<table>
<thead>
<tr>
<th>Topics</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viscosity</td>
<td>60</td>
</tr>
<tr>
<td>Surface Atomic Concentrations</td>
<td>61</td>
</tr>
<tr>
<td>Degree of Deacetylation</td>
<td>62</td>
</tr>
<tr>
<td>Bioadhesivity</td>
<td>63</td>
</tr>
<tr>
<td>Chitosan+IL-12 Co-formulation</td>
<td>64</td>
</tr>
<tr>
<td>Cell Culture</td>
<td>65</td>
</tr>
<tr>
<td>Tumor Cell Injections and Confirmation of Tumor Establishment</td>
<td>66</td>
</tr>
<tr>
<td>Intravesical Treatments</td>
<td>68</td>
</tr>
<tr>
<td>Tumor Re-challenge</td>
<td>68</td>
</tr>
<tr>
<td>Chitosan+IL-12 Vaccination of Mice</td>
<td>69</td>
</tr>
<tr>
<td>Euthanasia</td>
<td>69</td>
</tr>
<tr>
<td>Statistical Analyses</td>
<td>69</td>
</tr>
<tr>
<td>Results</td>
<td>69</td>
</tr>
<tr>
<td>NtNP Effectively Sterilized Chitosan</td>
<td>69</td>
</tr>
<tr>
<td>NtNP Reduced CS Viscosity</td>
<td>71</td>
</tr>
<tr>
<td>NtNP Altered DD, Not Surface Atomic Concentrations</td>
<td>71</td>
</tr>
<tr>
<td>CS Bioadhesion Unchanged with NtNP Sterilization</td>
<td>72</td>
</tr>
<tr>
<td>Orthotopic Tumors Established in Most Animals</td>
<td>73</td>
</tr>
<tr>
<td>CS+IL-12 Eliminated Tumors in Most C57Bl/6 Mice</td>
<td>74</td>
</tr>
<tr>
<td>Survival Unchanged by NtNP Sterilization</td>
<td>76</td>
</tr>
<tr>
<td>Re-challenge After Tumor Regression</td>
<td>77</td>
</tr>
<tr>
<td>Vaccination</td>
<td>79</td>
</tr>
<tr>
<td>Discussion</td>
<td>79</td>
</tr>
<tr>
<td>Perspective and Significance</td>
<td>82</td>
</tr>
<tr>
<td>Conclusions</td>
<td>83</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>84</td>
</tr>
<tr>
<td>References</td>
<td>85</td>
</tr>
<tr>
<td>4. Effect of Plasma Sterilization on the Hemostatic Efficacy of a Chitosan Hemostatic Agent in a Rat Model</td>
<td>92</td>
</tr>
<tr>
<td>Abstract</td>
<td>93</td>
</tr>
<tr>
<td>Introduction</td>
<td>95</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>98</td>
</tr>
<tr>
<td>Materials</td>
<td>98</td>
</tr>
<tr>
<td>Morphologic Characterization</td>
<td>99</td>
</tr>
<tr>
<td>Sterilization and Surface Modification with NtNP</td>
<td>100</td>
</tr>
<tr>
<td>Tissue Adhesion Studies</td>
<td>100</td>
</tr>
<tr>
<td>Surface Atomic Concentration Analysis</td>
<td>102</td>
</tr>
</tbody>
</table>
# FIGURES

<table>
<thead>
<tr>
<th>Figures</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Copper concentration in synaptic vesicles</td>
<td>5</td>
</tr>
<tr>
<td>2. Histologic copper localization in human temporal lobe tissue</td>
<td>5</td>
</tr>
<tr>
<td>3. Dynactin p62 levels in human temporal lobe</td>
<td>7</td>
</tr>
<tr>
<td>4. Dynactin p62 levels in blood plasma</td>
<td>7</td>
</tr>
<tr>
<td>5. Chemical structures of chitin, cellulose, and chitosan</td>
<td>14</td>
</tr>
<tr>
<td>6. Macroscopic images of chitosan flake and cryo-jet milled powder</td>
<td>40</td>
</tr>
<tr>
<td>7. Effect of cryo-jet milling on intrinsic viscosity and molecular</td>
<td>42</td>
</tr>
<tr>
<td>weight of chitosan</td>
<td></td>
</tr>
<tr>
<td>8. Effect of cryo-jet milling on degree of deacetylation of chitosan</td>
<td>43</td>
</tr>
<tr>
<td>9. Scanning electron micrographs of chitosan flake and cryomilled</td>
<td>45</td>
</tr>
<tr>
<td>powder</td>
<td></td>
</tr>
<tr>
<td>10. Fourier Transform Infrared Spectra of chitosan flake and powder</td>
<td>46</td>
</tr>
<tr>
<td>11. Effect of low pressure and atmospheric pressure plasma treatment</td>
<td>47</td>
</tr>
<tr>
<td>on viscosity of chitosan flake and powder</td>
<td></td>
</tr>
<tr>
<td>12. Log reductions in spore viability from biological indicators with</td>
<td>70</td>
</tr>
<tr>
<td>increasing exposure to plasma treatment</td>
<td></td>
</tr>
<tr>
<td>13. Intrinsic viscosity of micronized chitosan powder before and after</td>
<td>71</td>
</tr>
<tr>
<td>plasma sterilization</td>
<td></td>
</tr>
<tr>
<td>14. Bioadhesivity of native and plasma sterilized chitosan</td>
<td>72</td>
</tr>
<tr>
<td>15. Success rates of orthotopic tumor establishment in BALB/c and</td>
<td>74</td>
</tr>
<tr>
<td>C57Bl/6 mice</td>
<td></td>
</tr>
<tr>
<td>16. Longitudinal tumor regression after intravesical treatment</td>
<td>75</td>
</tr>
<tr>
<td>17. Antitumor activity of intravesical treatments</td>
<td>76</td>
</tr>
<tr>
<td>18. Mouse survival after tumor instillation</td>
<td>77</td>
</tr>
<tr>
<td>19. Instrument setup for testing of chitosan bioadhesivity</td>
<td>102</td>
</tr>
</tbody>
</table>
20. Experimental procedure for rat hemostasis studies ......................... 104
22. Effect of different low-pressure plasmas on bacterial spores over time ................................................................................................. 108
23. Effect of different low-pressure plasmas on bioadhesivity of chitosan ............................................................................................... 109
24. Average weights of rats in each experimental group ....................... 111
25. Time to hemostasis after puncture of rat femoral artery ................ 112
26. Time to hemostasis after transection of rat femoral artery and vein ... 113
27. Hemostasis scores after puncture of rat femoral artery ................. 114
28. Hemostasis scores after transection of rat femoral artery and vein ... 115
29. Deployment of chitosan pad to cut surface of porcine kidney under warm ischemia ............................................................................... 136
30. Retrograde ureteropyelography ...................................................... 139
31. Intraoperative, post-mortem in situ, and ex vivo sectioned views of chitosan pads ............................................................................... 141
32. Histologic appearance of kidney parenchyma in contact with plasma-sterilized chitosan ................................................................. 142
33. Chitosan engulfment by giant cells ............................................... 143
34. Germinal centers and immune cell infiltration ............................. 144
# TABLES

<table>
<thead>
<tr>
<th>Tables</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Mean, median and D95 values for cryomilled chitosan</td>
<td>41</td>
</tr>
<tr>
<td>2. Tumor establishment in tumor exposed and tumor naïve mice treated with chitosan containing IL-12</td>
<td>78</td>
</tr>
<tr>
<td>4. Experimental parameters for XPS measurements</td>
<td>102</td>
</tr>
<tr>
<td>5. Elemental composition of chitosan after different plasma sterilization treatments</td>
<td>110</td>
</tr>
<tr>
<td>6. Surgical data for porcine laparoscopic partial nephrectomy with chitosan hemostatic agent</td>
<td>137</td>
</tr>
<tr>
<td>7. Pig blood chemistry and cell counts between groups and pre-versus post-operatively</td>
<td>138</td>
</tr>
</tbody>
</table>
# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD</td>
<td>Alzheimer's Disease</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid Precursor Protein</td>
</tr>
<tr>
<td>ApP</td>
<td>Atmospheric Pressure Plasma</td>
</tr>
<tr>
<td>BC</td>
<td>Bladder Cancer</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacillus Calmette-Guerin</td>
</tr>
<tr>
<td>CAA</td>
<td>Cerebral Amyloid Angiopathy</td>
</tr>
<tr>
<td>CS</td>
<td>Chitosan</td>
</tr>
<tr>
<td>CS+IL-12</td>
<td>Chitosan and interleukin-12</td>
</tr>
<tr>
<td>psCS+IL-12</td>
<td>Plasma-sterilized chitosan and interleukin-12</td>
</tr>
<tr>
<td>usCS+IL-12</td>
<td>Unsterilized chitosan and interleukin-12</td>
</tr>
<tr>
<td>cSt</td>
<td>Centistokes</td>
</tr>
<tr>
<td>Cu</td>
<td>Copper</td>
</tr>
<tr>
<td>DD</td>
<td>Degree of Deacetylation</td>
</tr>
<tr>
<td>EB</td>
<td>Electron Beam</td>
</tr>
<tr>
<td>EBN2</td>
<td>Electron Beam with nitrogen plasma treatment</td>
</tr>
<tr>
<td>EBL</td>
<td>Estimated Blood Loss</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier Transform Infrared Spectroscopy</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric Acid</td>
</tr>
<tr>
<td>IL-12</td>
<td>Interleukin-12</td>
</tr>
<tr>
<td>IRP2</td>
<td>Iron-regulatory Protein 2</td>
</tr>
<tr>
<td>rMuIL-12</td>
<td>Recombinant murine interleukin-12</td>
</tr>
<tr>
<td>LPN</td>
<td>Laparoscopic partial nephrectomy</td>
</tr>
<tr>
<td>LpP</td>
<td>Low pressure plasma</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>-------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>MCI</td>
<td>Mild Cognitive Impairment</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular Weight</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium Hydroxide</td>
</tr>
<tr>
<td>NCI</td>
<td>National Cancer Institute</td>
</tr>
<tr>
<td>NtNP</td>
<td>Non-thermal Nitrogen Plasma</td>
</tr>
<tr>
<td>N2</td>
<td>Nitrogen gas or nitrogen plasma</td>
</tr>
<tr>
<td>N2O</td>
<td>Nitrous oxide plasma</td>
</tr>
<tr>
<td>SWI</td>
<td>Susceptibility-weighted imaging</td>
</tr>
<tr>
<td>TTH</td>
<td>Time to Hemostasis</td>
</tr>
<tr>
<td>TUR</td>
<td>Transurethral Resection</td>
</tr>
<tr>
<td>WIT</td>
<td>Warm Ischemia Time</td>
</tr>
</tbody>
</table>
ABSTRACT OF THE DISSERTATION

Chitosan Decontamination with Non-Thermal Nitrogen Plasma to Enable Internal Use

by

Andrew R. Crofton

Doctor of Philosophy, Graduate Program in Anatomy
Loma Linda University, March 2016
Dr. Wolff M. Kirsch, Chairperson

Chitosan (CS) is a ubiquitous biopolymer and is recognized as a promising biomedical material. Potential medical applications for chitosan are extensive and many have shown impressive results. However, chitosan is clinically only used as a topical hemostatic agent due to an inability to sterilize and depyrogenate chitosan without negatively altering its physical and biological properties with conventional techniques. We hypothesized that non-thermal nitrogen plasma (NtNP) would sterilize and depyrogenate chitosan while preserving its biological properties. Since plasma is a surface treatment, we micronized chitosan using cryo-ball and cryo-jet milling to increase its surface to volume ratio. Cryo-jet milling produced the smallest mean particle size (16.05 μm) and reduced molecular weight 36.5% (MW) and degree of deacetylation (DD) 9.6%. NtNP treatment of the resultant chitosan powder produced a sterile chitosan with endotoxin levels <2.50 EU/g, but further reduced MW 40% and DD 6.3%. Nitrogen content and bioadhesivity were unaltered. We tested biologic functionality in a murine orthotopic bladder cancer model in which a previous study showed chitosan dramatically enhanced anti-tumor properties of
interleukin-12 (IL-12). We found that bladder tumors regressed – and mice survived to 90 days – in 88% (n=16) and 92% (n=12) of C57Bl/6 mice treated with unsterile chitosan+IL-12 and plasma-sterilized chitosan+IL-12, respectively, but only 8% (n=12) and 0% (n=3) of mice treated with saline and CS-alone, respectively. All plasma-sterilized chitosan+IL-12 (n=11) and 92% of unsterile chitosan+IL-12 (n=13) treated mice rejected tumors upon re-challenge. These results are equivalent to the previously published chitosan+IL-12 study suggesting NtNP sterilization preserved the biological properties of chitosan. A rat model showed that hemostatic chitosan pads sterilized with electron beam irradiation and NtNP stopped femoral artery and artery/vein bleeding better than pads treated with either treatment alone and equivalent to unsterilized pads. A porcine kidney bleeding model showed that NtNP-sterilized pads had superior hemostatic properties compared to e-beam sterilized pads. No complications associated with implanting the pads were observed 14-weeks post-operatively. Histologic examination demonstrated tissue and inflammatory reactions characteristic of foreign body implants. Knowledge gained in these studies will allow clinical testing of parenteral applications of chitosan, including immediate trials of chitosan+IL-12 in bladder cancer patients.
CHAPTER ONE
INTRODUCTION

Background

Alzheimer's Disease

Dr. Kirsch’s laboratory has been studying Alzheimer's disease (AD) since the early 2000s after 2 amino acids his laboratory discovered in the early 1980s, aminomalonic acid and beta carboxyaspartic acid, were subsequently linked to iron homeostasis and aging. (Christy, 1981; Christy, 1982; Van Buskirk, 1984; Wheelan, 1989-A; Wheelan, 1989-B) The link was based on the finding that iron will bind to iron regulatory protein 2 (IRP2) under conditions of sufficient iron levels in the cell, which catalyzes an oxidative modification of IRP2 that converts a cysteine residue to aminomalonic acid. (Kang, 2003) This modification triggers proteasomal degradation of IRP2. This discovery led Dr. Kirsch to apply for an R01 grant from the NIH, which was funded, to study how IRP2 might be involved in perturbations in iron hemostasis that had been observed in AD.

Dr. Kirsch’s study of IRP2 led to the discovery that polymorphisms in the gene encoding IRP2 (IREB2) are significantly associated with AD, but expression levels and localization of the IRP2 protein are not altered in the hippocampus of AD patients. (Coon, 2006; Magaki, 2007) However, loosely bound iron was decreased in the hippocampus of AD patients and increased in brains of transgenic IRP2 knockout (-/-) mice. (Magaki, 2007-A; Magaki, 2007-B) This finding of decreased iron in the AD hippocampus directly contradicted many other studies in the literature. Since the human studies from Dr. Kirsch’s
laboratory were performed on human brains categorized on both AD and presence and severity of cerebral amyloid angiopathy (CAA), a common vascular co-morbidity with AD not commonly examined in AD studies, it was hypothesized that iron was increased in CAA patients due to microhemorrhages and this skewed findings of previous studies quantitating iron in post-mortem AD brains. Initial evidence supporting this hypothesis came in the form of susceptibility-weighted imaging (SWI), a magnetic resonance imaging (MRI) sequence exceptionally sensitive for detecting iron, that showed greater numbers of microbleeds in the brains of CAA than AD patients. (Schrag, 2010; Ayaz, 2010) A meta-analysis by Schrag et al subsequently demonstrated that a series of publications from one laboratory had reported significantly higher iron levels in AD brain than other studies. (Schrag, 2011) When those studies were removed from the analysis, there was no longer a statistically significant difference in iron levels in AD brains compared to age-matched controls. However, copper (Cu) remained significantly decreased (p=0.0003).

Copper

In the course of the iron studies, a link had been discovered between IRP2 and Cu in the IRP2 knockout mice mentioned above. Specifically, it was discovered that IRP2 knockout mice had significantly lower Cu levels in multiple brain regions, including the hippocampus, at 6 weeks of age compared to wild-type mice. (Mueller, 2009) This decrease in hippocampal Cu was correlated with increased expression of amyloid precursor protein (APP), a known Cu-binding
protein thereby suggesting that a possible mechanism for this observed change in Cu might be a change in APP due to IRP2 dyshomeostasis.

When I joined Dr. Kirsch’s laboratory, studies measuring Cu and iron concentrations in serum from AD patients and controls were ongoing. In these studies, we found that the ratio of Cu to non-heme iron predicted which patients with mild cognitive impairment (MCI) would progress to AD over a 5-year period. (Mueller, 2012) We also correlated these findings with longitudinal studies of serum protein markers, which showed changes in the complement system. (Zabel, 2012) Subsequent studies on complement found a switch in the mechanism of amyloid clearance from the brains of CAA patients compared to AD and control that involved complement proteins. (Zabel, 2013) We believe this mechanism might explain the vascular fragility associated with CAA. As such, studies aimed at developing a targeted therapy for abnormal complement activation on cerebral microvessels are currently underway in Dr. Kirsch’s laboratory.

To strengthen our initial studies on iron and Cu in the blood of AD patients, we performed a meta-analysis to examine changes in oxidative stress in the blood of AD and MCI patients. In this study, we found that markers of lipid peroxidation, which can occur with abnormalities in transition metal homeostasis, are elevated in the blood in AD and MCI patients. Furthermore, we found evidence of Cu metabolism abnormalities and reduced total antioxidant capacity. (Schrag, 2013) Thus, there was strong evidence to continue studying changes in Cu in AD and CAA brains.
Our initial studies found that Cu was decreased in post-mortem brains of both AD and AD+CAA patients compared to age-matched controls. Cu was significantly deposited on the vasculature in CAA as well. (Schrag, 2011) Thus, it appeared that Cu was perturbed in both AD and CAA, though the observed changes were different for the two diseases, suggesting different pathophysiological mechanisms.

Studies on Cu were expanded to begin understanding the extent of Cu dyshomeostasis in AD. Those studies were the focus of my early work in Dr. Kirsch’s laboratory. The primary results showed that Cu was significantly decreased in synaptic vesicles of AD brains (Figure 1), but a fluorescent probe for monovalent Cu showed a staining pattern that indicated an increase in intracellular Cu (Figure 2).
Figure 1. Copper concentration in synaptic vesicles. Synaptic vesicles were isolated via ultra-centrifugation from age-matched control, AD-only, and AD+CAA temporal lobe (n=4 each group) and Cu concentration measured via atomic absorption spectrometry. Values are mean ± standard deviation (SD). Significance calculated with Student's t-test.

Figure 2. Histologic copper localization in human temporal lobe. Histologic sections of human brain from temporal lobe were stained with a fluorescent probe selective for monovalent copper (CS3) and visualized by confocal microscopy.
As we studied this phenomenon, we began hitting the technological limits of techniques for studying neurological metallobiology. Specifically, after much trial and error, as well as consultation with an outstanding Swiss scientist from Georgia Tech University, we came to the conclusion that the fluorescent probe (CS3) we had been using for imaging monovalent Cu in histologic brain sections in fact stains lipids and might not stain Cu at all.

**Dynactin p62**

Despite this significant issue with Cu probes, our preliminary studies have led to the discovery that Dynactin subunit p62, a multi-subunit protein associated with intracellular transport that binds to ATP7B, a known Cu-binding protein, is decreased in the brains (Figure 3) and increased in the blood (Figure 4) of AD patients. We believe this indicates a degradation process in which p62 is broken down in the brain and released in the blood.
Based on the data presented in Figures 1-4, we hypothesized that breakdown of Dynactin p62 results in Cu transport abnormalities in the brain. Thus, our laboratory is currently working on characterizing a number of novel, non-commercially available monovalent Cu-staining probes that have recently been described by laboratories at UC Berkeley as well as Georgia Tech.
University in an effort to characterize tissue and cellular level alterations in Cu in AD and CAA. Additionally, we have attempted studies using a commercially available siRNA system for knocking down p62 expression in neuron-like cells, but the siRNA failed to establish knock down, and in fact increased p62 expression, albeit non-significantly. Thus, we plan to develop a p62 siRNA so we can characterize the effects of p62 knockdown on Cu and select proteins in brain cells like neurons, astrocytes, and microglia.

**Aβ Immunotherapy**

Another aspect of my early work on the involvement of Cu in AD involved the effect of amyloid-beta (β) immunotherapy. Specifically, Elizabeth Head and colleagues at UC Irvine were studying the effects of anti-Aβ immunotherapy on cognitive measures in aged beagles. We obtained dog brain tissue from Dr. Head and subsequently investigated the effects of Aβ immunotherapy on brain Cu levels. Our hypothesis was that Aβ immunotherapy would reduce Cu levels in the brain since Aβ has an exceptionally strong affinity for Cu. We also studied the effects of Aβ immunotherapy in canine brains on levels of iron and zinc. We found that there were no significant alterations in Cu or iron in dogs treated with immunotherapy (data not shown). Large, multi-center human clinical trials investigating the efficacy of Aβ immunotherapies in treating AD failed to demonstrate meaningful benefit and caused significant morbidity and even worsening of symptoms with anti-Aβ immunotherapy. These disappointing results led to rapid and significant changes in the types of vaccines being used for
human studies. Thus, we decided not to pursue the dog studies further since the Aβ immunotherapies that had been used on the dogs were quickly becoming replaced by new and improved versions that demonstrated vastly different results than the originals.

**Shift from AD and Copper to Chitosan and Nitrogen Plasma**

As our laboratory was working on the aforementioned studies on AD and Cu, we were subsequently increasing our involvement in research on a biopolymer called chitosan (CS), pronounced kite-o-san. Our interest in CS stemmed from Dr. Kirsch’s experience as a neurosurgeon. He was interested in developing a novel hemostatic agent for neurosurgical operations since currently available hemostatic agents have many negative characteristics that make obtaining intraoperative hemostasis a highly challenging endeavor. CS is known to have exceptional hemostatic properties – in addition to many other highly desirable properties from a biomedical perspective – but CS-based hemostatic agents can only be used topically.

Dr. Kirsch determined that the issue with gaining FDA approval for using CS as an implantable hemostatic agent is inadequate methods for reducing endotoxin contaminants in CS while simultaneously maintaining its biological properties. Dr. Kirsch, in collaboration with Dr. Samuel Hudson from North Carolina State University, identified non-thermal nitrogen plasma as a novel technique that might effectively reduce endotoxin contaminants in CS without
negatively affecting its biological properties. Thus, Drs. Kirsch and Hudson filed and obtained a patent on the idea.

In early 2012, Dr. Kirsch obtained an SBIR grant from the NIH to study the effects of nitrogen plasma on the hemostatic efficacy of CS in a porcine model of laparoscopic partial nephrectomy (LPN). As a result of the funding, I switched my focus to CS full-time from the AD and Cu work. However, by early 2013, the relationship between Dr. Kirsch and other co-founders of the small business that had received the SBIR grant had fallen apart and Dr. Kirsch was forced to return the remaining funds from the SBIR grant to the NIH. Thus, my attention shifted back to AD and Cu.

As previously described, our Cu work was not progressing due to technical challenges. Additionally, an LLU alumnus, Dr. James Gulley, M.D., Ph.D., who spent time in Dr. Kirsch’s laboratory in the 1990s had visited our laboratory and told me about work he was doing with collaborators on CS at the National Cancer Institute (NCI) of the National Institutes of Health (NIH) where he was the Director of Clinical Trials and Deputy Chief of the Laboratory of Tumor Immunology and Biology. Specifically, they had demonstrated outstanding anti-tumor properties of CS and interleukin-12 (CS+IL-12) in the treatment of murine bladder cancer. (Zaharoff, 2009) The NIH had been moving rapidly towards a clinical trial to test this combination in human bladder cancer patients, but their efforts were stymied when they were unable to obtain a sterile CS with sufficiently low endotoxins for parenteral use and intact biologic functionality. He was pleased to hear that we were working on solving this exact issue.
My conversation with Dr. Gulley led Dr. Kirsch and I to begin searching for a U.S. CS manufacturer so that we could obtain CS to sterilize and depyrogenate with nitrogen plasma in order to move forward the implantable hemostat and bladder cancer immunotherapy indications. We quickly identified Scion Cardiovascular, Inc. as the only U.S.-based CS manufacturer. We contacted Scion and entered into a relationship with them. Shortly thereafter, we wrote and submitted an SBIR grant application with the goal of characterizing the effects of nitrogen plasma decontamination on CS with an emphasis on the preservation of its biologic properties. We proposed to test the biologic functionality of plasma-treated CS in the same mouse model of bladder cancer that the NIH had used for its initial studies on CS+IL-12. This SBIR grant, officially given to Scion Cardiovascular, Inc., was funded in the fall of 2014. Thus, I once again shifted my full attention to studies on nitrogen plasma decontamination of CS and the studies presented herein are the result of that work.

**Chitosan**

*History and Derivation*

The precursor of CS is chitin, which was first described in 1811 by a professor of natural history in France named Henri Braconnot when he discovered that a fraction of mushrooms are insoluble in alkaline solution. (Nwe, 2011; Muzzarelli, 2012) Braconnot named this substance “fungine.” Amazingly, chitin was the first polysaccharide ever described. In fact, Braconnot discovered chitin 30 years before cellulose was first described. (Muzzarelli, 2012) The name
fungine was replaced in 1823 with “chitin” when Antoine Odier isolated a fraction of insect cuticles (i.e. exoskeleton) that was also insoluble in basic solutions and named it chitin after the Greek word “chiton,” meaning tunic or covering. We now know that chitin is a highly abundant biopolymer – only surpassed in abundance by cellulose – found in shells of crustaceans, cuticles of insects, cell walls of fungi, and microalgae. (Nwe, 2011)

Nearly 50 years after chitin was discovered, chitosan was first described in 1859 by C. Rouget when he found that boiling insect-derived chitin in potassium hydroxide altered it in a way that made it soluble in acids. (Baldrick, 2010; Buschmann, 2013) F. Hoppe-Seiler then gave the name “chitosan” to this substance in 1894. (Badawy, 2011) It took until 1950 before the chemical structure of CS was described. (Baldrick, 2010)

In the 1970s, research into CS was stimulated by a need to control waste generated by the seafood industry. This led to the first industrial extractions of CS from exoskeletons of crustaceans. (Buschmann, 2013; Badawy, 2011) Today, industrially produced chitin continues to be extracted primarily from crustacean shells in an effort to continue controlling shellfish waste. (Nwe, 2011) Because crustacean shell waste generated by the crab, lobster, and shrimp meat industries is such a major environmental problem, extracting chitin and subsequently converting it to CS is a highly economical, “green” process that is helping ameliorate a major environmental issue. (Dutta, 2004)
**Scientific Background**

Methods for isolating chitin are similar regardless of the source; the extraction procedure generally involves deproteinization with warm NaOH, demineralization with a strong acid like HCl, and an optional de-colorization step. (Nwe, 2011) Chitin from different organisms varies in secondary structure. α-Chitin, found in crustacean shells, has a secondary structure of anti-parallel chains and is the most abundant form of chitin in nature. β-Chitin, found in squid beaks, forms a secondary structure of parallel chains with intrasheet hydrogen bonding. γ-Chitin, found in cells wells of fungi, has a secondary structure with both parallel and anti-parallel chains (i.e. both α- and β-chitin). (Jang, 2004) The functional effects of these structural differences have not been widely investigated, but it has been shown that α-chitin is more resistant to chemical treatment than β- or γ-chitin due to inter-sheet hydrogen bonding. (Pillai, 2009)

The chemical structure of chitin is similar to that of cellulose with both polysaccharides consisting of linear chains of D-glucose moieties bonded via β-(1-4)-glycosidic linkages (Figure 5). The difference is chitin, with the chemical names N-acetyl-D-glucosamine or N-acetamido-2-deoxy-D-glucopyranose, has an acetylated amino group (–NHRCH3) bonded at the C2 position in each D-glucose unit whereas cellulose has a hydroxyl group (–OH). CS is unique amongst naturally occurring polysaccharides since it is basic, whereas most others, including cellulose, are acidic. This gives CS many unique properties.
CS is formed by deacetylating chitin with hot, often boiling, concentrated NaOH. The amount of time chitin is exposed to hot NaOH dictates the degree of deacetylation (DD) of the resultant CS. Generally, CS is defined as chitin that is >50% deacetylated, meaning the C2 functional groups of at least half of the D-glucose units are primary amines (-NH₂) (Fig. 5). (Pillai, 2009) However, disagreement exists over naming chitin and CS based on DD and this has generated a proposal to name chitin and CS based on acetic acid solubility.
(Badawy, 2011; Kumar, 2000) Of course, CS dissolves in aqueous acetic acid and chitin does not. The former definition for CS is used in this study.

The aforementioned primary amines are positively charged and make CS polycationic. This positive charge is responsible for virtually every advantageous biomedical characteristic of CS, which include being biocompatible, biodegradable, non-toxic, non-antigenic, bioadhesive, hemostatic (even in anti-coagulated blood), easily manipulated, anti-oxidative, immune modulatory, an analgesic, and a wound healing enhancer. (Bumgardner, 2003; Di Martino, 2005; Dodane, 1999; Dutta, 2004; Khor, 2003; Lang, 1989; Nwe, 2011; Rao, 1997; Yen, 2007; Yen, 2008) These attributes have led to investigations of a wide range of clinical applications for CS including as a(n) drug and/or gene delivery vehicle, immune adjuvant, pharmaceutical excipient, hemostatic agent, tissue scaffold, and wound dressing. (Di Martino, 2005; Dutta, 2004; Enriquez de Salamanca, 2006; Harish Prashanth, 2007; Kenaway, 2005; Khor, 2002; Khor, 2003; Kind, 1990; Mitra, 2001; Nettles, 2002; Nwe, 2011; Rao, 1997; Read, 2005; Sandri, 2004; Subar, 1992; Sugano, 1980; Vinsova, 2011; Whang, 2005; Yen, 2008; Zivanovic, 2005)

As described previously, our laboratory’s interest in CS was sparked first by its exceptional hemostatic properties that occur independent of the clotting cascade via strong adhesion to negatively charged tissues and electrostatically driven agglutination of red blood cells and platelets. (Chou, 2003; Whang, 2005) Our interest was subsequently piqued by CS’s outstanding potential as a drug delivery vehicle. We quickly learned that clinical uses of CS remain limited to
topical hemostats and wound dressings in spite of CS’s outstanding biomedical potential and the large number of CS-related publications in the biomedical literature. We then identified inadequacy of conventional sterilization methods for sterilizing and especially depyrogenating CS as the primary reason for limited clinical use of CS. These conventional sterilization methods include dry/wet heat, radiation, and chemical sterilants and they cause alterations such as caramelization of the polysaccharide, chain scissions, and/or may leave residual toxic residues in the material. (Whang, 2005; de Oliveira Cardoso Macedo, 2013; Franca, 2013; Lim, 1998; Lim, 1999; Marreco, 2004; Norzita, 2013; Rao, 1995; Rosiak, 1992; San Juan, 2012) These alterations to the physical and chemical properties of CS cause changes in the biological properties as well. Thus, these methods are not optimal, or appropriate, for sterilizing CS. We hypothesized that non-thermal nitrogen plasma would effectively sterilize and depyrogenate CS and the biological properties of CS would simultaneously be preserved.

**Plasma**

*Scientific Background*

Plasma is the oft forgotten 4th state of matter – after solid, liquid, and gas – and has emergent properties that gases do not such as conductivity, magnetism and quasi-neutrality. (Cornelius, 2007) Ironically, 99% of the matter in the universe is in the plasma state since most stars in the universe, including Earth’s sun, primarily consist of plasmas. (Tendero, 2006) These extraterrestrial plasmas are fully ionized and therefore consist entirely of ionized gas molecules and
electrons. (Moisan, 2001) Due to their very high temperatures (5,000-10,000 Kelvin), these plasma are termed hot plasmas. (Yokoyama, 1990)

On Earth, the most common forms of naturally occurring plasmas include lightning and auroras (borealis/australis). (Moisan, 2001; Shintani, 2007) Many artificially generated plasmas have become commonplace as well, especially in industries such as the aeronautical sector (e.g. plasma arc welders, a hot plasma) and microelectronics/semiconductor sector (e.g. low pressure cold plasma for altering material surfaces). (Tendero, 2006) To generate a plasma, the kinetic energy of the molecules of a gas must exceed their ionization energy. Once this occurs, the outer-most electron exits the electron cloud thereby making the atom positively charged. Although an ion has formed, there is no overall charge of the plasma because the parent species, the gas, was uncharged. This is called the principle of quasi-neutrality; there are areas of positive charge (ions) and negative charge (electrons), but overall the charges are equal and the plasma is not charged. (Cornelius, 2007)

For medical applications of plasma, a "cold," or non-thermal plasma, generally defined as having a temperature ≤ 40°C, is desired. (Kong, 2009) Non-thermal plasmas consist of only partially ionized gas. Thus, non-thermal plasmas contain ionized gas molecules, free electrons, free radicals, neutral byproducts, and photons. Such plasmas are generated with a weak electric field. (Moisan, 2001; Messerer, 2005; Morfill, 2009) The number of excited, or hot, atoms and molecules is small enough that the heated gas species and their derivatives (i.e. electrons) quickly collide with non-excited, or "cold," gas molecules. These
collisions de-excite and resultantly cool the excited species. When this occurs, photons are emitted. In the case of plasma generated from nitrogen gas, called non-thermal nitrogen plasma (NtNP), a rapidly pulsed electric field can be used to generate the plasma because ionization of nitrogen species occurs rapidly via Townsend discharge, a form of electron avalanche where excited electrons collide with non-excited atoms causing more electrons to ionize, which then collide with more non-excited atoms. (Massines, 2005)

**Plasma Decontamination**

Many studies have demonstrated plasma effectiveness in killing or inactivating bacteria, bacterial spores, viruses, fungi, pyrogens, and prions. (Claire, 2006; Deilmann, 2008; Ehlbeck, 2011; Kelly-Wintenberg, 1998; Klampfl, 2012; Kong, 2009; Koulik, 1999; Kvam, 2012; Laroussi, 1996; Laroussi, 2005; Messerer, 2005; Mogul, 2003; Moisan, 2001; Rauscher, 2010; Rossi, 2009; Shintani, 2007; Sureshkumar, 2010; Ulbin-Figlewicz, 2014; Vicoveanu, 2008; Yang, 2009) As such, non-thermal plasma has been used in food processing and beverage bottling to sterilize plastics in an economical manner. (Adler, 1998; Deilmann, 2008; Messerer, 2005) However, the only plasma systems that have been employed in the medical field are “plasma-assisted” devices in which a germicidal gas vapor, usually hydrogen peroxide or formaldehyde, are ionized in order to enhance the microbicidal properties of the vapor. (Laroussi, 2005; Okpara-Hofmann, 2005) It is noteworthy that plasma-assisted hydrogen peroxide
sterilization is thought to sterilize via oxidation, a different mechanism than true gas plasmas. (Krebs, 1998; Lerouge, 2000)

The mechanisms by which plasmas inactivate spores and other biological molecules like endotoxins and prions are debated, but it is agreed that UV plays a significant role in spore inactivation, as does chemical etching. Studies have shown that there is a triphasic effect of plasma on bacterial spores. During the first phase, which is fast, it is postulated that UV radiation, through DNA damage, inactivates the first layer of spores on a surface. During the second phase, which is much slower, etching takes place. During this process, layers of spores are physically "etched" away by bombardment of the surface with charged particles and synergistic reactions of active species with the surface molecules of the material being treated. The third phase, which is fast like the first, is thought to be when the last monolayer of spores are inactivated by UV radiation. (Rauscher, 2010; Klampfl, 2012; Kvam, 2012; Moisan, 2001; Vicoveanu, 2008; Von Keudell, 2010) For pyrogens, studies suggest that the inactivation occurs via physico-chemical interactions with reactive species in the plasma that result in either chemical alterations to the molecules rendering them unrecognizable by immune cells or physical degradation through etching that ultimately leads to volatility and, thus, removal from the material. (Von Keudell, 2010)

NtNP has been shown to be the most efficient, least material damaging plasma in terms of sterilization and depyrogenation because nitrogen gas is difficult to energize to atomic nitrogen. (Shintani, 2007) Thus, we focused our studies on nitrogen-based non-thermal plasmas. Our studies presented herein
represent the largest characterization of NtNP sterilization and depyrogenation of medical devices or pharmaceuticals to date.

**Plasma Treatment of Chitosan**

To our knowledge, there are no reports in the literature of sterilization and depyrogenation of CS with NtNP or any other type of plasma. The reports on plasma treatment of CS that do exist focus on surface modifications induced by plasma treatment. For example, in a study by Silva et al, CS films were treated with nitrogen and argon plasmas and changes in physico-chemical properties were characterized. (Silva, 2008) They went on to determine the effect of the physico-chemical changes on the biological properties of CS. Similar studies have examined the physico-chemical changes to CS films induced by H₂, NH₃, He/NH₃, and argon plasmas. (Ogino, 2008; Wanichapichart, 2009) Another study examined how argon, methane, and oxygen plasmas affect the anti-microbial properties of CS. (de Oliveira Cardoso Macedo, 2013) One study, by Luna et al, showed that NtNP treatment increased cell adhesion to CS. (Luna, 2011) This led us to hypothesize that NtNP treatment would enhance the hemostatic and drug delivery properties of CS by increasing its bioadhesivity.

**The Present Research**

The present studies sought to characterize the physico-chemical and biological effects of NtNP decontamination of CS. Our goal was to enable new clinical applications of CS that have been hindered by the inadequacy of
conventional sterilization methods in decontaminating CS. The studies were intended to serve as the pre-clinical foundation necessary for the commencement of human safety tests of CS as an implantable material. Two major clinical applications of CS were targeted in our studies. First, we sought to demonstrate that NtNP-sterilized CS is safe and effective as a drug delivery vehicle. Second, we sought to demonstrate that NtNP-sterilized CS maintains its hemostatic efficacy and can be safely implanted.

A secondary goal of the project was to advance development of NtNP as a sterilization and depyrogenation procedure for thermolabile polymers. These materials are becoming increasingly utilized in medical products and require improved sterilization techniques.

**Approach of the Studies**

For all of the studies, raw CS flake was obtained from Scion Biomedical, Inc. The CS Scion manufactured is a medical-grade CS derived from crab shells from one haul of one fishing boat. All NtNP treatments were performed on either a low-pressure plasma (LpP) instrument manufactured and operated by PVA-TePla America, Inc. or an atmospheric pressure plasma (ApP) instrument built and operated by the Department of Nuclear Engineering at North Carolina State University. The majority of the plasma treatments were performed on the LpP system due to concerns regarding the age, consistency, and durability of the ApP system. Additionally, since this project was focused on translatability of the research results, the commercial availability of the LpP system was viewed as a
major advantage over the ApP system. Prior to the commencement of these studies, there were no definitive results that had been published showing that either type of plasma, LpP or ApP, was superior at achieving sterilization and depyrogenation. (Klampfl, 2012; Koulik, 1999; Laroussi, 1996)

**Study 1**

Since the raw Scion CS was in a flake form and NtNP is only a surface treatment, we first micronized the CS into a powder via a cryo-milling technique to increase its surface to volume ratio and improve its solubility. Two cryo-milling techniques were analyzed and the physico-chemical properties of CS were characterized before and after milling (Study 1). The effect of NtNP sterilization on the physico-chemical properties of the CS flake and powder were then determined.

**Study 2**

Once a sterile CS powder was generated via NtNP treatment, preservation of the biological properties of the CS was characterized in a mouse model of bladder cancer. This model was chosen based on the results of a previous pre-clinical study performed at the NCI in which it was found that CS drastically enhanced the anti-tumor efficacy of interleukin-12 (IL-12) in a murine orthotopic bladder cancer model. (Zaharoff, 2009) The NCI prepared a phase I human clinical trial based on the results of this study, but it was stymied by an inability to obtain a sterile CS with sufficiently low endotoxins and intact biologic
functionality. Thus, we reasoned that if NtNP-sterilized CS+IL-12 demonstrated similar anti-tumor properties as the NCI observed, the phase I trial could move forward. Since intravesical administration of drugs into the bladder is considered a parenteral route of administration, but there is limited direct contact with the blood, we reasoned that initially testing a parenteral preparation of CS in the bladder would be an exceptionally safe approach.

**Study 3**

In Study 3, we shifted our attention to the hemostatic properties of CS. There has been immense interest in developing CS as a hemostatic agent since 1983 when Mallette and colleagues discovered that CS could induce a rapid coagulum in both normal and anti-coagulated blood. (Malette, 1983) This is reflected in the large number of 100% CS topical hemostatic agents currently on the market. However, not a single implantable CS hemostatic agent has been approved for human use. The primary barriers preventing approval of implantable CS hemostatic agents include pyrogen levels that exceed regulatory limits and insufficient evidence demonstrating CS is safe to implant.

For Study 3, Scion’s FDA approved topical hemostatic agent (Clo-Sur P.A.D.™), which consists of a lyophilized CS pad, was sterilized with NtNP and its hemostatic efficacy subsequently evaluated in an established rat model of bleeding. (Ersoy, 2007) Unsterilized and electron beam (EB) sterilized pads were used as controls to evaluate whether NtNP sterilization had a negative effect, positive effect, or no effect on the hemostatic properties of CS. Effect of NtNP
sterilization on select physico-chemical properties of the pads were also characterized.

**Study 4**

For study 4, NtNP-sterilized pads were tested in a porcine LPN model of bleeding. For this model, the lower pole of one kidney was removed using a laparoscopic approach. CS was then used as the sole hemostatic agent for achieving hemostasis. EB sterilized pads were used as the benchmark for evaluating the effect of NtNP sterilization on the hemostatic properties of CS. Hemostatic effectiveness was determined by the number of pads needed to achieve hemostasis, estimated blood loss (EBL), and warm ischemia time. The animals were then monitored for complications for 14 weeks and histologic examinations were performed to evaluate the surgical site with the implanted CS for presence of urinoma, hematoma, degree of CS resorption, and tissue reactivity. We hypothesized that the NtNP-sterilized CS would have superior hemostatic properties and tissue compatibility compared to the ES-sterilized CS.

**Significance of the Studies**

As discussed previously, CS has numerous potential parenteral uses including as a(n) resorbable hemostatic agent, immune adjuvant, and drug or gene delivery vehicle. Overcoming the primary barrier that has prevented these applications from achieving clinical use would represent a major advancement. As such, a sterile CS with endotoxin levels below the regulatory mandates for
parenteral drugs and Class III medical devices with intact biological properties
will enable immediate clinical testing of CS as a drug delivery vehicle and
implantable hemostatic agent. For example, the NCI will be able to move forward
with a phase I clinical trial testing the safety and efficacy of CS+IL-12 for the
treatment of non-muscle invasive bladder cancer. Since other murine tumors
have also shown responsiveness to CS+IL-12, human trials for these other
malignancies could also commence. Additionally, wide availability of a
pharmaceutical-grade, sterile/depyrogenated CS would potentially eliminate, or
at least reduce, the inflammatory and immunologic artifacts secondary to
endotoxin contamination that plague pre-clinical studies of CS. (Gorbet, 2005;
Lieder, 2013)

The significance of an effective new treatment for urinary bladder cancer
(BC) cannot be understated. BC is the 5th most common cancer in the US with
over 70,000 new cases diagnosed each year. It is also the most expensive
cancer to treat per patient due to high recurrence rates. (Jemal, 2010; Siegel,
2012; Siegel, 2013; Siegel, 2011; Van Der Heijden, 2009; van Rhijn, 2009) Over
90% of BC cases are transitional cell carcinomas (TCC) and 80% of TCCs are
classified as superficial, or more appropriately non-muscle invasive, tumors
(stages Ta, Tis, or T1). (Kirkali, 2005; Pasin, 2008) Notably, it has been over 30
years since a new treatment for non-muscle invasive BC was approved. (Jarow,
2014) The current standard of care for non-muscle invasive BC is transurethral
resection (TUR) of the tumor with post-operative immunotherapy provided by
inducing an intravesical infection with bacillus Calmette-Guerin (BCG).
Askeland, 2012; Hall, 2007) Between 20-30% of patients do not respond to BCG therapy and 20-50% of BCG-responsive patients develop recurrent tumors within 5 years. (Parkin, 1999; Malmstrom, 1999; Van Der Heijden, 2009; van Rhijn, 2009)

As with BC, an implantable hemostatic agent could have a tremendous clinical impact. Hemostasis remains a significant challenge in many surgical operations and traumas. An economical, resorbable hemostatic agent that retains its effectiveness in the presence of anti-coagulant therapy would greatly improve the management of intraoperative hemostasis. (Whang, 2005)

Finally, there will be many uses for a sterilization/depyrogenation technique like NtNP in medicine. Many delicate materials are increasingly being used for medical devices and traditional sterilization methods either reduce the effectiveness and/or durability of the devices or preclude their use entirely. Thus, an effective decontamination technique that retains the physico-chemical properties of such materials would be widely used. Replacing techniques that use highly toxic compounds like ethylene oxide and formaldehyde would improve the safety for workers that must routinely sterilize medical supplies.
CHAPTER TWO

FORMULATION AND CHARACTERIZATION OF A PLASMA STERILIZED, PHARMACEUTICAL GRADE CHITOSAN POWDER

Andrew Crofton\textsuperscript{a,c}, Samuel Hudson\textsuperscript{d}, Kristy Howard\textsuperscript{c}, Tyler Pender\textsuperscript{c}, Abdelrahman Abdelgawad\textsuperscript{d}, Daniel Wolski\textsuperscript{d}, Floyd Petersen\textsuperscript{c}, and Wolff Kirsch\textsuperscript{b,c}

acrofton@llu.edu, shudson@ncsu.edu, khoward@llu.edu, aabelg@ncsu.edu, dwolski@ncsu.edu, wkirsch@llu.edu

\textsuperscript{a}Department of Anatomy, \textsuperscript{b}Division of Biochemistry, and \textsuperscript{c}Neurosurgery Center for Research, Training, and Education, School of Medicine, Loma Linda University
11234 Anderson St.
Medical Center A537
Loma Linda, CA 92350

\textsuperscript{d}College of Textiles, North Carolina State University
2401 Research Dr.
Raleigh, North Carolina 27695

*Corresponding Author
+1-909-558-7071
fax: +1-909-558-0472
wkirsch@llu.edu

Carbohydrate Polymers, 2016
**Abstract**

Chitosan has great potential as a pharmaceutical excipient. In this study, chitosan flake was micronized using cryo-ball and cryo-jet milling and subsequently sterilized with nitrogen plasma. Micronized chitosan was characterized by laser diffraction, scanning electron microscopy (SEM), conductometric titration, viscometry, loss on drying, FTIR, and limulus amebocyte lysate (LAL) assays. Cryo-jet milling produced mean particle size of 16.05 μm, 44% smaller than cryo-ball milling. Cryomilled chitosan demonstrated increased hygroscopicity, but reduced molecular weight and degree of deacetylation (DD). SEM imaging showed highly irregular shapes. FTIR showed changes consistent with reduced DD and an unexplained shift at 1100 cm$^{-1}$. Plasma treated chitosan was sterile with <2.5 EU/g after low-pressure plasma and <1.3 EU/g after atmospheric pressure plasma treatment. Plasma treatment decreased the reduced viscosity of chitosan flake and powder, with a greater effect on powder. In conclusion, pharmaceutical grade, sterile chitosan powder was produced with cryo-jet milling and plasma sterilization.

**Keywords:** Chitosan, Cryomilling, Jet Milling, Ball Milling, Cryo-ball Milling, Cryo-jet milling, Plasma Sterilization

**Abbreviations:** CS – chitosan; NtNP – non-thermal nitrogen plasma; ApP – atmospheric pressure plasma; LpP – low-pressure plasma; DD – degree of deacetylation;
Introdução

Chitosan (CS) em presença de umidade é um polímero biológico macio e tenso com grande potencial como material biológico, especialmente como excipiente e adjuvante imunológico. CS é obtido comercialmente por desacetilamento de chitina extraída de cascas de crustáceos por extração química ou fungos por extração enzimática e consiste de β-1,4-ligado 2-amino-2-deoxi-D-gluco-2-deoxi-D-glucopiranose e N-acetamido-2-deoxi-D-glucopiranose moieties that are randomly distributed throughout the polymer chain. Since chitin is the second most abundant biopolymer on Earth and crustacean shell waste generated by the seafood industry is an environmental problem in coastal areas, CS is a widely available, green, and economical biomaterial. Chitin becomes CS once at least 50% of the D-glucosamine moieties in the polymer chain are deacetylated (Pillai, Paul & Sharma, 2009). However, there is disagreement over the naming of chitin and CS based on degree of deacetylation, which has resulted in a proposal for naming chitin and CS based on solubility in acetic acid (Badawy & Rabea, 2011; Kumar, 2000). CS is soluble in aqueous acetic acid, whereas chitin is not.

As an excipiente, CS enhances drug penetration through tissues and epithelial barriers by loosening gap junctions, maintains drug in the area of interest through bioadhesion between cationic amino groups of CS and anionic tissues, and controls drug release over time by keeping drugs bound until physical degradation (Artursson, Lindmark, Davis & Illum, 1994; Dodane, Amin Khan & Merwin, 1999; Jameela, Misra & Jayakrishnan, 1994; Kristl, Šmid-Korbar, Štruc, Schara & Rupprecht, 1993; Luessen, de Leeuw, Langemeyer, de
The biological functions of CS are dependent on its physical properties, including molecular weight (MW), degree of deacetylation (DD), salt form, and pH at which it is used (Dodane & Vilivalam, 1998; Kumiarska, Weinhold, Thöming & Stepnowski, 2011). This physical-functional relationship necessitates careful characterization of CS formulations. Since powder and hydrogel forms of CS – especially those composed of particles in the low micron range – have been identified as the ideal form of CS for pharmaceutical applications and micronization of materials is a common process in pharmaceutical manufacturing, the goal of the present study was to formulate and characterize a pharmaceutical grade CS powder that can also serve as a precursor to a hydrogel when dissolved in dilute acids (van der Lubben, Verhoef, van Aelst, Borchard & Junginger, 2001).

A powder form of CS was also desired to enhance the effectiveness of a novel sterilization method for CS based on non-thermal nitrogen gas plasma (NtNP) (de Oliveira Cardoso Macedo, de Macedo, Gomes, de Freitas Daudt, Rocha & Alves, 2013). Plasma is considered the fourth state of matter and consists of an ionized gas that has emergent properties the gas alone does not, such as magnetism and conductivity. Use of NtNP for sterilization of CS is necessitated by the fact that conventional sterilization methods like dry/wet heat, radiation, and chemical sterilants cause caramelization of the polysaccharide,
chain scissions, and/or may leave residual toxic residues in the material (de Oliveira Cardoso Macedo, de Macedo, Gomes, de Freitas Daudt, Rocha & Alves, 2013; Franca et al., 2013; Lim, Khor & Koo, 1998; Lim, Khor & Ling, 1999; Marreco, da Luz Moreira, Genari & Moraes, 2004; Norzita, Norhashidah, Maznah, Nurul Aizam Idayu Mat, Nor Akma & Norafifah Ahmad, 2013; Rao & Sharma, 1995; Rosiak, Ułański, Kucharska, Dutkiewicz & Judkiewicz, 1992; San Juan et al., 2012). These physicochemical changes result in changes in the biologic properties of CS making these methods non-optimal for sterilizing CS. Since NtNP is a surface treatment, it benefits from a large surface to volume ratio, which is accomplished when a material is micronized.

Knowledge on micronizing hard and crystalline materials is extensive, but lacking on softer materials. It is vital to characterize particle size and size distribution of powders since these properties influence flowability, dissolution, release kinetics, and more (Koennings, Sapin, Blunk, Menei & Goepferich, 2007; Miranda, Millán & Caraballo, 2007; Mullarney & Leyva, 2009). Reports in the literature of techniques for generating micronized CS powders are especially sparse. Techniques for micronizing CS that have been reported include dense gas anti-solvent precipitation, supercritical-assisted atomization, microsphere precipitation, high speed planetary ball mill, and ultrafine milling (Chien, Li, Lee & Chen, 2013; Gimeno, Ventosa, Boumghar, Fournier, Boucher & Veciana, 2006; Reverchon & Antonacci, 2006; Yao, Peng, Yin, Xu & Goosen, 1995; Zhang, Zhang, Jiang & Xia, 2012; Zhang, Zhang & Xia, 2014). We identified cryomilling as an optimal method for generating a CS powder based on previous studies that
show it preserves functional properties of proteins and starches by reducing the energy input needed to fractionate particles into smaller sizes (Dhital, Shrestha, Flanagan, Hasjim & Gidley, 2011; Ehmer, 2010; Tran, Shelat, Tang, Li, Gilbert & Hasjim, 2011). Additionally, cryomilling overcomes the soft ductile nature of CS, which reduces the effectiveness of traditional milling techniques in micronizing soft materials like CS (Garmise et al., 2006; Saleem & Smyth, 2010).

In the present study, two cryomilling techniques were tested, cryo-jet and cryo-ball milling. Ball and jet mills were chosen for this study since they are the only milling machines commonly used to reduce particles to 5 μm or less in dry conditions. (Vatsaraj et al 2003) Both ball and jet mills are thought to reduce particle sizes using the same mechanism(s), which is by breaking particles along cracks or fractures that already exist at the micro- or nanoscale. Although jet milling is the most commonly used technique for producing particles in the lower micrometer range and is the gold standard for manufacture of inhalable particles of small molecular drugs, this study is the first reported use of cryo-jet milling for micronizing CS (Ehmer, 2010). Once the optimal CS powder formulation was identified, defined as the micronized powder with the smallest mean particle size, it was sterilized with NtNP to form a sterile, pharmaceutical grade CS powder. Physicochemical properties of the CS were characterized before and after both milling and NtNP sterilization.
Materials and Methods

Reagents

All chemical reagents including glacial acetic acid, lactic acid, sodium acetate, hydrochloric acid, sodium hydroxide, and tryptic soy agar were obtained from Sigma Aldrich or Fisher Scientific and were of analytical grade.

Cryomilling

CS derived from crab shells in the form of 1-10 mm flakes was obtained from Scion Biomedical, Inc. (Miami, FL). CS flakes were filtered with fine mesh to remove flakes > 8 mm in diameter and approximately 30 g of filtered CS flake was placed in a 25 mL zirconium oxide jar. Six milling balls made of either zirconium oxide (10 mm diameter) or stainless steel (5 mm) were added to the jar and the CS flakes were subsequently milled under cooling with liquid nitrogen at 30 Hz for up to 30 minutes in the Retsch CryoMill system (Verder Scientific, Inc., Newtown, PA). For cryo-jet milling, CS flakes were filtered, as described above, to remove flakes > 8 mm, 470 g of filtered CS was placed in the Micron-Master jet pulverization system (The Jet Pulverization Co., Inc., Moorestown, NJ), cooled with liquid nitrogen, and milled with a jet stream of liquid nitrogen for 30 minutes.

Particle Sizing

Particle size analyses were performed on powders by suspending CS in water and measuring particle size with a Horiba LA-930 laser diffraction analyzer (HORIBA Instruments, Inc., Irvine, CA).
**Degree of Deacetylation**

Degree of deacetylation (DD) of both CS flake and powder was determined by conductometric titration. Conductometric titration was performed by dissolving dried CS sample of known mass (about 0.100 g) into 10 mL of 0.1 N hydrochloric acid (HCl) then 90 mL of distilled water. The CS solution was then titrated with a standard 0.1 N sodium hydroxide (NaOH) solution using a 10 mL buret while the solution conductivity was monitored as a function of the volume of NaOH added with an Orion Benchtop Conductivity Meter (Model 162) equipped with an Orion Conductivity Cell (Model 013030) (Raymond, Morin & Marchessault, 1993). During the titration, the temperature of the solution was kept constant (30 °C) by using a water bath since the conductivity is a function of temperature. In a typical conductometric titration curve, there are two deflection points. The first deflection point corresponds to the neutralization of excess H⁺ ions of the strong acid, HCl. After all excess H⁺ ions are neutralized, then the neutralization of the weak acid, the ammonium salt in CS, starts. After the ammonium is completely neutralized, the conductivity again goes up with a higher value of slope due to the excess of OH⁻ ions of NaOH added, which is the second deflection point. Thus, the range between the first and the second deflection points corresponds to the neutralization of the protonated amine groups of CS. As a result, the number of moles of NaOH used between the first and second deflection points equals the number of moles of amine groups of the CS sample. The percent DD was calculated by the following equation:
\[
\% \text{DD} = \frac{(v_2 - v_1) \times M_{\text{NaOH}}(\text{mol}L^{-1}) \times 161.16}{\text{Mass of chitosan sample (g)}} \times 100
\]  

(1)

where \((v_2 - v_1)\) is the difference in volume in liters between the two deflection points, \(M_{\text{NaOH}}\) is the molarity (mol/L) of standard NaOH solution, and 161.16 is the molar mass of chitosan.

**Morphologic Characterization**

CS particles were imaged via scanning electron microscopy (SEM) using a Phenom G1 (Model 800-03103-02) (FEI Co., Netherlands) microscope. Prior to imaging, a small amount of CS powder or flake was fixed on conductive carbon tape and mounted on the support. The sample was then sputtered with an approximately 6 nm layer of gold/palladium (Au/Pd) using a Quorum Technologies SC7620 Mini Sputter Coater (Laughton, East Sussex, UK), which deposits 10 nm coating/45 sec.

**Hygroscopicity**

Hygroscopicity of CS flake and powder was measured by loss on drying using a 120VAC Moisture Balance IL-50.001 (Summit Measurement Inc., South Deerfield, MA), an automatic moisture balance with a capacity of 50 g and resolution of 0.001 g. CS samples were placed on the balance and the masses before and after drying via vacuum were recorded. Loss on drying was calculated as the percent difference between the initial and final mass.
Molecular Weight

Viscosity of CS samples was measured using a 2-10 centistoke glass capillary ASTM 1 Ubbelohde viscometer (Fisher Scientific, Pittsburgh, PA). Viscosity measurements were performed by dissolving chitosan in a solvent containing 0.3 M acetic acid and 0.2 M sodium acetate (0.3 M AcOH/0.2 M NaOAc) to a final CS concentration of 1-2 mg/mL (0.1%-0.2%). The AcOH protonates the amino group which solubilizes CS and the NaOAc salt screens electrostatic interactions between different CS chains. The 0.3 M AcOH/0.2 M NaOAc solvent was chosen since previous studies have shown it prevents aggregate formation, unlike other solvents like 0.1 M AcOH/0.2 M NaCl (Rinaudo, Milas & Dung, 1993; Roberts & Domszy, 1982). Efflux times were measured for each of 3 concentrations (1 mg/mL, 1.5 mg/mL, and 2 mg/mL) and the following viscosities were determined using the given equations:

Relative viscosity \[ n_{\text{rel}} = \frac{t_{\text{CS}}}{t_{\text{sol}}} \] (2)

Specific Viscosity \[ n_{\text{sp}} = n_{\text{rel}} - 1 \] (3)

Reduced viscosity \[ n_{\text{red}} = \frac{n_{\text{sp}}}{c} \] (4)

where \(t_{\text{CS}}\) represents efflux time for chitosan, \(t_{\text{sol}}\) represents efflux time for the solvent, and \(c\) represents chitosan concentration in g/mL. Intrinsic viscosity ([n]) was calculated using the Huggins plot in which the reduced viscosities for the 3
chitosan concentrations were extrapolated to infinite dilution (i.e. zero concentration). Intrinsic viscosity is related to molecular weight by the Mark-Houwink equation:

$$[\eta] = KM^a$$

where K and a are constants for the specific polymer, solvent, and temperature combination. The K and a values for CS with a %DD of approximately 80% and the solvent 0.3 M AcOH/0.2 M NaOAc at 25 °C are 0.074 and 0.80, respectively (Brugnerotto, Desbrières, Roberts & Rinaudo, 2001).

**FTIR**

Molecular composition of CS flake and powder was assessed via Fourier Transform Infrared Spectroscopy (FTIR). To generate FTIR spectra, films of CS salts were cast by dissolving the CS flake or powder in 1% acetic acid. The films were then converted to the free amine form of CS by washing films in alcoholic base prior to imaging with a Nicolet iS50 FT-IR instrument (Thermo Fisher Scientific, Madison, WI) using a diamond attenuated total reflectance (ATR) attachment and 32 scans at 4 cm⁻¹ resolution.

**Plasma Sterilization**

CS samples were sterilized via NtNP treatment with either atmospheric pressure plasma (ApP) or low-pressure plasma (LpP) systems. The ApP
instrument is a custom built instrument housed at North Carolina State University that has been described in detail previously and was operated at 4.8 kW power and 5 kHz frequency (Cornelius, 2007). Helium was used as the carrier gas at a flow rate of 10 L/min. The LpP instrument is the IoN40 plasma system (PVA-TePla America, Corona, CA) and the gas used was medical grade nitrogen, power was 500 Watts, pressure was maintained at 300 mTorr, and gas flow was 0.5 L/min.

Prior to NtNP treatment, CS samples were weighed and placed into paper-backed sterilization pouches. The pouches used in the ApP instrument were sealed and subsequently flushed with 100% nitrogen gas whereas the pouches used in the LpP instrument were left unsealed. The CS-containing pouches were placed into the ApP instrument or directly on the metal tray of the IoN40. For the LpP, the chamber was evacuated down to <100 mTorr and flushed with 100% nitrogen gas 3 times prior to beginning the NtNP treatment. Evacuating and flushing the chamber served to ensure the CS-containing pouches were filled completely with 100% N₂ gas instead of air.

Previous studies showed that sterility doses, defined as a 6-log reduction in bacterial spores, were achieved at 15 mins with both the ApP and LpP instruments when using nitrogen gas. As is commonly done in medical device and pharmaceutical manufacturing, an overkill dose of 2X the sterility dose, 30 minutes in this case, was used in these studies. Sterility of each NtNP-treated CS sample was confirmed by diluting each sample 40X with pyrogen-free water to overcome the anti-microbial properties of CS and subsequently cultured in
standard polystyrene petri dishes containing tryptic soy agar at 37°C for 72 hours. Colony-forming units were enumerated by manually counting each culture plate.

**Endotoxins**

Endotoxins are measured in endotoxin units (EU), a unit of biological activity based on the United States Pharmacopoeia Reference Endotoxin Standard, since different types of endotoxins elicit varying degrees of immune responses. Endotoxins were extracted from CS by incubating CS samples in a 40X volume of pyrogen-free water (w/v) for 2 hours at 37°C. The extracts were then syringe filtered through a 0.22 μm mixed cellulose esters filter to remove any residual CS that can cause false positives with Limulus amebocyte lysate (LAL) assays. The endotoxin concentration in the filtrate was subsequently quantitated using a handheld LAL device (Endosafe PTS device, Charles River Laboratories, Charleston, South Carolina) and FDA-certified 0.5-0.005 EU/mL PTS cartridges. The LAL assay is the current gold standard method for quantitating endotoxins in drugs and medical devices.

**Results and Discussion**

**Particle Size Analysis**

Cryomilling raw CS flake with either a cryo-ball or a cryo-jet mill resulted in a fine, white powder, as shown in Figure 6, consisting of mostly <100 μm particles. Average particle size and size distribution, including median and the
diameter larger than 95% of the particles (D95), for each milling machine and parameter are presented in Table 1. Cryo-ball milling CS flakes for 15 minutes with 10 mm zirconium oxide balls generated CS powder with the largest mean particle size. Doubling the grind time to 30 minutes reduced the mean particle size by 21% and D95 by 15%. Using 50% smaller stainless steel balls reduced the mean particle size and D95 by an additional 57% and 55%, respectively. The smallest mean particle size generated by cryomilling (28.5 μm) was 24% smaller than the mean particle size generated by a standard ball mill after 8 hours (37.53 μm) in a previous study (Zhang, Zhang & Xia, 2014).

Figure 6. Macroscopic images of CS flake (left) and cryo-jet milled powder (right).
Cryo-jet milling CS for 30 minutes produced the smallest particles, with a mean particle size (16.05 μm) that was 44% smaller than the smallest mean particle size produced by cryo-ball milling (28.5 μm). Although the cryo-jet mill proved to be the superior method for milling CS into powder in the present study, a previous study showed that CS powder with a particle size distribution similar to cryo-jet milling can be produced with a cryo-ball mill when coupled with thermocatalytic destruction of CS (Laka & Chernyavskaya, 2006). Furthermore, another study found that CS flakes can be reduced to submicron sizes using an ultrafine milling technique (Zhang, Zhang, Jiang & Xia, 2012).

**Viscosity and Molecular Weight**

Cryomilling reduced the intrinsic viscosity and molecular weight (MW) of CS as shown in Figure 7. This reduction is a result of scissions of the chitosan
polymer chain. A detailed discussion of this mechanism is provided in section 3.5. Since MW has a dramatic influence on the biological properties of CS, additional studies are needed to understand the influence of powders with different particle size distributions on biologic properties (Kumirska, Weinhold, Thöming & Stepnowski, 2011).

Figure 7. Effect of cryo-jet milling on intrinsic viscosity (A) and molecular weight (B) of CS. Flake is raw CS and powder is milled CS. Values in A are the intrinsic viscosity (measured in centistokes (cSt)) calculated as the y intercept at zero concentration of the reduced viscosities of 3 CS concentrations. Values in B are the MW of CS calculated by the Mark-Houwink equation.

**Degree of Deacetylation**

Changes in the DD are shown in Figure 8. The 9.6% reduction in DD after cryo-jet milling appears to be another consequence of the scissions in the CS polymer chain that occur during cryomilling. Specifically, we postulate that chain breaks lead to changes in functional groups in CS that may lead to chemical reactions. However, other mechanisms are possible. For example, high
temperatures are known to occur, albeit for very short times (< 10^6 seconds), at the fracture sites during cryo-jet milling and this might cause slight caramelization of the CS (Rumpf, 1973; Weichert & Schönert, 1976). Thus, additional studies are needed to better understand the mechanism(s) responsible for this change in %DD with cryomilling.

![Figure 8](image.png)

Figure 8. Effect of cryo-jet milling on degree of deacetylation of CS. Flake is raw CS and powder is milled CS. Values represent degree of deacetylation expressed as a percent off all the glucosamine moieties comprising the CS polymer.

**Hygroscopicity**

The hygroscopicity of CS was increased by 25% after milling with loss on drying of the cryo-jet milled CS at 11.12% compared to 8.34% for the CS flake, reflecting the increase in surface area of the cryomilled material. The increase in hygroscopicity of CS after cryomilling is similar to the % loss on drying for the thermocatalytic ball milled CS powder reported by Laka and Chernyavskaya. The
high hygroscopicity of CS powder must be taken into account when making CS solutions, which are commonly used for biomedical, and especially pharmaceutical, applications (Ahmadi, Oveisi, Samani & Amoozgar, 2015; Heffernan, Zaharoff, Fallon, Schlon & Greiner, 2011).

**Morphologic Characterization**

Morphologic characterization of the micronized CS via SEM imaging showed highly irregular particle shapes after cryo-jet milling, as shown in Figure 9. These irregular particle shapes are thought to be a result of the fact that most polymers at room temperature exhibit considerable tensile toughness or work to rupture, compared to glassy, brittle materials such as inorganic glasses. The appearance of the milled CS suggests the CS flakes are torn apart in a random fashion, leaving highly diverse particle shapes and sizes. The majority of particles appeared either sphere-like or spindle shaped. The size of these shapes is small enough that individual polymer chains are likely broken in the milling process, which leads to the reduced intrinsic viscosity and MW shown in Figure 7. For example, if we take 5.15 x 10^{-10} m as the diameter of the glucopyranose sugar ring, and assume that there is a degree of polymerization of 1000, then the CS chain is 0.5 μm in length and the particles range in diameter down to 16 μm and smaller.
Figure 9. SEM images of CS flake before cryo-jet milling at 500X magnification (A) and resultant CS powder after cryo-jet milling at 1000X (B) and 5000X (C) magnification.

**FTIR**

FTIR spectra for CS flake and powder are shown in Figure 10 and reveal changes in the molecular composition of CS after cryomilling. Specifically, the amine peak at 3400 cm\(^{-1}\) is lower in the CS powder compared to the CS flake, which correlates with the conductometric titration data and indicates loss of amine groups (is this the best way to state what the peak reduction indicates). There is no change in the amide I peak at 1640 cm\(^{-1}\), but the CS powder shows a rightward shift at 1100 cm\(^{-1}\) compared to the CS flake. The significance of this peak shift is not currently understood.
Figure 10. FTIR spectra of CS flake and powder showing changes in molecular composition caused by cryo-jet milling. Blue represents chitosan flake, red represents chitosan powder. Both flakes and powder were analyzed in the free amine form.

Plasma Decontamination

Plasma treatment resulted in a sterile CS with endotoxin levels of 1.26 EU/g (ApP) and 2.46 EU/g (LpP). This is a very low level of endotoxins. Most medical or pharmaceutical grades of CS contain > 100 EU/g, although most are certified to be < 100 EU/g. Plasma sterilization also resulted in a significant reduction in viscosity of both flake and powder forms of CS, as shown in Figure 11.
Figure 11. Effect of LpP and ApP treatment on CS viscosity. Values are % reduced viscosity retained after LpP or ApP treatment of CS flakes (left grouping) or powder (right grouping) with untreated CS serving as the control (i.e. 100%).

**Solubility**

In the process of making CS hydrogels for the tests described in previous sections, it was noted that cryomilled CS powder solubilized significantly faster and better in dilute acids than CS flakes. Thus, milling increased the solubility of CS. This is again a result of the increased surface area of the milled CS compared to the raw CS flake.

**Conclusion**

Cryo-ball and cryo-jet milling are effective methods for producing micron-sized CS particles and increasing the surface to volume ratio of CS. Cryo-milling also increases the hygroscopicity and decreases the %DD, intrinsic viscosity, and therefore the molecular weight, of CS. Cryo-jet milling produced a 44%
smaller mean particle size than cryo-ball milling after 30 minutes of milling. However, in the case of cryo-ball milling, smaller grinding balls may produce much smaller mean particle sizes, potentially within the range of cryo-jet milling, but it is unclear whether there would be any advantage to cryo-ball milling over cryo-jet milling.

Plasma-treated CS was sterile with <2.5 EU/g, but NtNP treatment caused a significant reduction in CS MW. This effect was greater with micronized CS powder, which is predicted to be due to the increased surface area of the powder. Future studies must be aimed at elucidating how different particle sizes influence biological properties of CS.

Acknowledgements

Research reported in this publication was supported by the National Cancer Institute of the National Institutes of Health under SBIR award number R43CA186374. This grant was awarded to Scion Cardio-Vascular, Inc. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The authors thank Jackie Knecht and Louis Rose for administrative assistance. The authors also thank Demetri Chrysostomou, Michael Barden, Luke Turalitsch, Eduardo Mateo, and James Bond for assistance with the IoN40 plasma instrument.
References


CHAPTER THREE

INTRAVESICAL IMMUNOTHERAPY OF ORTHOTOPIC MURINE BLADDER CANCER WITH NITROGEN PLASMA STERILIZED CHITOSAN+INTERLEUKIN-12

Andrew Crofton\textsuperscript{a,c}, Samuel Hudson\textsuperscript{d}, Matthew Dopp\textsuperscript{c}, Tyler Pender\textsuperscript{c}, Kristy Howard\textsuperscript{c}, Nicholas Sanchez\textsuperscript{c}, Floyd Petersen\textsuperscript{c}, Abdelrahman Abdelgawad\textsuperscript{d}, and Wolff Kirsch\textsuperscript{*b,c}

acrofton@llu.edu, shudson@ncsu.edu, mdopp@llu.edu, tpender@llu.edu, khoward@llu.edu, nsanchez@llu.edu, fpetersen@llu.edu, aabdelg@ncsu.edu, wkirsch@llu.edu

\textsuperscript{a}Department of Pathology and Human Anatomy, \textsuperscript{b}Division of Biochemistry, and \textsuperscript{c}Neurosurgery Center for Research, Training, and Education, School of Medicine, Loma Linda University
11234 Anderson St.
Medical Center A537
Loma Linda, CA 92350
+1-909-558-7070

\textsuperscript{d}College of Textiles, North Carolina State University
2401 Research Dr.
Raleigh, North Carolina 27695
+1-919-515-6545

\textsuperscript{*Corresponding Author}
Wolff M. Kirsch
Professor of Biochemistry and Neurological Surgery
Loma Linda University, School of Medicine,
11234 Anderson St., Medical Center A537
Loma Linda, CA 92354
+1-909-558-7071
fax: +1-909-558-0472
wkirsch@llu.edu

Biomaterials (Submitted)
Abstract

Chitosan co-formulated with interleukin-12 (CS+IL-12) has shown remarkable efficacy treating bladder cancer. Translating this and other CS-containing therapeutics is hampered by difficulty sterilizing CS without altering its biological properties. We hypothesized that low-pressure nitrogen plasma would sterilize CS while preserving its in vivo functionality. To test this, we plasma sterilized CS and characterized physicochemical changes and preservation of in vivo efficacy of CS+IL-12 in a murine orthotopic bladder cancer model (MB-49 tumors). Plasma treatment produced sterile CS with < 2.50 EU/g and reduced CS viscosity 36.5% and degree of deacetylation 6.3%, but did not alter atomic nitrogen content or bioadhesivity. Tumors regressed – and mice survived to 90 days – in 88% (n=16) and 92% (n=12) of C57Bl/6 mice treated with unsterile CS+IL-12 and plasma-sterilized CS+IL-12, respectively, but only 8% (n=12) and 0% (n=3) of mice treated with saline and CS-alone, respectively. Upon tumor re-challenge, 100% of plasma-sterilized CS+IL-12 (n=11) and 91% of unsterile CS+IL-12 (n=11) treated mice rejected tumors. Tumors established in 25-50% of CS+IL-12 pre-treated mice, but 92% of tumor naïve mice (n=64). Thus, nitrogen plasma sterilizes CS while preserving its biologic functionality. Plasma-sterilized CS will enable human trials to test CS-containing therapeutics including CS+IL-12 for bladder cancer and other malignancies.

Keywords: chitosan, bladder cancer, interleukin-12 (IL-12), plasma sterilization, non-thermal nitrogen plasma (NtNP), immunotherapy, cryo-jet milling
Abbreviations: CS – chitosan; NtNP – non-thermal nitrogen plasma; LpP – low-pressure plasma; IL-12 – interleukin-12; DD – degree of deacetylation; psCS+IL-12 – plasma-sterilized CS+IL-12; usCS+IL-12 – unsterile CS+IL-12
Introduction

Chitosan (CS) is the deacetylated derivative of chitin, a ubiquitous biopolymer, and has long been recognized as a promising biomedical material due to its biocompatibility, biodegradability, hemostatic effectiveness, and antimicrobial properties. (Domb, 2011; Kumirska, 2011) Potential medical applications for CS are extensive and include gene and drug delivery. One particularly promising drug delivery application for CS involves local delivery of cytokines for treating solid tumor cancers. (Heffernan, 2011; Yang, 2013) For example, recent studies have shown that immune responses generated by local delivery of interleukin-12 (IL-12), a potent antitumor cytokine that generates significant toxicity when administered systemically, are robust and durable. (Zaharoff, 2010; Zaharoff, 2009; Smith, 2015; Vo, 2014; Xu, 2012)

IL-12 has been thought to be a highly promising cancer immunotherapeutic agent since the early 1990s, just a couple of years after its discovery in 1989. (Lieberman, 1991; Kobayashi, 1989) Recently, IL-12 was even ranked third among agents with high potential for use in treating cancer by the National Cancer Institute (NCI) based on extensive input from academia, industry, the US Food and Drug Administration (FDA), and the NCI. (Cheever, 2008) However, translating IL-12 to clinical usefulness has been hampered by toxicities associated with systemic administration that prevents sufficient dosages from being achieved. (Atkins, 1997; Cohen, 1995; Gollob, 2000; Gollob, 2003; Hurteau, 2001; Lenzi, 2007; Lenzi, 2002; Motzer, 2001; Robertson, 1999; Weiss, 2003; Younes, 2004) This issue has spurred interest in delivery systems for IL-12
that can localize IL-12 to the site of interest while also increasing the concentration and duration at the site of action.

CS has emerged as a leading drug delivery candidate for IL-12 based on recent studies showing that coformulation of IL-12 with CS (CS+IL-12) significantly enhances IL-12 efficacy in treating murine bladder, breast, colorectal, ovarian, and pancreatic tumors. (Zaharoff, 2009; Vo, 2014; Smith, 2015; Yang, 2013; Zaharoff, 2010) In the case of murine orthotopic bladder tumors, CS+IL-12 resulted in an 88-100% survival rate compared to a 37.5% survival rate with IL-12 treatment alone and 0% survival rate with BCG, the current standard treatment for non-muscle invasive bladder tumors. Despite these outstanding results, which were generated with unsterilized CS containing endotoxin levels that exceed the levels required for parenteral uses in humans, CS+IL-12 has yet to be tested in human clinical studies due to issues with obtaining sterile CS with sufficiently low endotoxin levels and intact biologic functionality. Standard sterilization techniques like dry/wet heat (i.e. autoclave), γ-irradiation, electron beam irradiation, and ethylene oxide gas alter the physical properties of CS such that its functional efficacy is significantly attenuated. (Franca, 2013; Lim, 1998; Lim, 1999; Marreco, 2004; Rao, 1995; Rosiak, 1992; San Juan, 2012)

As a precursor to clinical studies testing the safety and efficacy of CS+IL-12 in humans, the present study sought to generate a sterile CS that would meet FDA requirements for use in humans while preserving the biologic functionality of CS. A novel sterilization technique, non-thermal nitrogen plasma (NtNP), was
employed as a terminal sterilization method for CS in this study. Plasma, considered the fourth state of matter after solids, liquids, and gas, consists of an ionized gas that has emergent properties the gas alone does not. For example, plasmas are conductive, conductive, and quasi-neutral. Quasi-neutrality means there are pockets of positive charges and negative charges, but the overall charge of the plasma is neutral. (Cornelius, 2007; Laroussi, 2005; Moisan, 2001)

We hypothesized that NtNP would adequately sterilize and depyrogenate CS for parenteral use, increase the nitrogen content of CS thereby increasing its bioadhesivity, and preserve the biologic functionality of CS. This hypothesis was tested in an orthotopic murine bladder cancer model. By employing this model, the study would not only provide insight into the biologic activity of NtNP sterilized CS, but also an independent replication and expansion of previous pre-clinical mouse studies of CS+IL-12 for bladder cancer therapy. (Zaharov, 2009)

Materials and Methods

All reagents were obtained from Sigma-Aldrich unless stated otherwise.

Chitosan

Raw CS flake, with individual flakes ranging in diameter from ~1-15 mm, was obtained from Scion Biomedical, Inc. After filtering out particles > 8mm in diameter, the CS flakes were milled into a powder with a cryo-jet mill (Micron-Master Jet Pulverization System, The Jet Pulverization Co., Moorestown, NJ,
USA). CS was micronized to increase surface to volume ratio – since NtNP is a surface treatment – and to enhance solubility and in vivo efficacy.

**Plasma Decontamination**

NtNP treatment was performed with a low-pressure plasma (LpP) system (IoN40 Gas Plasma System, PVA-TePla America, Inc., Corona, CA, USA). Treatment times and parameters required to achieve a 6-log reduction in bacterial spores – considered a sterility dose – with the chosen NtNP system were determined by treating bacterial indicator (BI) spore strips containing 3.12 X $10^6$ G. stearothermophilus spores and 5.5 x $10^6$ B. atrophaeus spores (VERIFY™ Dual Species BI, Steris Corporation, Mentor, OH, USA) with NtNP. The power input for the plasma system was maintained at 500 watts, pressure at 300 mTorr, and gas flow rate at 0.5 L/min. After NtNP treatment, the paper spore strips were dispersed in sterile water to release the spores from the paper and the spores were subsequently cultured in tryptic soy agar for 48 and 72 hours at 37°C and 58°C, respectively, to allow growth for each of the 2 spore types.

Prior to NtNP treatment, the micronized CS powder was packaged in standard sterilization pouches and treated with an overkill dose (2X sterility dose) of NtNP. CS sterility was confirmed by culturing BIs co-treated with the CS as described above and diluting the NtNP treated CS powder 1:100 with pyrogen-free water, to overcome the anti-microbial properties of CS, and subsequently culturing it in tryptic soy agar at 37°C for 72 hours.
Endotoxin levels were quantitated by incubating CS powder in a 40X (w/v) volume of pyrogen-free water for 2 hours at 37°C, filtering the extract through a 0.22 μm mixed cellulose esters filter to remove any residual CS that can cause false positives, and testing the extract for endotoxins using a handheld Limulus amebocyte lysate (LAL) assay system (Endosafe-PTS device, Charles River, Inc., Charleston, SC, USA) and FDA-certified 0.5-0.005 EU/mL PTS cartridges. A recent multi-center trial showed the PTS device is effective in measuring endotoxin concentrations in medical devices and pharmaceuticals. (Gee, 2008)

Note that endotoxins are measured in endotoxin units (EU), which is a unit of biological activity based on a reference endotoxin standard. This allows for standardization of the biologic significance of endotoxin quantities based on concentration (pg/mL) because different endotoxins elicit varying degrees of immune responses. (Adner, 1991) The LAL assay is the current gold standard for quantitating endotoxins and we deviated slightly from the US Pharmacopaeial and FDA recommendations on LAL endotoxin testing by extracting the endotoxins at 37°C, instead of room temperature, based on previous experiments in our laboratory that have shown significantly higher extraction rates at 37°C (data not shown). (US Dept. of Health and Human Services, 2012)

**Viscosity**

It is important to characterize the viscosity of CS for any pharmaceutical applications since viscosity is a surrogate measure of molecular weight (MW) and the biological properties of CS are dependent on its MW. (Dodane, 1998;
Rinaudo and colleagues have described methods for viscometric determinations of CS in detail and those studies formed the basis for the techniques used here. (Brugnerotto, 2001; Rinaudo, 1993)

Briefly, the intrinsic viscosity of the native and NtNP-sterilized CS powder was determined by measuring efflux times of CS solutions using a 2-10 centistoke glass capillary ASTM 1 Ubbelohde Viscometer (Fisher Scientific, Pittsburgh, PA, USA). For these measurements, CS was dissolved in 0.3 M acetic acid and 0.2 M sodium acetate (0.3 M AcOH/0.2 M NaOAc) for final concentrations of 1, 1.5, and 2 mg/mL (0.1%, 0.15%, and 0.2%). Efflux times were measured for each of the 3 concentrations and intrinsic viscosity was determined using the Huggins plot, in which the reduced viscosities for the 3 concentrations are extrapolated to zero concentration. Reduced viscosity for each concentration was calculated by the following equation:

\[
\text{Reduced Viscosity } n_{\text{red}} = \left( \frac{t_{\text{cs}}}{t_{\text{sol}}} - 1 \right) \div c \tag{1}
\]

where \(t_{\text{cs}}\) represents efflux time for CS, \(t_{\text{sol}}\) represents efflux time for the solvent (0.3 M AcOH/0.2 M NaOAc), and \(c\) represents CS concentration in g/mL.

**Surface Atomic Concentrations**

Because previous studies have shown changes in the elemental composition of plasma-treated CS films after plasma treatment, which may have an effect on the bioadhesivity of CS, the elemental composition of NtNP-treated and native CS powder was analyzed via X-ray Photoelectron Spectroscopy.
We hypothesized that NtNP may add nitrogen groups to the CS. Since the amine group is the source of CS’s cationic charge, adding nitrogen may increase the positive charge of CS and resultantly, its bioadhesivity. XPS characterization was performed using a Kratos AXIS ULTRA\textsuperscript{DLD} XPS system equipped with an Al K\textsubscript{α} monochromated X-ray source and a 165-mm radius electron energy hemispherical analyzer. Vacuum pressure was kept below 3 x 10\textsuperscript{-9} torr during acquisition. CS powder was affixed to a piece of tape during the XPS scanning process to prevent movement of the particles in the acquisition chamber.

\textbf{Degree of Deacetylation}

As with viscosity/MW, degree of deacetylation (DD) must be characterized for any biomedical application of CS since DD has a significant impact on the biological properties of CS. (Kumirska, 2011; Dodane, 1998) DD determinations were carried out by conductometric titration following the protocol reported by Raymond and colleagues. (Raymond, 1993) Briefly, dried CS sample of known mass (about 0.100 g) was dissolved in 10 mL of 0.1 N hydrochloric acid (HCl) and 90 mL of distilled water. The CS solution was subsequently titrated with a standard sodium hydroxide (NaOH) solution using a 10 mL buret while the conductivity of the solution was monitored as a function of the volume of NaOH added with an Orion Benchtop Conductivity Meter (Model 162) equipped with an Orion Conductivity Cell (Model 013030). The temperature of the solution was kept constant (30°C) during the titration using a water bath since the conductivity
is a function of temperature. There are two deflection points in the conductometric titration curve, the first corresponding to the neutralization of excess H\(^+\) ions of the strong acid, HCl, and the second corresponding to the point at which the weak acid, the ammonium salt in CS, has been completely neutralized. The range between the 2 deflection points corresponds to the neutralization of the protonated amine groups of CS. Since the number of moles of NaOH used between the 2 deflection points equals the number of moles of amine groups of the CS sample, the DD was calculated by the following equation:

\[
\text{%DD} = \frac{(v_2-v_1) \times M_{NaOH}(\text{mol} / \text{L}) \times \frac{161.16 g}{\text{mol}}}{\text{Mass of chitosan sample (g)}} \times 100
\]  

(2)

where \((v_2-v_1)\) is the difference in volume in liters between the two deflection points, \(M_{NaOH}\) is the molarity (mol/L) of standard NaOH solution, and 161.16 is the molar mass of CS. Titrations were performed in duplicate on each sample.

**Bioadhesivity**

One of the mechanisms by which CS is thought to enhance IL-12 efficacy is through adhesion to tissues based on the cationic charge of CS and the anionic charge of tissues. (Sinha, 2004; Zaharoff, 2007; Yang, 2013; Zaharoff, 2009) Thus, the effect of NtNP sterilization on bioadhesion of CS was assessed. The technique we employed for measuring bioadhesion was adapted from a study by Hurler and Skalko-Basnet. (Hurler, 2012) For this technique, CS powder was first solubilized with 1% acetic acid overnight at 4°C to form 1% CS.
solutions. Fresh pig bladders were obtained on the day of testing and kept on ice until measurements. Immediately prior to testing, bladder samples were rinsed with 70% ethanol and allowed to dry for 60 seconds at room temperature. For testing, a polystyrene culture plate (Fisher Scientific, Inc., Pittsburgh, PA, USA) was centered on a standard laboratory balance with a 0.001-100g range (Fisher Scientific, Inc.) and a 50 μL volume of 1% CS acetate solution was dispensed into the center of the plate and the weight recorded. A piece of pig bladder that fully covered the CS solution was placed over the CS and a 200g weight applied for 30 seconds. After 30 seconds, the weight was removed followed by the bladder. Care was taken to ensure the bladder was removed using the same technique for each sample. The weight on the balance was again recorded. The % loss in mass of the CS solution after applying and removing the bladder was considered a surrogate measure for bioadhesivity. Each solution was tested twice, with a fresh piece of pig bladder for each test, and 3 samples from each treatment group were tested.

**Chitosan+IL-12 Co-formulation**

NtNP-sterilized CS powder was aseptically transferred to sterile 50 mL polypropylene tubes (Fisher Scientific, Inc.) and solubilized with sterile-filtered 1.25% lactic acid (Sigma Aldrich, St. Louis, MO, USA) in 1X phosphate buffered saline (PBS) (Thermo Fisher Scientific, Inc., Waltham, MA, USA) for a final CS concentration of 1.25%. Immediately prior to use, the CS lactate solution was combined with recombinant murine IL-12 (rMuIL-12) (Peprotech, Inc., Rocky Hill,
NJ, USA) that had previously been reconstituted with 1X PBS to achieve final concentrations of 20 μg/mL rMuIL-12, 1% CS, and 1% lactic acid.

**Cell Culture**

MB-49$^{\text{lucSH+}}$ cells, an aggressive murine transitional cell carcinoma (TCC) tumor line that shares many characteristics of human bladder cancer including cell surface markers, sensitivity to apoptosis, and immunologic profile, were obtained by material transfer agreement from the National Cancer Institute (generous gift of Jeffrey Schlom and Jack Griener). (Loskog, 2005; O’Donnell, 2004; Loskog, 2004) The MB-49$^{\text{lucSH+}}$ cells had been previously stably transfected with a plasmid encoding a synthetic CpG-free firefly luciferase zeocin resistance fusion gene (pSELECT-zeo-LucSh; InvivoGen, Inc., San Diego, CA, USA). MB-49$^{\text{lucSH+}}$ cells were maintained in Dulbecco’s Modified Eagle Medium supplemented with GlutaMAX, 10% fetal bovine serum, 1% penicillin/streptomycin, and 200 μg/mL zeocin (InvivoGen, Inc.). Zeocin acted as the selection agent for the plasmid-expressing cells. All cell culture reagents were obtained from Thermo Fisher Scientific, Inc. unless otherwise specified.

On the day of cell injections, MB-49$^{\text{lucSH+}}$ cells were prepared by trypsinizing the cells, re-suspending in culture media for a final concentration of 1.5 x 10$^6$ cells/mL, and placing 1.5 mL aliquots in 2 mL sterile microcentrifuge tubes until intravesical injection.
Tumor Cell Injections and Confirmation

Female C57Bl/6 and BALB/c mice were obtained from The Jackson Laboratory (Sacramento, CA, USA) after obtaining approval from the Loma Linda University Institutional Care and Use Committee and used at 7-10 weeks of age. Mice were cared for in compliance with The Guide for Care and Use of Laboratory Animals (National Research Council). These two mouse strains were chosen for this study to elucidate the impact of different immune phenotypes on the efficacy of CS+IL-12. Specifically, BALB/c mice have a Th2 polarized response to invading pathogens with low levels of IL-12 production whereas C57Bl/6 have a predominantly Th1 polarization. (Lichtman, 2013; Schulte, 2008; Mills, 2000; Chen, 2005) However, this long-held belief has come under scrutiny recently with a study showing that BALB/c mice have a Th1 polarized response induced by BCG vaccination. (Garcia-Pelayo, 2015) We hypothesized that BALB/c mice might be less responsive to IL-12 therapy than C57Bl/6 mice since IL-12 stimulates a Th1 response.

For establishment of orthotopic bladder tumors, methods were adapted from previous studies. (Dobek, 2011; Kasman, 2013) Mice were anesthetized with 2-3% isoflurane, placed supine, and 24 G ¾ inch SURFLO Teflon catheters (Terumo Medical Corporation, Somerset, NJ, USA), coated with water-based lubricating jelly and introducer needles removed, were inserted into the urethra while the vulva was gently pulled antero-inferiorly at 45 degrees from horizontal with surgical forceps to straighten the curved murine urethra. After catheterization, urine was drained into the catheter by applying gentle manual
pressure to the bladder and suctioning the resultant urine from the catheter tip with a pipette.

Fifty microliters of 0.1 μg/mL poly-L-lysine (PLL) (molecular weight 70,000-150,000; Sigma Aldrich) in sterile water for injection was then injected and held in place for 10 minutes by drawing 80 μL of PLL solution into the SURFLOW introducer needle with a 1 mL syringe, inserting the needing into the catheter until slight resistance was met, expelling the solution in the introducer needle unit until the meniscus reached the proximal tip of the needle, and taping the syringe in place. Care was taken to avoid introducing air into the bladder. After 10 minutes, the PLL solution was suctioned from the bladder, the introducer needle removed while the catheter was left in place, 80 μL of 1.5 x 10⁶ cells/mL MB-49lucSH⁺ cell suspension was drawn into a new introducer needle, and the needle placed into the catheter as before with 50 μL of the cell suspension (75,000 cells) subsequently injected into the bladder (30 μL deadspace in the needle) and held in place for 35 minutes.

Tumor presence was confirmed and monitored over time via bioluminescent imaging with an IVIS Lumina Series III Pre-clinical In Vivo Imaging System (Perkin-Elmer, Waltham, MA, USA). For bioluminescent imaging, 15 mg/kg D-Luciferin potassium salt (Sigma Aldrich) in 1X PBS was given via intraperitoneal injection 15 minutes prior to imaging. Mice were imaged for 1 minute with large binning and medium f/stop.
**Intravesical Treatments**

Intravesical treatments with PBS, CS hydrogel alone (CS-only), plasma-sterilized CS+IL-12 (psCS+IL-12), or unsterile CS+IL-12 (usCS+IL-12) were administered to tumor bearing mice using the same technique described above for the PLL and MB-49\textsuperscript{lucSH+} cell injections, with 35 minute dwell times. All CS-containing treatments contained 1% CS lactate and all IL-12 containing treatments contained 20 μg/mL IL-12 for a total of 1 μg rMuIL-12 per mouse per treatment. Treatments were administered every 3-5 days with a total of 4 treatments per animal.

To monitor for treatment-related toxicity, a group of tumor naïve mice received CS+IL-12 treatments, but were never challenged with tumors.

**Tumor Re-challenge**

Mice that became tumor-negative on bioluminescent imaging for more than 3 consecutive weeks after the conclusion of the treatment regimen were re-challenged with tumor cells 8 weeks after the initial tumor cell instillation, which was 5 weeks after the 4\textsuperscript{th} treatment was administered. The MB-49\textsuperscript{lucSH+} cell instillations were again performed as described above. At the time of re-challenge, tumor naïve mice were also challenged with tumor cells to ensure appropriate percentages of tumor establishment were achieved.
**CS+IL-12 Vaccination of Mice**

Groups of tumor naïve mice received psCS+IL-12 (n=4) or usCS+IL-12 (n=4) treatments prior to tumor challenge. The treatments were administered as described above for tumor-bearing mice. Tumor instillation was performed 5 weeks after the final treatment was given.

**Euthanasia**

Mice were sacrificed via cervical dislocation after inducing anesthesia with 3% isoflurane.

**Statistical Analyses**

Non-parametric data (tumor establishment, tumor regression) were analyzed by Pearson’s chi-squared test with p<0.05 considered significant. Additionally, survival data were analyzed by the log-rank test with p<0.05 considered significant. Parametric data were analyzed by two-tailed student’s t-test (bioadhesion) with p<0.05 considered significant.

**Results**

**NtNP Effectively Sterilized CS**

Cryo-jet milled CS powder had an average particle diameter of 16.05 μm, a significant increase in surface to volume ratio compared to the raw CS flakes. It was also observed that the CS powder solubilized faster with fewer insolubles than CS flake. NtNP achieved a 6-log reduction in spores after 15 minutes.
Figure 12. Log reductions in spore viability from BIs with increasing exposure to non-thermal $N_2$ plasma treatment. A sterilization dose represents a 6-log reduction in spore viability. Data points represent log reductions in mean colony forming units; n=3 each time point.
**NtNP Reduced CS Viscosity**

Viscometric measurements showed that NtNP sterilization decreased the intrinsic viscosity of CS by 36.5% (Figure 13). The change in viscosity was also observed in the time it took for the catheters to fill with psCS+IL-12 versus usCS+IL-12 during animal treatments.

![Intrinsic Viscosity Graph](image.png)

Figure 13. Intrinsic viscosity of micronized CS powder before and after NtNP sterilization. Values are the y-intercept of reduced viscosities for 3 concentrations of CS (n=3 per concentration, measured in duplicate) when extrapolated to zero concentration on Huggins Plot.

**NtNP Altered DD, Not Surface Atomic Concentrations**

According to the conductometric titrations, CS DD was reduced from 73.75% to 69.11% with NtNP sterilization. XPS measurements revealed that there was no change in the nitrogen content of the CS powder. There was a 1%
decrease in oxygen content and 1% increase in carbon content, which might be explained by residual air in the plasma chamber or absorbed on the CS surface.

**CS Bioadhesion Unchanged with NtNP Sterilization**

The bioadhesion studies showed that there was no significant change, whether positive or negative, in the bioadhesivity of CS after NtNP sterilization (Fig. 14). These data correlate with the XPS data as we had hypothesized that NtNP treatment would increase the bioadhesion of CS through increasing the nitrogen content.

![Figure 14. Bioadhesivity of native and NtNP sterilized CS. Values are mean ± standard error of the mean (SEM); n=3 each group, measured in duplicate. Statistical significance (p<0.05) was not reached.](image-url)
Orthotopic Tumors Established in Most Animals

Figure 15 shows success rates in tumor establishment in both BALB/c and C57Bl/6 mouse strains. The tumor establishment rates we achieved with injections of 75,000 cells in C57Bl/6 mice were in between rates achieved by Loskog et al in which both higher and lower numbers of cells (60,000 and 100,000) were used for tumor instillation. (Loskog, 2005) Loskog achieved a 100% tumor establishment rate when injecting 100,000 cells. Taken together, these data indicate 100,000 cells should be injected for establishing orthotopic MB-49 tumors in C57Bl/6 mice.

Although tumor establishment rates were not significantly different between strains, tumors spontaneously regressed in all but one of the BALB/c mice in all treatment groups, including the control PBS-treated group (data not shown). Interestingly, the sole BALB/c mouse that retained its tumor developed a large abdominal tumor secondary to bladder puncture that inadvertently occurred during MB-49 cell instillation. The spontaneous tumor regression observed in BALB/c mice is likely explained by the fact that the MB-49 cell line is derived from C57Bl/6 mice, which have an MHC H2b haplotype whereas BALB/c mice have an H2d haplotype. This MHC mismatch appears to lead to transplant rejection in the BALB/c mice since the rejections generally occurred in all groups, including the PBS control, within 10-14 days, a timeline similar for transplant rejection (7-10 days). (Janeway, 2001) On the other hand, the MB-49 cell line is derived from male mice and used in female mice to enable urethral catheterization, yet the HY antigen appears to be innocuous. (Loskog, 2005) We had hypothesized that the...
immunosuppressive nature of the tumor microenvironment might prevent transplant rejection in BALB/c mice based on the MHC mismatch.

![Graph](image)

**Figure 15.** Success rates of orthotopic tumor establishment in BALB/c and C57Bl/6 mice. Values represent percent of mice that were tumor positive (lower, dotted bars) and tumor negative (upper, striped bars) in each strain after tumor cell instillation. Difference was not statistically significant at p<0.05 by Pearson’s chi-squared test.

**CS+IL-12 Eliminated Tumors in Most C57 Mice**

In C57Bl/6 mice, the CS+IL-12 treatment groups showed outstanding response rates to treatment with 11 of 12 usCS+IL-12 treated mice and 14 of 16 psCS+IL-12 treated mice becoming tumor negative within 60 days of the start of therapy (Fig. 16). It was observed that we had technical difficulties with treatment administration in all 3 mice that failed to respond to therapy. These issues ranged from difficulty catheterizing the urethral to treatment leakage around the catheter.
due to an abnormally large urethra. Tumors regressed in only 1 of 12 PBS treated mice (Fig. 16) and 0 of 3 CS-alone treated mice. Thus, MB-49 tumors persist in the absence of treatment and CS-alone completely lacks anti-tumor properties.

![Graph](image)

**Figure 16.** Longitudinal tumor regression after intravesical treatment. Data points represent percentage of mice imaging positive for tumors with bioluminescent imaging. Both psCS+IL-12 (n=16; square data points) and usCS+IL-12 (n=12; diamond data points) treatment groups had significantly less tumor positive mice than the saline control group (n=12; triangle data points) from 30-90 days (p<0.001). There was no significant difference between psCS+IL-12 and usCS+IL-12 at any time point. Statistical significance at or below p<0.05 calculated by Pearson’s chi-squared test.

Over 75% of the C57Bl/6 mice treated with CS+IL-12 were tumor negative within 30 days of administering the first treatment. In fact, approximately 50% of the mice were tumor negative after only 2 treatments. As such, Figure 17 shows
representative bioluminescent scans of tumors in 1 mouse from each treatment group over the course of treatment.

Figure 17. Anti-tumor activity of intravesical treatments. Images show longitudinal bioluminescent tracking of tumors in C57Bl/6 mice bearing orthotopic MB-49\textsuperscript{LucSH}\textsuperscript{+} tumors during treatment with saline (upper left image panel), psCS+IL-12 (upper right image panel), and usCS+IL-12 (lower image panel). Imaging took place on day of intravesical instillation of treatments. Post-treatment imaging took place 4 days after receiving treatment 4. Tx – treatment; usCS+IL-12 – unsterilized CS+IL-12; psCS+IL-12 – plasma sterilized CS+IL-12.

\textbf{Survival Unchanged by NtNP Sterilization}

A Kaplan Meier survival curve is presented in Figure 18 showing percent survival out to 90 days after tumor instillation. There was no significant difference in survival between the CS+IL-12 treatment groups (p>0.05), but a very significant difference compared to the saline group (p<0.001). Additionally, the survival rates for both psCS+IL-12 (88\% survival) and usCSIL-12 (92\% survival)
match the results found previously by Zaharoff and colleagues (88-100% survival). (Zaharoff, 2009) However, the median survival time for saline treated animals was significantly longer ($p<0.01$) in our study (55 days) than in the Zaharoff study (18 days). This difference is likely explained by the subjective nature of determining when mice must be sacrificed due to tumor-related morbidity or differences in location or severity of orthotopic tumor establishment. No adverse events or toxicity were noted as a result of CS+IL-12 therapy.

Figure 18. Survival after tumor instillation. C57Bl/6 mice were treated with psCS+IL-12 ($n=16$), usCS+IL-12 ($n=12$), or phosphate buffered saline ($n=12$) starting 7 days after tumor instillation. Survival time was significantly longer for both CS+IL-12 treatment groups compared to saline control ($p<0.001$; log-rank), but there was no significant difference between CS+IL-12 groups.

Re-challenge After Tumor Regression

A major problem with superficial bladder cancer is high recurrence rates.

In fact, bladder cancer is the most expensive cancer to treat due to the high
recurrence rates. (Van Der Heijden, 2009; van Rhijn, 2009) Approximately 20-50% of patients with bladder cancer who are treated successfully with the current standard of care (transurethral resection followed by intravesical BCG) will have recurrent tumors within 5 years. (Herr, 2003; Kamel, 2009; Lamm, 2000; Malmstrom, 1999) These recurrent tumors are frequently resistant to BCG. Thus, we re-challenged the “cured” mice with tumor cells 8 weeks after initial MB-49 cell instillation. We found that 100% of psCS+IL-12 treated and 91% of usCS+IL-12 treated mice rejected the tumors outright (Table 2). Tumors established in 100% of the tumor naïve mice. The one treated mouse that imaged tumor positive had received usCS+IL-12 and survived 30 days after tumor re-challenge before sacrifice with evidence of a bladder tumor. Thus, there appears to be a strong anti-tumor immunity generated by CS+IL-12 in the gross majority of animals.

Table 2. Tumor establishment in tumor-exposed and tumor naïve mice treated with plasma sterilized and unsterilized CS+IL-12.

<table>
<thead>
<tr>
<th></th>
<th># of Mice Tumor Positive</th>
<th># of Mice Tumor Negative</th>
<th>Total # of Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor Naïve</td>
<td>6</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>psCS+IL-12</td>
<td>0</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>usCS+IL-12</td>
<td>1</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>psCS+IL-12vax</td>
<td>1</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>usCS+IL-12vax</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>
**Vaccination**

Pre-treated, or “vaccinated,” mice were given 4 treatments of CS+IL-12 while tumor naïve and then were challenged with MB-49 cells 5 weeks after their 4\(^{th}\) treatment. This treatment regimen resulted in 25% of mice developing tumors after psCS+IL-12 pre-treatment (psCS+IL-12vax) and 50% of mice developing tumors after usCS+IL-12 pretreatment (usCS+IL-12vax) (Table 2). These data and the fact that the tumor establishment rate for CS+IL-12-naïve mice was 92.2% overall suggest that administration of CS+IL-12, even prior to tumor instillation, results in tumor protection, though the effect is profoundly improved with treatment after tumor establishment.

**Discussion**

Our results show that NtNP treatment effectively sterilized and depyrogenated CS while preserving its efficacy as an excipient for immune molecules like IL-12. This indicates that potential contaminants present in unsterilized CS do not enhance the efficacy of CS+IL-12 treatment by, for example, bolstering the immune response elicited by CS+IL-12. This is an important finding since previous studies have found that many of the biologic properties of CS, especially those related to the immune response it triggers, are influenced by contaminants in CS such as endotoxins. (Lieder, 2013; Nolte, 2014)

Previous studies have also shown that MW influences the bioadhesion of polymers, including CS, with bioadhesivity decreasing as MW decreases.
(Honary, 2009; Roy, 2010) However, we did not find a reduction in bioadhesivity of CS after NtNP sterilization despite a 36.5% reduction in intrinsic viscosity, which is a surrogate measure of MW. This phenomenon might be explained by previous studies demonstrating that bioadhesivity usually becomes significantly reduced once MW falls below 100 kDa. (Roy, 2010) The NtNP-treated CS produced in this study still had a MW >250 kDa, based on the Mark-Houwink equation and K and α values of 0.074 and 0.80, respectively. (Brugnerotto, 2001)

Since the cationic charge of CS is responsible for its bioadhesive properties and the NtNP sterilization did not significantly alter bioadhesivity, it is reasonable to conclude that the cationic charge of CS was also not changed. This suggests that CS exerts its effect on the delivery of IL-12 in the bladder through its cationic charge. The specific mechanism is likely a combination of prolonging residence time of IL-12 on the surface of the bladder transitional epithelium through binding to negatively charged sialic acid residues, glycosaminoglycans, mucin, and proteoglycans on the cell surface and enhancing penetration of IL-12 through the bladder wall by increasing the permeability of epithelial barriers. (Mi, 2002; Sinha, 2004; Illum, 1994) This conclusion is supported by a study that has effectively demonstrated the dependence of CS’s cationic charge on enhancing epithelial permeability through redistribution of cytoskeletal F-actin and zonula occludens-1 (ZO-1). (Schipper, 1997) The cationic charge not only mediates adhesion of CS to tissues and penetration of epithelial barriers, but is also responsible for virtually every other advantageous biomedical characteristic CS possesses including its
biocompatibility, biodegradability, anti-microbial, wound healing, and analgesic properties. (Domb, 2011) Thus, preservation of the cationic charge of CS after sterilization is paramount.

Survival rates of CS+IL-12 treated C57Bl/6 mice, even when sterilized with NtNP, in the present study mirrored the results found by Zaharoff et al at the NCI in which CS+IL-12 dramatically prolonged the survival rates of MB-49 tumor-bearing mice and eliminated tumors in at least 88% of the mice. (Zaharoff, 2009) Thus, we have independently confirmed the exceptional anti-tumor properties of CS+IL-12 in treating orthotopic murine bladder tumors and extended the results to a CS that complies with sterility and pyrogen levels required for parenteral use of CS.

The very low number of animals that established tumors upon re-challenge also confirmed the findings by Zaharoff et al that durable immunity is achieved with CS+IL-12 therapy. Moreover, the vaccination effect we observed suggests that immunity can be generated even in the absence of tumor. Thus, prophylactic treatment, or vaccination, with CS+IL-12 may be effective in preventing the development of bladder cancer. Such a vaccination could be useful for individuals at high risk of developing bladder cancer like smokers, the genetically predisposed, and workers with certain occupational exposure hazards. (Kirkali, 2005; Pasin, 2008)
Perspective and Significance

Bladder cancer is a significant burden both in the US and abroad. It is well established as the 5th most common cancer in the US with over 70,000 new cases diagnosed each year. (Jemal, 2010; Siegel, 2012; Siegel, 2013; Siegel, 2011) As the most costly cancer to treat due to high recurrence rates, new approaches for bladder cancer therapy are needed. (Van Der Heijden, 2009; van Rhijn, 2009) Significantly, the current standard of care for treating bladder cancer, which consists of transurethral resection followed by intravesical BCG therapy, has been such for over 30 years despite major advances in the treatment of other urologic malignancies. (Askeland, 2012) A major issue with BCG that is currently plaguing urologists and bladder cancer patients is an ongoing shortage of intravesical BCG. According to the FDA CBER-Regulated Products: Current Shortages webpage, this shortage has been ongoing since May 2012. This shortage has a real impact on the treatment of bladder cancer patients (personal communication).

Taken together, our results presented herein and those previously reported by other laboratories suggest that human trials testing CS+IL-12 for the treatment of non-muscle invasive bladder cancer should commence with NtNP-sterilized CS+IL-12. No toxicity or side effects of the CS+IL-12 were noted in the current study or previous studies. There is also extensive animal and human data suggesting that CS will be a safe pharmaceutical excipient in humans. (Baldrick, 2010) Moreover, a phase I human clinical trial showed that intravesical IL-12 therapy is safe in humans. (Weiss, 2003) In fact, that trial failed to establish the
max tolerated dose of intravesical IL-12 in humans despite a dose-escalation design. Beyond bladder cancer, we believe human trials for treating other tumors of epithelial origin, including breast, colorectal, ovarian, and pancreatic tumors, with CS+IL-12 are indicated based on promising results in mice. (Vo, 2014; Yang, 2013; Zaharoff, 2010; Hurteau, 2001; Lenzi, 2002) Moreover, CS should be considered as a more general drug delivery vehicle for not only other cytokines and peptides, but also other chemotherapeutic agents. (Kato, 2005)

Development of a sterile, depyrogenated CS that is functional and safe for parenteral use will have indications beyond cancer therapy. Numerous studies have shown that CS-containing vaccines produce superior immune responses to those containing traditional adjuvants like aluminum hydroxide. (Zaharoff, 2007; Chua, 2015; Li, 2015; Powell, 2015) CS also enables novel routes of delivery for vaccines, including nasal and oral, that are non-invasive and convenient compared to the traditional injection-based vaccines. (Xia, 2015)

Conclusions

NtNP treatment produced CS that was sterile with endotoxin levels that are below the level required for parenteral indications. Biologic functionality of CS is also preserved with NtNP sterilization as demonstrated by therapeutic efficacy of psCS+IL-12 in treating orthotopic murine bladder tumors equivalent to usCS+IL-12. Human trials testing the safety and efficacy of NtNP sterilized CS+IL-12 are indicated and treating individuals at high risk of developing bladder cancer with a CS+IL-12 “vaccine” should be considered.
Acknowledgements

Research reported in this publication was supported by the National Cancer Institute of the National Institutes of Health under SBIR award number R43CA186374. This grant was awarded to Scion Cardio-Vascular, Inc. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The authors express their gratitude to Audomaro Flores, Billy Daniel, and Dr. David Wolf, DVM, for assistance with the animal studies. The authors also thank Jackie Knecht and Louis Rose for administrative assistance. The authors also thank Demetri Chrysostomou, Michael Barden, Luke Turalitsch, Eduardo Mateo, and James Bond for assistance with the IoN40 plasma instrument.
References


Cheever MA. Twelve immunotherapy drugs that could cure cancers. Immunol Rev. 2008;222:357-68.


Chen X, Oppenheim JJ, Howard OM. BALB/c mice have more CD4+CD25+ T regulatory cells and show greater susceptibility to suppression of their CD4+CD25- responder T cells than C57BL/6 mice. Journal of leukocyte biology. 2005;78:114-21.

Garcia-Pelayo MC, Bachy VS, Kaveh DA, Hogarth PJ. BALB/c mice display more enhanced BCG vaccine induced Th1 and Th17 response than C57BL/6 mice but have equivalent protection. Tuberculosis. 2015;95:48-53.


CHAPTER FOUR

EFFECT OF PLASMA STERILIZATION ON THE HEMOSTATIC EFFICACY OF A CHITOSAN HEMOSTATIC AGENT IN A RAT MODEL

Andrew Crofton\textsuperscript{a,e}, John Chrisler\textsuperscript{c}, Samuel Hudson\textsuperscript{i}, Serkan Inceoglu\textsuperscript{d}, Floyd Petersen\textsuperscript{e}, and Wolff Kirsch\textsuperscript{b,e}

\texttt{acrofton@llu.edu, jchrisler@llu.edu, shudson@ncsu.edu, sinceoglu@llu.edu, fpetersen@llu.edu, wkirsch@llu.edu}

\textsuperscript{a}Department of Anatomy, \textsuperscript{b}Division of Biochemistry, \textsuperscript{c}Animal Care Facility, \textsuperscript{d}Department of Orthopedic Surgery, and \textsuperscript{e}Neurosurgery Center for Research, Training, and Education, School of Medicine, Loma Linda University
11234 Anderson St.
Medical Center A537
Loma Linda, CA 92350

\textsuperscript{i}College of Textiles, North Carolina State University
2401 Research Dr.
Raleigh, North Carolina 27695

*Corresponding Author
Wolff M. Kirsch
Professor of Biochemistry and Neurological Surgery
Loma Linda University, School of Medicine,
11234 Anderson St., Medical Center A537
Loma Linda, CA 92354
\texttt{+1-909-558-7071}
fax: \texttt{+1-909-558-0472}
\texttt{wkirsch@llu.edu}

Advances in Therapy, 2016
Abstract

Introduction: The U.S. military has had success with chitosan-based hemostatic agents to control trauma-induced hemorrhages. Despite the positive reviews, additional physical forms of chitosan may enhance its hemostatic efficacy. Additionally, standard sterilization techniques may negatively affect the hemostatic efficacy of chitosan. We studied the effects of a chitosan-based hemostatic pad, the Clo-Sur P.A.D., on severe femoral vessel bleeding in a rat model. The effects of different sterilization techniques on the bioadhesivity, surface atomic concentrations, and hemostatic efficacy of the P.A.D. were also evaluated.

Methods: Hemostatic efficacy, bioadhesivity, and surface atomic concentrations of the P.A.D. were evaluated in its unsterilized form, after sterilization with standard e-beam treatment, and after sterilization with one of three types of non-thermal nitrogen plasma, nitrogen gas, air, or nitrous oxide plasma. After standardized puncture of the femoral artery or transection of the femoral vessels, rats were treated with either a chitosan P.A.D. or gauze pad.

Results: The Clo-Sur P.A.D., regardless of sterilization technique, stopped arterial and mixed arterial/venous bleeding in all cases in < 90 seconds with the time to hemostasis (TTH) significantly less for all P.A.D. treatment groups (p<0.001; n=4-5/group) compared to gauze-treated controls (n=3). E-beam sterilized P.A.D.s consistently showed non-significant trends toward increased
TTH and worse hemostasis scores compared to unsterilized and plasma-sterilized P.A.D.s. Treating e-beam sterilized P.A.D.s with N₂ plasma reverted the hemostatic efficacy to levels equivalent to native, unsterilized PADs.

**Conclusion:** A chitosan-based hemostatic pad successfully controlled severe bleeding in a rat model with combined e-beam+plasma sterilized P.A.D.s showing the most promising results. Further studies are warranted.

Keywords: chitosan, plasma sterilization, hemostasis, hemostatic agents, non-thermal nitrogen plasma, rat model

Abbreviations: CS – chitosan; NtNP – non-thermal nitrogen plasma; TTH – Time to Hemostasis;
ApP – atmospheric pressure plasma; LpP – low-pressure plasma; DD – degree of deacetylation; DFSD – dry fibrin sealant dressing; RDH – rapid deployment hemostat; HC – HemCon Bandage
Introduction

Nearly 10% of the U.S. population (30,888,063 people) experienced non-fatal injuries in 2013, the most recent year for which data are available from the CDC. An additional 192,945 people suffered fatal injuries in 2013 according to the CDC making unintentional injury the leading cause of death in the U.S. for individuals aged 1-44 years and the largest cause of years of potential life lost before age 65. (WISQARS, 2013) Hemorrhage is the second leading cause of death after injury, trailing behind only central nervous system (CNS) damage. (Kauvar, 2006) In the war theater, hemorrhage is the leading cause of death for combat casualties. (Champion, 2003)

In contrast to CNS injury, many methods are available to reduce or stop bleeding. There are currently four main hemostatic agents used to control hemorrhage in the prehospital setting. These four hemostatic agents include the dry fibrin sealant dressing (DFSD), rapid deployment hemostat (RDH), HemCon Bandage (HC), and QuickClot. DFSD is a lyophilized polygalactin mesh containing human fibrinogen, human thrombin, and calcium chloride, RDH is a lyophilized poly-N-acetyl-glucosamine bandage, HC Bandage is a lyophilized chitosan pad, and QuikClot is a granular zeolite powder. The only one of these four hemostatic agents that meet all 7 criteria for the ideal hemostatic dressing for prehospital use is chitosan. (Pusateri, 2006)

Chitosan (CS) is the deacetylated derivative of chitin, the second most abundant biopolymer on Earth. CS is derived from crustacean shells, insect exoskeletons, fungi, and algae and has been studied for its hemostatic properties
since 1983 when Malette and colleagues showed that blood, even when
defibrinated and heparinized, forms a coagulum when in contact with CS.
(Malette, 1983) Numerous animal and human studies have confirmed in vivo that
the hemostatic efficacy of CS is superior to gauze and at least equivalent to other
advanced hemostatic agents both under normal and anti-coagulant conditions.
(Kind, 1990; Subar, 1997; Brandenberg, 1984; Kourelis, 2012; Klokkevold, 1991;
Klokkevold, 1992; Klokkevold, 1999; Xie, 2008; De Castro, 2012; Rajiv, 2013;
Hattori, 2010; Dailey, 2009; Ahuja, 2006; Huang, 2015; Pusateri, 2003;
Wedmore, 2006) Studies have also shown that topical use and implantation of
CS in mammals does not produce toxicity or tissue reactivity and CS is rapidly
resorbed after implantation. (Xie, 2012; Kean, 2010; Onishi, 1999; Richardson,
1999)

The extensive animal studies on CS culminated in the U.S. Office of the
Surgeon General mandating in September 2005 that every soldier carry a CS
hemostatic dressing (HC Bandage, HemCon Medical Technologies, Portland,
OR). Combat Lifesavers and combat medics were mandated to carry 3 and 5 CS
bandages, respectively. (Cordts, 2008) After 42 of 44 uses of the HemCon
bandage in a combat setting were successful with no adverse effects
documented, a report proposed adding two CS based hemostatic agents, Celox
and ChitoGauze, to the Tactical Combat Casualty Care Guidelines. (Bennett,
2014) The same report describes widespread use of CS-based hemostats by
NATO militaries, emergency medical service (EMS) agencies and law
enforcement agencies in addition to the U.S. military. Although human studies
and roughly a decade of use have shown that CS dressings are effective hemostatic agents, there is significant room for improvement. (Pusateri, 2006; Alam, 2005)

Some of the issues identified with current CS-based hemostatic agents include issues with batch to batch variability in molecular weight (MW) and degree of deacetylation (DD) - both of which influence hemostatic efficacy - and low malleability that prevents easy manipulation to fit deep or jagged wound surfaces. (De Castro, 2012; Whang, 2005; Ahuja, 2006) Variations in MW and DD are likely a result of natural variability in the source of CS, which is typically crustacean shells, and inconsistencies in converting raw chitin extracts from crustacean shells to CS. (Kumirska, 2011; Kumar, 2004) However, sterilization methods also play a role since conventional sterilization techniques reduce CS MW. For example, electron beam sterilization reduced the MW of CS by 56% and gamma irradiation induced main chain scissions in CS fibers and films that decreased the MW of CS by 25%. (Dutkiewicz, 1989; Lim, 1998) Additional issues regarding sterilization of CS via traditional methods include chemical alterations of CS and residual toxic residues. (Franca, 2013; Lim, 1999; Marreco, 2004; Rao, 1995; Ułański, 1992)

Reduction in the MW of CS reduces its adhesion strength to tissues by limiting chain flexibility for interpenetration and entanglement of tissue proteins and mucus. (Whang, 2005) This effect is demonstrated by the failure of low molecular weight CS to form a firm coagulum when exposed to blood in vitro. (Lee, 1975) Other factors that affect the bioadhesivity of CS, like DD and degree
of ionization, also may reduce its hemostatic efficacy since it is postulated that CS induces hemostasis via red blood cell agglutination and a “velcro-like” adhesion to tissue surfaces. (Whang, 2005; Dowling, 2011)

The aim of the present study was to improve the hemostatic efficacy of a topical CS hemostatic agent (Clo-Sur P.A.D.™, Scion Cardio-Vascular, Inc., Miami, FL) by using non-thermal nitrogen gas plasma (NtNP), a non-conventional sterilization method. Though low-temperature plasmas have been shown to effectively sterilize materials, including wound care bandages, they have not been used widely in medicine. (Moisan, 2001; Messerer, 2005; Roth, 2010; Rossi, 2009; Shintani, 2007; Morfill, 2009) Non-thermal gas plasmas have typically been used to alter the surface chemistry of plastics and metals. For example, the surface characteristics of plastic microtiter plates for cell culture are frequently altered using NtNP to enhance cellular adhesion. We hypothesized that NtNP would not reduce bioadhesivity or MW of chitosan like conventional sterilization methods since it is only a surface treatment and is not composed of ionizing radiation. Additionally, we hypothesized that NtNP would increase the nitrogen content, and thus the bioadhesion, of the Clo-Sur P.A.D. since NH₃ gas plasma exposure increases nitrogen content of CS films. (Ogino, 2008)

**Materials and Methods**

**Materials**

Unsterile and E-beam sterilized Clo-sur P.A.D.s were obtained from Scion Cardio-Vascular, Inc. The P.A.D.s consist of lyophilized CS with each P.A.D.
measuring 4 cm x 4 cm x 1 cm. The P.A.D.s are normally sterilized with a standard dose of electron beam irradiation. Specifications for the Clo-Sur P.A.D. are given in Table 3.

Table 3. Properties of chitosan-based Clo-Sur P.A.D.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitin Source</td>
<td>Crab Shell</td>
</tr>
<tr>
<td>Endotoxin Quantity</td>
<td>28 EU/g</td>
</tr>
<tr>
<td>Loss on Drying</td>
<td>12%</td>
</tr>
<tr>
<td>Ash</td>
<td>2%</td>
</tr>
<tr>
<td>Viscosity</td>
<td>4,000 cP</td>
</tr>
<tr>
<td>Molecular Weight</td>
<td>500 kDa</td>
</tr>
<tr>
<td>Turbidity</td>
<td>150 NTU</td>
</tr>
<tr>
<td>Degree of Deacetylation</td>
<td>80%</td>
</tr>
<tr>
<td>Insolubles</td>
<td>2%</td>
</tr>
<tr>
<td>Residual Protein</td>
<td>500 μm</td>
</tr>
<tr>
<td>Heavy Metals</td>
<td>5 ppm</td>
</tr>
</tbody>
</table>

**Morphologic Characterization**

The Clo-Sur P.A.D. was imaged with a Phenom G1 (Model 800-03103-02) (FEI Co., Netherlands) scanning electron microscope (SEM). Before imaging, a small sample of the P.A.D. was mounted on the support. The sample was then sputtered with an approximately 6 nm layer of gold/palladium (Au/Pd) using a Quorum Technologies SC7620 Mini Sputter Coater (Laughton, East Sussex, UK), which deposits 10 nm coating/45 sec.
Sterilization and Surface Modification with NtNP

Unsterile P.A.D.s were treated with NtNP using the IoN40 plasma system (PVA-TePla America, Corona, CA) using medical-grade nitrogen gas, ammonia gas, nitrous oxide gas, or compressed air. Power was 500 Watts, pressure was maintained at 300 mTorr, and gas flow rate was 0.5 L/min. The sterilization dose for each gas was determined first by treating paper spore strips containing $2 \times 10^6$ B. subtilis and G. stearothermophilis spores (Steris Corp., Mentor, OH) and subsequently culturing spore extracts in tryptic soy agar plates for 72 hours following USP chapter 55 guidelines. Minimum sterilization doses, defined as a 6-log reduction in CFUs, for each gas were identified and an overkill dose, defined as 2x the minimum sterilization dose, were used for sterilizing the CS P.A.Ds.

P.A.D.s were placed directly on the metal tray of the IoN40 for sterilization and aseptically flipped every 5 minutes to ensure even plasma exposure. At the end of the sterilization treatment, P.A.D.s were aseptically transferred to a sterile vial for transport and storage until use. After initial tests showed increased brittleness of the P.A.D.s after plasma treatment due to drying, sterile gauze pads soaked with 5 mL of sterile water were added to the vials containing the plasma-treated P.A.D.s to increase malleability.

Tissue Adhesion Studies

Bioadhesion studies were performed using pig bladders and a universal materials testing machine (Electropuls E10000, Instron Inc., Norwood, MA, USA).
Pig bladders were obtained (Farmer John, Los Angeles, CA, USA), drained of urine, rinsed with cold 1X phosphate buffered saline, kept on ice, and splayed open with a sterile scalpel immediately prior to use. Each pig bladder (n=3) was cut into pieces such that enough pieces were available for testing one of each type of P.A.D. Cut bladder pieces were placed on the base of the testing machine and a stainless steel sheet with a 3.5 cm circular cutout was placed on top of the bladder and fixed to the base with tape (Figure 19 A & B). The stainless steel plate with a circular opening ensured consistent surface area contact of tissue with P.A.D. samples. Clo-sur P.A.D.s were cut into 2 cm x 2 cm squares and attached to the upper moveable arm of the testing machine using double-sided tape (Figure 19 C). The P.A.D.s were brought into contact with the tissue and compressed with 30 N of force for 30 seconds. After 30 seconds, the P.A.D.s were removed from the tissue surface at a rate of 1 mm/s and the peak force achieved when removing the P.A.D.s was recorded. 3 PADs were tested from each group. Care was taken to ensure the tape fully immobilized the P.A.D. and tissue during the compressing and peeling phases of the experiment.
Figure 19. Instrument setup for testing of CS bioadhesivity. Porcine bladder tissue was placed on stage of Electropuls E10000 under a stainless steel plate with a circular cutout to expose a standard surface area of tissue (A & B). The stainless steel plate was fixed to the stage with tape to prevent tissue movement. CS P.A.D. was fixed with double-sided tape to a plate attached to the moveable arm (C).

Surface Atomic Concentration Analysis

X-ray photoelectron spectroscopy (XPS) characterization was carried out using a Kratos AXIS ULTRA DLD XPS system equipped with an Al Kα monochromated X-ray source and a 165-mm mean radius electron energy hemispherical analyzer. Vacuum pressure was kept below $3 \times 10^{-9}$ torr during acquisition. Other data acquisition parameters are listed in Table 4.

Table 4. Experimental parameters for XPS measurements.

<table>
<thead>
<tr>
<th>Element</th>
<th>Start</th>
<th>End</th>
<th>Step</th>
<th>Dwell</th>
<th>Sweep</th>
<th>P.E.</th>
<th>Sensitivity Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survey</td>
<td>1200 eV</td>
<td>-5 eV</td>
<td>1.0 eV</td>
<td>100 ms</td>
<td>1</td>
<td>80</td>
<td>Al. mono.</td>
</tr>
<tr>
<td>N 1s</td>
<td>410 eV</td>
<td>390 eV</td>
<td>0.1 eV</td>
<td>200 ms</td>
<td>10</td>
<td>20</td>
<td>0.477</td>
</tr>
<tr>
<td>O 1s</td>
<td>540 eV</td>
<td>520 eV</td>
<td>0.1 eV</td>
<td>200 ms</td>
<td>5</td>
<td>20</td>
<td>0.780</td>
</tr>
<tr>
<td>C 1s</td>
<td>300 eV</td>
<td>275 eV</td>
<td>0.1 eV</td>
<td>200 ms</td>
<td>5, 10</td>
<td>20</td>
<td>0.278</td>
</tr>
</tbody>
</table>
Hemostasis Procedure

The hemostatic procedure used for this study was based on a previous study assessing the hemostatic efficacy of Microporous Polysaccharide Hemospheres® in rats. (Ersoy, 2007) After receiving approval for the study from the Loma Linda University Institutional Animal Care and Use Committee, 30 2-month old Sprague-Dawley rats (16 female, 14 male) were obtained (Charles River, Inc., Charleston, SC, USA). All procedures for the handling and care of the animals were carried out in accordance with the 1996 National Research Council’s “Guide for the Care and Use of Laboratory Animals.” Rats were housed 2 per cage with food and water ad libitum. Immediately prior to the procedure, rats were weighed, anesthetized with 3% isoflurane, and maintained with 2% isoflurane. Once anesthetized, the rats were placed in a supine position, the hind paws were immobilized such that the legs were maximally stretched inferolaterally, and the groin region shaved.

A rostral to caudal incision in the epidermis and dermis was made with a surgical scalpel blade No. 10 at the mid-clavicular line. The underlying fascia and fat pad were removed using scissors and blunt dissection. The left femoral artery was separated from the vein by removing the vasa vasorum using microsurgical instruments under an operating microscope. A 25 G needle was inserted into the left femoral artery approximately 2 mm distal to the deep femoral artery (Figure 20, middle panel). The 2 cm x 2 cm Clo-sur P.A.D. or cotton gauze was then centered over the needle, and the needle removed. Once bleeding was confirmed, a 200 g brass weight was placed on the P.A.D. and timing began. The
weight was held in place manually ensuring no additional weight was applied. The weight was removed every 30 seconds for a period of 15 seconds to observe the wound for bleeding. Excess blood was removed using gauze to enable visualization of the wound site, if necessary. The brass weight was placed back on the P.A.D. after each 15-second observation period until hemostasis was achieved (Figure 20, right panel). Time to hemostasis was recorded, as was the severity of bleeding after each 30-second compression period. If hemostasis was not achieved after 180 seconds, the artery was ligated using sutures to prevent exsanguination.

Figure 20. Experimental procedure for rat hemostasis studies. The left panel shows microsurgical scissors around the femoral vessels immediately prior to transection with the P.A.D. centered over the wound site for application after transection. The middle panel shows a needle within the femoral artery immediately prior to removal. The right panel shows the Clo-Sur P.A.D. in the wound site after a femoral artery puncture. Note that hemostasis has been achieved with very little bleeding.
After obtaining hemostasis on the left side, the right femoral vessels were dissected as described above for the left side. Once both the femoral artery and vein were isolated from the femoral nerve approximately 2 mm distal to the deep femoral artery, microsurgical scissors were placed around the vessels and the Clo-sur P.A.D. or cotton gauze was centered over the scissors (Figure 20, left panel). The femoral vessels were then fully transected. Once bleeding was visually confirmed, the 200 g weight was placed on the P.A.D. or gauze as described above. In cases where hemostasis was not achieved after 180 seconds, the vessels were ligated with sutures or clips.

Arterial puncture and artery/vein transection were both performed in order to model different wound types. Trauma wounds are highly complex with many different types of vascular injury, many of which behave differently. For example, vasoconstriction and vessel retraction can have a significant impact on bleeding after transection, whereas puncture wounds exhibit this phenomenon to a lesser degree, if at all.

After achieving hemostasis on both sides, the P.A.D.s were carefully removed to assess clot stability first on the arterial puncture side followed by the transection side. Assessing clot stability is important since re-bleeding is frequently an issue during pre-hospital transport. Animals were then euthanized by pentobarbital injection into the left ventricle.
**Hemostasis Scoring**

A hemostasis scoring system was devised to characterize the extent of hemorrhage at each 30-second time point. The four point scoring system was as follows: (4) severe, easily observable bleeding; (3) mild, easily observable bleeding; (2) minimal, difficult to observe bleeding; and (1) no observable bleeding (i.e. hemostasis).

**Statistical Analyses**

Data were analyzed via one-way ANOVA and Tukey’s Post-hoc test with p < 0.05 considered significant.

**Results**

**PAD Morphology**

SEM imaging of the P.A.D., shown in Figure 21, revealed a highly porous, honeycomb-like structure. This porosity gives the P.A.D. a very large surface to volume ratio, theoretically enhancing its hemostatic potential by maximizing intercalation and entrapment of blood cells.
Figure 21. SEM images of chitosan PAD. Morphologic analysis shows the PAD has a porous, honeycomb-like structure. The images show the PAD at 78X magnification (A), 500X magnification (B), and 1000X magnification (C).

**Plasma Dosing**

Three of the four nitrogen-based plasmas achieved sterility doses (i.e. 6 log reduction in bacterial spores) within 15 minutes of treatment. Figure 22 shows the kill curves for each plasma type over 15 minutes. Notably, ammonia plasma only reached a 5-log reduction even after 30 minutes, which means it failed to achieve a sterility dose. Due to the inefficiency of ammonia plasma, we elected not to test the bioadhesivity or hemostatic efficacy of ammonia plasma sterilized P.A.D.s. For the hemostasis studies, P.A.D.s were treated with nitrogen gas plasma for 30 minutes and air or nitrous oxide gas plasma for 20 minutes, since these doses constituted overkill doses.
Figure 22. Effect of different low-pressure plasmas on B. subtilis and G. stearothermophilis spores over time. Each data point represents the average of 3 spore strips. Sterility dose is defined as 6 log reduction in spores.

**Bioadhesivity**

Bioadhesion results are shown in Figure 23. Although the bioadhesion studies failed to demonstrate statistically significant results due to high intra-group variability, the 30-minute N\textsubscript{2} gas plasma treated P.A.D.s showed a trend toward higher bioadhesivity than all other samples tested. Additionally, air and nitrous oxide gas plasma treatment for 20 minutes trended toward lower bioadhesivity than all other treatments.
Figure 23. Effect of different low-pressure plasmas on bioadhesivity of chitosan. Data represent mean ± standard error of the mean (n=3 each group). None of the results reached statistical significance.

**Surface Atomic Concentrations**

Table 5 shows that contrary to our hypothesis, air and nitrous oxide plasma treatment for 20 minutes increased nitrogen content in the P.A.D.s by 4% and 9% respectively, whereas N₂ plasma treatment for 30 minutes only increased nitrogen content by 2%.
Table 5. Elemental composition of chitosan after different plasma sterilization treatments.

<table>
<thead>
<tr>
<th>Sample</th>
<th>% Oxygen</th>
<th>% Carbon</th>
<th>% Nitrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>No plasma</td>
<td>32</td>
<td>57</td>
<td>4</td>
</tr>
<tr>
<td>20 mins N₂O Plasma</td>
<td>36</td>
<td>43</td>
<td>13</td>
</tr>
<tr>
<td>20 mins Air Plasma</td>
<td>33</td>
<td>48</td>
<td>8</td>
</tr>
<tr>
<td>30 mins N₂ Plasma</td>
<td>25</td>
<td>59</td>
<td>6</td>
</tr>
</tbody>
</table>

*Hemostatic Efficacy*

**Time to Hemostasis**

As shown in Figure 24, rats in each experimental group weighed 286-324 g on average, with no statistical difference between groups. Figure 25 shows that time to hemostasis (TTH) after femoral artery puncture was significantly lower (p<0.001) in the Clo-sur P.A.D. groups compared to gauze controls. This result is further strengthened by the fact that bleeding was consistently greater in the male rats compared to the females and the 3 gauze treated rats were all females and hemostasis was still not achieved in even one of them within 180 seconds.
Figure 24. Average weights of rats in each of the experimental groups. Data presented as mean ± standard error of the mean. The mean values of the groups were not statistically significantly different. Group numbers were as follows: EB and Gauze (n=3); No plasma, N2O, EBN2, and Air (n=4); and N2 (n=5).

AIR – air plasma sterilization group; EB – electron beam sterilization group; N2O – nitrous oxide plasma sterilization group; EBN2 – electron beam + N2 plasma sterilization group.
Figure 25. Time to hemostasis after puncture of femoral artery. Data represent mean ± standard error of the mean. Group numbers were as follows: EB and Gauze (n=3); No plasma, N2O, EBN2, and Air (n=4); and N2 (n=5). CS compared to gauze was statistically significant for all CS samples (p <0.001).

AIR – air plasma sterilization group; EB – electron beam sterilization group; N2O – nitrous oxide plasma sterilization group; EBN2 – electron beam + N2 plasma sterilization group.

Although there was no statistically significant difference between the P.A.D. groups, electron beam sterilized P.A.D.s trended toward a longer TTH than unsterilized, native P.A.D.s. Interestingly, this trend was reversed when e-beam sterilized P.A.D.s were treated with N2 gas plasma for 30 minutes. (Fig. 25)

TTH results after femoral transection are shown in Figure 26. TTH was significantly reduced (p<0.001) compared to gauze control for the femoral vessel transection challenge with the N2 plasma treated P.A.D.s trending toward the lowest TTH. Once again, there was no statistically significant difference in TTH
between the P.A.D. groups. Treating E-beam sterilized P.A.D.s with 30 minutes of N₂ gas plasma again resulted in a lower mean TTH.

Figure 26. Time to hemostasis after transection of femoral artery and vein. Data represent mean ± standard error of the mean. Group numbers were as follows: EB and Gauze (n=3); No plasma, N2O, EBN2, and Air (n=4); and N2 (n=5). CS compared to gauze was statistically significant for all CS samples (p <0.001).

AIR – air plasma sterilization group; EB – electron beam sterilization group; N2O – nitrous oxide plasma sterilization group; EBN2 – electron beam + N2 plasma sterilization group.

**Hemostasis Score**

Hemostasis scores shown over the course of the experiment are shown in Figures 27 and 28. Both N₂ plasma and E-beam sterilized P.A.D.s trended toward worse hemostasis scores at 30 seconds in the arterial puncture model compared to the other P.A.D. groups. E-beam sterilized P.A.D.s retained this
trend for the transection model, but N₂ sterilized P.A.D.s reversed this trend and, in fact, demonstrated the lowest hemostasis score at 30 seconds. It is evident that hemostasis scores for the e-beam sterilized P.A.D.s returned to levels achieved by unsterilized P.A.D.s. when also treated with 30 mins of N₂ plasma.

Figure 27. Hemostasis scores after puncture of femoral artery. Data at each time point represent mean values. Group numbers were as follows: EB and Gauze (n=3); No plasma, N₂O, EBN2, and Air (n=4); and N₂ (n=5). AIR – air plasma sterilization group; EB – electron beam sterilization group; N₂O – nitrous oxide plasma sterilization group; EBN2 – electron beam + N₂ plasma sterilization group.
Figure 28. Hemostasis scores after transection of femoral artery and vein. Data at each time point represent mean values. Group numbers were as follows: EB and Gauze (n=3); No plasma, N2O, EBN2, and Air (n=4); and N2 (n=5).

AIR – air plasma sterilization group; EB – electron beam sterilization group; N2O – nitrous oxide plasma sterilization group; EBN2 – electron beam + N2 plasma sterilization group.

**Clot Stability**

There was little, and in many cases, no re-bleeding when P.A.D.s were removed from wound sites after hemostasis had been achieved. Additional manual manipulation of the clot site rarely resulted in re-bleeding indicating robust clotting had occurred.

**Discussion**

Our results provide strong evidence that the Clo-sur P.A.D., a 100% CS hemostatic agent, is a superior hemostatic agent compared to gauze for arterial and combined arterial/venous hemorrhage, which is contrary to findings reported
by Fischer et al in 2004. That group reported that Clo-sur P.A.D. was worse than cotton gauze in controlling hemorrhage in a porcine spleen-bleeding model. A major limitation in comparing these two studies, of course, is the hemorrhages produced by rat femoral vessels are certainly different in nature than hemorrhages from pig spleens. However, this limitation does not weaken the conclusion that the Clo-Sur P.A.D. is an effective hemostatic agent.

The currently used sterilization technique for Clo-Sur P.A.D.s, e-beam irradiation, lengthens TTH in both arterial and combined arterial/venous hemorrhage, though not statistically significantly. N₂ plasma sterilized P.A.D.s trended toward a lower TTH compared to e-beam sterilized P.A.D.s, but this result also failed to reach statistical significance and was not entirely consistent between the arterial and arterial/venous hemorrhages. The most consistent result we obtained was with P.A.D.s treated with both e-beam and 30 minutes of N₂ plasma. This combination consistently produced the lowest TTH and hemostasis scores that were at least equivalent to native, non-sterilized PADs. Thus, although plasma does not enhance the hemostatic efficacy of the native, unsterilized CS P.A.D. as we initially hypothesized, it does return the hemostatic efficacy of the P.A.D.s to baseline after e-beam treatment. This suggests that a combined e-beam+plasma sterilization protocol might be optimal.

It is presently unclear why combined e-beam+plasma treatment generates the best hemostatic results. We postulate that e-beam treatment initially alters the CS functional groups, which produces a minor reduction in hemostatic efficacy, but N₂ plasma treatment then alters the functional groups again such
that the initial hemostatic efficacy is reestablished. Effects due to heating might play a role in the alterations induced by both e-beam and plasma. (Lim, 1999) Future studies will seek to better characterize the changes in CS functional groups that occur after e-beam treatment, N₂ plasma treatment, and the combination of both.

The XPS data represent initial evidence showing that functional group alterations occur after plasma treatment. However, the changes induced by air, nitrous oxide, and nitrogen plasmas deviated from the predicted outcomes. Specifically, it is surprising that air and nitrous oxide plasmas demonstrated greater increases in nitrogen content than nitrogen plasma. Surface alterations to polymers induced by plasmas remains a poorly understood phenomenon. (Cornelius, 2007; Ogino, 2008; Messerer, 2005)

The bioadhesion data produced in the present study suggest that overall nitrogen content is not the driving factor in determining CS bioadhesion, but rather the type of nitrogen functional groups present. For example, the degree of acetylation dictates the charge density of CS and thus the electrostatic interactions between CS and tissues. Thus, if plasma sterilization induces changes in the degree of deacetylation, perhaps through heating and resultant caramelization of the polymer, this may alter the bioadhesivity, and therefore the hemostatic properties, of CS. Follow-up studies using FTIR and/or mass spectrometric techniques will aid in clarifying this issue.

In the course of conducting these hemostasis studies it was noted that there was no difficulty in fitting the P.A.D.s to the wound site. The P.A.D.s are
pliable and easy to manipulate. This is important since one of the seven key attributes of the ideal hemostat for pre-hospital use is that it should simple to apply. Notably, the CS P.A.D.s tested in the present study also meet the other 6 criteria for the ideal pre-hospital hemostatic dressing, including being able to stop large-vessel arterial and venous bleeding within 2 minutes of application, ready to use without special preparation, lightweight and durable, stable and functional at room temperature for at least 2 years, posing no risk of injury to tissue or transmission of bacteria or viruses, and inexpensive. (Pusateri, 2006) Additionally, P.A.D.s produced robust clots that may aid in reducing re-bleeding that may occur during pre-hospital transport.

Conclusion

We demonstrated superior hemostatic efficacy of a chitosanic hemostat (Clo-Sur P.A.D.) compared to gauze in a rat model of severe arterial bleeding and combined arterial/venous bleeding. Nitrogen gas plasma sterilization of these P.A.D.s maintains the bioadhesive and hemostatic properties of the unsterilized P.A.D.s. Finally, treating e-beam sterilized chitosan P.A.D.s. with 30 minutes of nitrogen gas plasma reverts the hemostatic efficacy to levels equivalent to native, unsterilized PADs.
Acknowledgements

Funding

Research reported in this publication was supported by the National Cancer Institute of the National Institutes of Health under SBIR award number R43CA186374. This grant was awarded to Scion Cardio-Vascular, Inc. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The authors would like to thank Jackie Knecht and Louis Rose for administrative assistance. The authors also thank Demetri Chrysostomou, Michael Barden, Luke Turalitsch, Eduardo Mateo, and James Bond for assistance with the IoN40 plasma instrument.

Authorship

All named authors meet the International Committee of Medical Journal Editors (ICMJE) criteria for authorship for this manuscript, take responsibility for the integrity of the work as a whole, and have given final approval to the version to be published.

Disclosures

Andrew Crofton, Samuel Hudson, John Chrisler, Serkan Inceoglu, Floyd Petersen, and Wolff Kirsch declare that they have no conflicts of interest.
Compliance with Ethics Guidelines

All institutional and national guidelines for the care and use of laboratory animals were followed in the conduct of this study.
References


Kourelis K, Shikani AH. Effectiveness of chitosan-based packing in 35 patients with recalcitrant epistaxis in the context of coagulopathy. Clinical otolaryngology : official journal of ENT-UK ; official journal of Netherlands


Ersoy G, Kaynak MF, Yilmaz O, Rodoplu U, Maltepe F, Gokmen N. Hemostatic effects of Microporous Polysaccharide Hemosphere® in a rat model with

CHAPTER FIVE

EVALUATION OF A CHITOSAN HEMOSTAT IN A PORCINE
LAPAROSCOPIC PARTIAL NEPHRECTOMY MODEL: A PILOT STUDY

Andrew Crofton, B.A.\textsuperscript{a,d}, Duane Baldwin, M.D.\textsuperscript{c}, Muhamad Alsyouf, M.D.\textsuperscript{c}, Daniel Faaborg, M.D.\textsuperscript{c}, Kristine Myklak, M.D.\textsuperscript{c}, Kerby Oberg, M.D., Ph.D.\textsuperscript{a}, Matthew Dopp\textsuperscript{d}, Javier Arenas, M.D.\textsuperscript{c}, Nazih Khater, M.D.\textsuperscript{c}, Samuel Hudson, M.D.\textsuperscript{e}, Craig Zupan, M.D.\textsuperscript{a}, and Wolff Kirsch, M.D.\textsuperscript{b,d}

\texttt{acrofton@llu.edu, dbaldwin@llu.edu, malsyouf@llu.edu, dfaaborg@llu.edu, kmyklak@llu.edu, koberg@llu.edu, mdopp@llu.edu, jarenas@llu.edu, nkhater@llu.edu, shudson@llu.edu, czupan@llu.edu, wkirsch@llu.edu}

\textsuperscript{a}Department of Pathology and Human Anatomy, \textsuperscript{b}Division of Biochemistry, \\
\textsuperscript{c}Department of Urology, and \textsuperscript{d}Neurosurgery Center for Research, Training, and Education, School of Medicine, Loma Linda University

11234 Anderson St.
Medical Center A537
Loma Linda, CA 92350
+1-909-558-7070

\textsuperscript{e}College of Textiles, North Carolina State University
2401 Research Dr.
Raleigh, North Carolina 27695
+1-919-515-6545

*Corresponding Author
Wolff M. Kirsch
Professor of Biochemistry and Neurological Surgery
Loma Linda University, School of Medicine,
11234 Anderson St., Medical Center A537
Loma Linda, CA 92354
+1-909-558-7071
fax: +1-909-558-0472
\texttt{wkirsch@llu.edu}

(Manuscript in preparation)
Abstract

Background and Objectives: No ideal hemostatic agent exists for laparoscopic partial nephrectomy despite the need for complete hemostasis and sealing of the urinary collecting system in this procedure. Chitosan is an emerging hemostatic agent, but standard sterilization techniques affect its functional and biological properties. This study sought to characterize the hemostatic efficacy and safety of an implanted chitosan hemostat sterilized with either electron beam irradiation or non-thermal nitrogen plasma in a porcine model of laparoscopic partial nephrectomy.

Methods: Laparoscopic partial nephrectomies were performed on 6 farm pigs and hemostasis achieved using only a chitosan hemostatic agent (Clo-Sur P.A.D.) that was e-beam (n=3) or plasma sterilized (n=3). Number of pads needed, estimated blood loss, operative time, nephrectomy size, warm ischemia time, and pig weight were measured. Animals were monitored for complications for 14 weeks. Retrograde ureteropyelography was performed to assess urine leakage. Post-mortem examination was followed by histologic analysis.

Results: Complete hemostasis and collection system sealing was achieved in both groups, but fewer pads were required (p = 0.056) and estimated blood loss was lower (p = 0.096) with plasma-sterilized pads. No complications were observed over 14 weeks and gross examination showed the chitosan was encapsulated in a fibrous capsule. Histologic analysis revealed normal renal
parenchyma, mild giant cell and macrophage infiltration into the fibrous capsule, and absence of infection or significant tissue reactivity.

**Conclusion**: Chitosan pads alone provided effective hemostasis in laparoscopic partial nephrectomy and were safe to implant. Plasma sterilization enhanced hemostatic efficacy whereas e-beam accelerated resorption of chitosan.

Keywords: chitosan, plasma sterilization, hemostasis, hemostatic agents, non-thermal nitrogen plasma, laparoscopic partial nephrectomy, LPN, electron beam, e-beam

Abbreviations: CS – chitosan; NtNP – non-thermal nitrogen plasma; LPN – laparoscopic partial nephrectomy; LpP – low-pressure plasma; DD – degree of deacetylation;
Introduction

Partial nephrectomy has become the reference standard treatment for small, clinical T1 tumors (<7 cm) in patients who are candidates for nephron-sparing surgery (NSS) since equivalent oncologic and superior functional outcomes have been demonstrated with partial nephrectomy compared to radical nephrectomy. (Gill, 2010; Lee, 2000; Fergany, 2000; Lau, 2000) Laparoscopic partial nephrectomy (LPN) is becoming the preferred approach to partial nephrectomy due to comparable outcomes with LPN compared to open partial nephrectomy (OPN). (Lane, 2007; Dominguez-Escrig, 2011) However, LPN is a technically challenging procedure and has shown higher rates of bleeding and urine leak complications. (Dominguez-Escrig, 2011; Aron, 2007; Gill, 2007) Current methods used in combination or alone for achieving hemostasis and sealing of the collecting system in LPN include electrocautery, harmonic scalpel, suturing, and hemostatic agents. Amongst hemostatic agents, Surgicel (Ethicon, Inc.) is the most commonly used, but there is no ideal hemostatic agent for LPN at present. (Breda, 2007) Thus, new hemostatic agents to improve hemostatic control and tissue sealing for LPN are needed.

In the present feasibility study, a chitosan (CS) hemostatic agent (Clo-Sur P.A.D.™, Scion Biomedical, Inc., Miami, FL) was evaluated in a hypertensive porcine survival model of LPN. CS is known as a highly effective hemostatic agent, but has yet to be used as an implantable hemostat due to concerns regarding pyrogen contamination and negative alterations to the material caused by conventional sterilization methods including dry/wet heat, irradiation, and
chemical sterilants. (Whang, 2005; Ułański, 1992; Dutkiewicz, 1989; Franca, 2013; Lim, 1998; Lim, 1999; Marreco, 2004; Rao, 1995) Thus, this study sought to characterize the hemostatic efficacy of a lyophilized CS hemostatic pad in LPN when sterilized by a conventional method – electron beam irradiation – or a novel method of non-thermal nitrogen plasma (NtNP).

Plasma is considered the fourth state of matter, after solid, liquid, and gas, and has emergent properties that gases do not like quasi-neutrality, collective behavior, and controlled motion by electromagnetism. (Cornelius, 2007) Plasma is used widely in a number of industries, but has only just begun being used in medicine as a dry decontamination technique. (Kong, 2009) Plasmas contain neutrals, ionized species, and excited atoms and molecules. The mechanism(s) by which NtNP kills microbes, and inactivates spores, viruses, and pyrogens is debated, but the leading hypothesis is that UV, reactive chemical species, and physical etching all play a role. (Moisan, 2001)

**Materials and Methods**

Lyophilized, foam-like CS pads that are currently manufactured for topical control of bleeding and infection control were obtained (Clo-Sur P.A.D. ™). Both e-beam sterilized and unsterilized pads were obtained. The unsterilized CS pads were sterilized with 30 minutes of non-thermal nitrogen plasma (NtNP) using the IoN 40 plasma system (PVA-TePla America, Inc., Corona, CA, USA). Sterility was confirmed by placing spore strips with $5.5 \times 10^6$ *B. atrophaeus* and *G. stearothermophilis* spores (Steris Corporation, Mentor, OH, USA) in the IoN 40
with the CS pads and subsequently culturing the spores with tryptic soy agar for 48-72 hours at 37°C and 58°C.

Six female farm pigs (S&S Farms, Santee, CA) with an average weight of 28.3 kg (range of 24-32 kg) were included in the study after obtaining approval for the study from the Loma Linda University Institutional Animal Care and Use Committee. All procedures for handling and care of the animals were carried out in accordance with the 1996 National Research Council’s “Guide for the Care and Use of Laboratory Animals.” Animals were split into two treatment groups, where electron beam sterilized (ES) pads were used to control hemostasis in one group and non-thermal nitrogen gas plasma sterilized (PS) pads were used in the other.

After acclimating for a minimum of 4 days, each animal was sedated with intramuscular telazole, intubated, and placed in the right lateral decubitus position. The animal was then sterilely prepped and draped in the typical fashion. Sedation was maintained with isoflurane or sevoflurane throughout the remainder of the case. IV antibiotics were then administered. Blood was drawn for preoperative chemistry panels and complete blood counts. Blood pressure was monitored with a cuff placed on the left arm. Heart rate and SpO₂ were monitored by a pulse oximeter placed on either the ear or tongue. A 14-gauge Veress needle was then inserted percutaneously into the abdomen and pneumoperitoneum was established superior to the umbilicus. Pneumoperitoneum was maintained between 12-15 mmHg throughout each case. A 12 mm port was then placed and used as the camera port. Three
additional 12 mm ports were subsequently placed under direct visualization. Following this, the bowel was mobilized medially with use of a hook electrode and the left renal vein and artery were identified. The entire renal unit including the renal artery and vein were then circumferentially dissected free of attachments with the hook electrode.

With hemostasis confirmed, a bulldog clamp (Aesculap, Inc., Center Valley, PA, USA) was placed over the left renal artery. The lower pole of the left kidney was then removed with sharp dissection. The collecting system was entered in one animal from each treatment group. CS pads were rolled, passed down a 12 mm port, and applied to the freshly cut surface of the kidney. At least 2 pads (4 cm X 4 cm) were applied to the cut surface of each kidney before the bulldog clamp was removed. Epinephrine (1 μg/mL) was administered to artificially raise the blood pressure and heart rate. Manual pressure was applied to the CS pads via use of the laparoscopic instruments. If there was continued bleeding observed after removing the bulldog clamp, then additional pads were passed through a port and applied to the site of bleeding until hemostasis was achieved. After complete hemostasis was achieved, the wound was observed for re-bleeding and blood was removed from the abdomen via suction. The kidney specimen was removed using the Endo Catch specimen pouch (Covidien, Inc., Minneapolis, MN). In 4 of 6 animals (2 in each group), peritoneum was pulled over the kidney and closed using non-absorbable hemo-clips. The CS pads were left in place. The 12 mm port sites were then closed under direct visualization with the use of a Carter-Thomason device. Subcutaneous tissue was then closed
with 4-0 Monocryl subcuticular sutures followed by Dermabond. The animal was awakened and extubated.

The primary outcome measures were time to hemostasis, estimated blood loss (EBL), number of pads required to achieve hemostasis, pre- versus postoperative blood counts/chemistries, complications (e.g. urinoma, re-bleeding, pyrogenic reaction, etc.) up to postoperative day 98, and histologic abnormalities. Additional outcome measures included operative time and warm ischemia time (WIT). Ultrasounds and blood counts/chemistries were performed immediately postoperatively and at postoperative day 6. Retrograde pyelography was performed immediately prior to euthanasia on day 98 by first inducing anesthesia as described above. A polyurethane ureteral catheter (C.R. Bard, Inc, Covington, GA, USA) was inserted using a standard cystoscope. Catheter placement was confirmed via guide wire and fluoroscopy. Iohexol (Omnipaque 300, GE Healthcare, Inc., Little Chalfont, Buckinghamshire, UK) was diluted 1:2 with normal saline and injected via the cystoscope into the ureter and urinary pelvis of each kidney. Integrity of the ureter, renal pelvis, and calices was determined by fluoroscopy after injection of iohexol.

After pyelograms were obtained, animals were sacrificed by lethal IV injection of sodium pentobarbital. The LPN site and implanted CS were then carefully examined grossly before harvesting the kidneys. Both kidneys were fixed in 10% neutral buffered formalin before histologic analysis. For histology, kidney samples were processed in a series of ethanol (70%-100%) washes followed by xylenes and paraffin. Samples were then embedded in paraffin and
sectioned at 7 μm. Sections were mounted on lysine-coated microscope slides and heat-set overnight.

For hematoxylin and eosin (H&E) staining, samples were cleared with 2 washes of xylenes at 5 minutes each, followed by 5 minute washes with 100%, 95%, and 70% ethanol. Slides were subsequently washed for 5 minutes with deionized water and then stained with hematoxylin Gill’s No. 1 (Sigma Aldrich, St. Louis, MO, USA) for 3 minutes, washed with tap water for 5 minutes, dipped 8-12x in acid ethanol (1 mL HCl + 400 mL 70% ethanol), rinsed with tap water for 2 minutes, and rinsed with deionized water for 2 minutes. Samples were then stained with eosin Y containing phelon (Sigma Aldrich) for 30 seconds, rinsed with 95% ethanol for 15 minutes, 100% ethanol for 15 minutes, and xylenes for 45 minutes. The slides were cover slipped using Permount (Thermo Fisher Scientific, Inc., Waltham, MA) and imaged with a standard light microscope. Two board-certified pathologists – who were blinded to the identity of the specimens – analyzed each of the stained sections.

Data are presented as mean ± standard error of the mean (SEM). Data were analyzed using Student’s t-test with p <0.05 considered statistically significant.

**Results**

Complete hemostasis was achieved in all 6 cases using only CS pads. The pads, which are foam-like, were rolled, successfully passed through a 12 mm port, unfurled intraabdominally, and applied to the resected renal surface (Figure 29). However, some difficulty was encountered initially with the PS pads
due to brittleness. This problem was solved in subsequent operations by raising the relative humidity in the packaging of the PS pads after NtNP treatment with water-soaked sterile gauze.

Figure 29. Deployment of the CS pad to the cut surface of the kidney under warm ischemia. The Clo-Sur P.A.D. was furled and passed through the 12 mm port (left) before being unfurled and applied to the cut surface of the kidney (right).

Complete hemostasis was achieved in all 6 animals, but fewer PS pads were required to achieve complete hemostasis than ES pads ($p = 0.0564$), as shown in Table 6. EBL was less in the PS pad treated group than the ES group (Table 6), but the difference was short of statistical significance ($p = 0.096$). Notably, 2 of the PS pad-treated animals had EBL < 20 cc and non-humidified PS pads were used in the one PS pad-treated animal that had increased EBL (110 cc). Table 1 also presents the nephrectomy specimen weights, WIT, and mean operative times, respectively, which were not significantly different

136
between groups. Blood chemistry values, which were also not significantly different between groups and preoperatively to postoperatively, are presented in Table 7. White blood cell counts were significantly lower postoperatively in the PS pad treated group than the ES pad treated group.

Table 6. Surgical data for porcine laparoscopic partial nephrectomy with chitosan hemostatic agent.

<table>
<thead>
<tr>
<th></th>
<th>ES Pads (n=3) (mean ± SEM)</th>
<th>PS Pads (n=3) (mean ± SEM)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Pads</td>
<td>3.67 ± 0.33</td>
<td>2.5 ± 0.29</td>
<td>0.056</td>
</tr>
<tr>
<td>EBL (mL)</td>
<td>201.67 ± 65.09</td>
<td>43.33 ± 33.33</td>
<td>0.096</td>
</tr>
<tr>
<td>WIT (mins)</td>
<td>11.14 ± 1.54</td>
<td>8.27 ± 0.81</td>
<td>0.174</td>
</tr>
<tr>
<td>Nephrectomy Weight (g)</td>
<td>6.65 ± 2.37</td>
<td>7.56 ± 1.83</td>
<td>0.776</td>
</tr>
<tr>
<td>Operative Time (hours)</td>
<td>2.49 ± 0.32</td>
<td>2.39 ± 0.15</td>
<td>0.799</td>
</tr>
</tbody>
</table>

EBL – estimated blood loss; WIT – warm ischemia time; SEM – standard error of the mean
Table 7. Blood Chemistry and Cell Counts Between Groups and Pre- Versus Postoperatively.

<table>
<thead>
<tr>
<th></th>
<th><strong>ES Pads</strong>&lt;br&gt;<strong>(n=3)</strong></th>
<th><strong>PS Pads</strong>&lt;br&gt;<strong>(n=3)</strong></th>
<th><strong>p-value</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC Pre</td>
<td>5.52±0.23</td>
<td>6.44±0.27</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>RBC Post</td>
<td>5.60±0.43</td>
<td>6.15±0.63</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Hg Pre</td>
<td>8.0±0.44</td>
<td>9.0±0.44</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Hg Post</td>
<td>8.23±0.48</td>
<td>8.23±0.49</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>HCT Pre</td>
<td>23.18%±1.05%</td>
<td>25.22%±1.16%</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>HCT Post</td>
<td>23.67%±1.47%</td>
<td>23.66%±1.08%</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>WBC Pre</td>
<td>14.33±1.72</td>
<td>13.23±1.47</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>WBC Post</td>
<td><strong>17.85±1.39</strong> *</td>
<td><strong>9.13±0.73</strong> *</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CRE Pre</td>
<td>1.03±0.07</td>
<td>1.0±0.1</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>CRE Post</td>
<td>0.80±0.0</td>
<td>0.93±0.12</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>ALB Pre</td>
<td>3.80±0.44</td>
<td>3.73±0.13</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>ALB Post</td>
<td>3.70±0.153</td>
<td>3.77±0.30</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>BUN Pre</td>
<td>5.0±1.0</td>
<td>4.33±1.20</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>BUN Post</td>
<td>5.0±1.0</td>
<td>4.33±1.20</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

RBC – red blood cell count; pre – preoperative; post – postoperative; Hg – hemoglobin; HCT – hematocrit; WBC – white blood cell count; CRE – creatinine; ALB – albumin; BUN – blood urea nitrogen *p<0.05

Postoperative ultrasound and paracentesis revealed urine in the abdomen of the ES pad treated animal in which the collecting system was breached, but blood chemistry values and behavior were normal throughout the post-operative period. Fluid was not observed via ultrasound in the abdomen of any animals treated with PS pads. All animals were negative for urine leaks at 14 weeks as assessed by retrograde ureteropyelography (Figure 30). However, minor hematuria was observed for 5 days postoperatively in the PS pad treated animal.
in which the collecting system was entered. There was no evidence of infection or pyrogenic responses in any animals up to 14 weeks after CS implantation.

Figure 30. Retrograde ureteropyelography. Representative pyelograms performed 14 weeks post-LPN of the right (images A and C) and left (images B and D) ureters and calyces from both ES pad (images A and B) and PS pad (images C and D) treated animals are shown. Urinary leakage was not observed in any animals.
Figure 31 shows representative examples from both treatment groups of the CS pads in relationship to the left kidney intraoperatively (A and D), *in situ* at sacrifice (B and E), and after removal from the body and longitudinal sectioning (C and F). Gross examination at sacrifice suggested modest breakdown of the pads in both treatment groups with pads appearing as yellow masses (Fig. 31 B and E). The CS was a paste-like consistency at sacrifice and appeared to be encapsulated within a capsule, which is part of the normal response to any foreign material implanted in a living organism (Fig. 31 C and F). (Anderson, 2001)
Figure 31. Intraoperative, post-mortem *in situ*, and *ex vivo* sectioned views of the CS pads. Images A-C show representative views of the ES pads and D-F show representative views of the PS pads from one animal each. Images A and D show the effect of epinephrine administration on hemostasis where epinephrine was given to the animal shown in A, but not to the animal in image D. Note the appearance of the CS implant after 98 days in B, C, E, and F.

The kidney parenchyma appeared normal upon histologic examination in all 6 animals (Fig. 32). Additionally, the tissue around the kidney showed a typical foreign body reaction in all 6 animals. The capsule that we observed surrounding the implanted CS macroscopically only had small amounts of collagen suggesting the capsule is primarily composed of an immune cell connective tissue matrix. There were large populations of giant cells present in this matrix that were frequently approximated to CS deposits or showing evidence of CS engulfment by presence of CS in the giant cell cytoplasm (Figure 33). In areas
with large numbers of giant cells there was less CS, suggesting degradation of CS by giant cells. Also present were lymphocytes with evidence of germinal centers likely consisting of B cells with small numbers of T cells (Figure 34). However, additional studies are needed to characterize the ratio of B cell to T cells. Leukocytes were observed, but in small numbers.

Infiltration of giant cells (and thus macrophages), lymphocytes, and leukocytes around implanted foreign materials is expected based on extensive literature reports on implantable medical devices. (Anderson, 2001) Notably, None of the animals showed evidence of infection, hematoma, urinoma, excessive tissue reactivity, or other pathologic abnormalities.

Figure 32. Appearance of kidney parenchyma in contact with plasma-sterilized CS. H&E section showing kidney parenchyma of pig treated with psCS. No abnormalities of the kidney parenchyma were observed in any animals, including areas with direct CS contact.
Figure 33. CS engulfment by giant cells. Images are representative examples of giant cells (marked by arrows) engulfing CS (stained pink) in H&E stained sections.
Discussion

A 100% CS pad (Clo-Sur P.A.D.™), currently marketed as an antimicrobial barrier and hemostatic agent for arterial catheterization procedures, was shown in this feasibility study to be an effective hemostatic agent in a porcine model of LPN. This is the first study to our knowledge to demonstrate that NtNP efficiently sterilizes and depyrogenates CS while improving its *in vivo* hemostatic efficacy compared to a conventional radiation-based sterilization procedure.
Due to the small sample size in this study, many of the primary endpoints failed to reach statistical significance, but the safety profile of the implanted CS was unequivocal.

A major complication of NtNP treatment identified in this study was dehydration of the CS, which led to brittleness making it difficult to roll the pad for placement down the laparoscopic ports. Simply increasing the relative humidity within the packaging by co-packaging with water-soaked sterile gauze not only reduced the brittleness, but actually enhanced the malleability of the pads from where it was prior to NtNP treatment. Since higher relative humidity accelerates and enhances hydrolytic depolymerization of CS within 6 months, alternative methods for increasing the malleability of CS are recommended. (Viljoen, 2014)

During the course of the LPN operations, it was noted that the PS pads demonstrated greater adhesion to the peritoneum and cut renal surface compared to ES pads. This enhanced bioadhesivity made unfurling the PS pads more difficult due to increased adherence to the peritoneum, but provided superior hemostasis by increasing adherence to the wound site and subsequently reducing the number of pads required to achieve hemostasis, EBL, and WIT. In agreement with previous porcine LPN studies using a similar lyophilized CS hemostatic agent produced by HemCon, Inc., the CS pads – from both treatment groups – effectively sealed the urinary collecting system. (Xie, 2008; Xie, 2012) However, since postoperative hematuria was observed in the PS pad treated animal in which the urinary collecting system was breached.
whereas uroadomen was observed in the ES pad treated animal with collecting system breach, it appears that PS pads are a better sealant.

Regardless of sterilization technique, the CS pads provided excellent hemostasis in this porcine model of LPN without additional hemostatic maneuvers. Inducing hypertension in the pigs via IV epinephrine bolus after applying the PADs led to active hemorrhaging through the CS pads in all 4 animals receiving epinephrine (2 each group), but blood loss remained low in these cases, including 3 with EBL ≤ 110 mL. Although there was a correlation between when the heart rate and blood pressure began decreasing and achievement of hemostasis, it remains unclear whether hemostasis was simply delayed by the epinephrine bolus or unable to be established until the blood pressure returned to a certain threshold. Regardless, improving the physical form of the CS, possibly to a fibrous form, may overcome the issue of bleeding through the CS agent in higher-pressure situations. Adding substances to the CS that enhance malleability may also enhance its hemostatic efficacy by improving its ease of manipulation and application.

It is important to note that pigs clot faster than humans due to higher levels of clotting factors V, VIII, IX, XI, and XII, which suggests that CS pads might not be sufficient when used alone in human LPN procedures. (Roussi, 1996) However, the aforementioned porcine LPN studies found that CS achieved hemostasis in porcine LPN procedures even after large doses of heparin were given preoperatively. (Xie, 2008; Xie, 2012) These data suggest that CS may in fact be effective as a lone hemostatic agent in humans as well. Alternatively,
improving the application techniques may prevent hemorrhage under hypertensive conditions.

It is unclear whether the marginal resorption of the pads observed after 14 weeks poses a potential problem. The HemCon CS hemostatic agent demonstrated a similarly slow resorption at 6 months and 1 year after porcine LPN and both the tissue reactivity and inflammatory response to the HemCon CS was similar to the reaction induced by Surgicel/Tisseel. (Xie, 2012) The CS pads used in the present study elicited tissue and inflammatory reactions similar to those observed with HemCon CS and Surgicel/Tisseel. Additional studies will focus on the significance of the germinal centers observed within the inflammatory cell matrix and time-course of the inflammatory reaction that is generated.

Although there was little tissue and immune reactivity to the CS in the kidney parenchyma at the 98-day mark, this could change over time since foreign bodies induce a chronic inflammatory environment. (Anderson, 2001) Thus, a physical form of CS that is resorbed within weeks or months might be ideal. In addition to alterations in physical form that may accelerate resorption, chemical alterations may be important as well. For example, a previous study in rats found that CS films with degree of deacetylation (DD) of 68.8% resorbed significantly faster than CS films with DDs ≥ 73.3%. (Tomihata, 1997) An in vitro study analyzing degradation of CS films by lysozyme recapitulated these findings by showing >50% reduction in mass of CS films after 4 weeks with DDs of 30-70%, but minimal degradation of CS films with DDs of >70%. (Freier, 2005) The CS
used in the present study has a DD of approximately 80% suggesting the DD may be responsible for the marginal resorption observed in the current study. Notably, the HemCon CS has a similar DD. (Xie, 2008)

Although zero ischemia approaches to LPN and robotic partial nephrectomy (RPN) are in development with promising results, hemostatic agents will remain a critical tool in the performance of these operations. (Lamoshi, 2015) For example, Gill and colleagues report using a Surgicel bolster with their zero ischemia RPN. (Gill, 2011) Perhaps a CS bolster would provide superior hemostatic control compared to the oxidized regenerated cellulose bolsters currently used making a complicated procedure like zero ischemia LPN and RPN procedures easier to perform and therefore achievable for more surgeons.

Weaknesses of the present study include the small number of animals and resultant small number of statistically significant findings. Use of a porcine model is also a potential weakness since renal vessels in the pig are generally smaller than human renal vessels and pigs have higher levels of clotting factors. These issues were addressed to some degree through induction of hypertension via epinephrine bolus. Since major collecting system breach was only performed in 2 animals, the ability of CS to seal the collecting system could be called into question. However, when the kidney is cut transversely, there will be open collecting tubules that must be sealed to prevent urinoma. Thus, it is unequivocal that CS sufficiently seals the proximal parts of the collecting system. Since serum CS antibody titers were unable to be assessed due to absence of a commercially
available anti-CS antibody, the ability to determine whether chitosan results in a significant systemic inflammatory response could not be assessed. Finally, without a comparison to gold standard hemostatic agents, the true hemostatic efficacy of CS for LPN cannot be adequately assessed. However, the data provide sufficient evidence to support additional studies of plasma-sterilized CS as a hemostatic agent for use in LPN procedures and provide proof-of-concept data for the efficacy of plasma sterilization and depyrogenation of CS. Several alterations to the CS may enhance its efficacy for LPN such as larger and or thinner pad sizes, use as a bolster, or even a different physical or chemical form entirely. Future studies will compare CS to currently used hemostats (e.g. FloSeal, Surgicel, and GelFoam) and will explore other physical forms of CS.

Conclusion

A 100% CS pad used alone is an effective hemostatic agent for LPN procedures and NtNP effectively sterilizes and depyrogenates CS while preserving or enhancing its in vivo hemostatic properties. Implantation of the CS pad, regardless of sterilization technique, did not produce complications and elicited tissue reactivity characteristic of foreign body implants. Marginal resorption of the CS pad was observed after 14 weeks with ES pads appearing further resorbed than PS pads. Future studies to further develop CS – NtNP-sterilized CS especially – for LPN are warranted.
Acknowledgements

Research reported in this publication was supported by the National Cancer Institute of the National Institutes of Health under SBIR award number R43CA186374. This grant was awarded to Scion Cardio-Vascular, Inc. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The authors express their gratitude to Audomaro Flores, Zachary Downs, and Dr. David Wolf, DVM for their assistance with the animal studies. The authors also thank Jackie Knecht and Louis Rose for administrative assistance. The authors are grateful to Demetri Chrysostomou, Michael Barden, Luke Turalitsch, Eduardo Mateo, and James Bond for assistance with the IoN40 plasma instrument.
References


Gill IS, Eisenberg MS, Aron M, Berger A, Ukimura O, Patil MB, Campese V,
CHAPTER SIX

DISCUSSION

In an effort to overcome the major barrier preventing translation of numerous promising clinical applications of CS, we hypothesized that NtNP would effectively sterilize and depyrogenate CS while preserving its biological properties.

To test our hypothesis, we first micronized CS via cryo-milling in order to maximize the surface to volume ration and to enhance the solubility of the raw CS flake we obtained from Scion Biomedical, Inc. (Chapter 2). We found that cryo-jet milling was superior to cryo-ball milling in reducing the particle size of CS. Although cryo-ball milling might be capable of generating similar mean and D95 values with alternative parameters, we used cryo-jet milled CS powder for all our future studies since it outperformed cryo-ball milling in our studies. Additionally, concerns were raised regarding potential metal contamination in the CS when micronized via cryo-ball milling since metal balls are used under very physically strenuous conditions and may shed metal particles during the milling procedure. Cryo-jet milling avoids this problem all together.

Since the biological properties of CS are dependent on its physico-chemical properties, we next characterized the physico-chemical properties of the CS flake and micronized CS powder. Cryo-jet milling produced a substantial reduction in the viscosity, and thus the MW of CS. This was expected since the raw CS flake is a very large MW and single chain breaks in the CS polymeric chain would be expected to reduce the MW quite substantially. We were,
however, surprised to find that DD was reduced by 9.6% after cryo-jet milling and the chemical spectra of CS had a shift at 1100 cm$^{-1}$. We hypothesize that these changes occur due to chain breaks that lead to alterations in the functional groups in CS and subsequently to chemical reactions. However, other mechanisms, like heat-induced caramelization of the CS, are possible. Heating of a cryogenically cooled CS might seem counterintuitive, but studies have shown that very high temperatures are generated for very short times ($< 10^{-6}$ s) during high-force collisions and micronization of materials, making this a plausible explanation. (Rumpf, 1973; Weichert, 1976). The changes we observed in the physico-chemical properties of CS did not alter the drug delivery efficacy of CS in the mouse model of bladder cancer (Chapter 3). This is a very significant finding since one of the primary goals of this project was to demonstrate preserved biologic properties of CS after NtNP sterilization.

Our results not only demonstrated preservation of the biologic functionality of cryomilled CS, but also of NtNP-sterilized CS. (Chapter 3) Notably, NtNP sterilization further reduced the DD, viscosity, and MW of CS, likely through mechanisms similar to those discussed above for cryo-milling, but preserved its bioadhesive properties. The fact that the bioadhesive properties of CS were preserved with NtNP sterilization suggests that the cationic charge of the CS was unchanged. It follows that the drug delivery properties of CS are dependent on the cationic charge and not so much on MW and DD. However, other studies suggest that reductions in MW and DD would eventually affect the drug delivery properties of CS once some unknown threshold is reached. (Roy, 2010)
Regardless, the finding that CS’s biologic properties are retained despite reductions in DD and MW is a very important observation since significant fluctuations in the MW and DD of CS can occur based on the individual crabs from which the chitin was extracted. (Kumirska, 2011) Thus, some fluctuations might be acceptable so long as minimum thresholds, in terms of MW and DD, are not crossed. Additional studies are needed to identify these thresholds.

Our studies on endotoxins were difficult to interpret. We found that endotoxin standards are not reliable, which dramatically reduced our ability to identify the optimal NtNP parameters for pyrogen inactivation. This conclusion is supported by a report on endotoxin testing of pharmaceuticals and medical devices by FDA and industry experts in endotoxin testing. (Bryans, 2004) Moreover, CS samples demonstrate highly variable endotoxin concentrations between samples. With the high between-sample variability in endotoxin concentrations and < 1-log reductions in endotoxins we were observing, it was impossible to determine with confidence the true magnitude of endotoxin reduction we were accomplishing. However, our results showed that ApP-sterilized CS consistently generated CS with endotoxins that were lower than LpP-sterilized CS. (Chapter 2) We believe this is a result of lower gas concentrations in the LpP system and reduced free radical generation in the LpP system due to superior removal of air contaminants. Thus, our future studies will focus on ApP systems.

For the purposes of this project, we decided not to further pursue optimization of NtNP parameters to reduce pyrogens in CS. The primary reason
was we found that neither the LpP nor the ApP systems we were using were suited for treating the physical forms of CS we were testing. The powder was especially difficult since it had to be packaged for plasma treatment. This prevented direct exposure to the plasma and might have prevented venting of volatilized contaminants, like the endotoxin lipopolysaccharide. It is believed that one mechanism by which plasma inactivates pyrogens, and other biological macromolecules, is through oxidation which leads to volatilization. (Von Keudell, 2010) However, if CS is packaged in a sterilization pouch where it is isolated from the gas flowing through the chamber, this mechanism might be hampered. Additionally, it is our opinion that movement of the particles during plasma treatment is needed to ensure all surfaces of the 3-dimensional structure of the CS particles are equally exposed to plasma. Thus, we have begun working with plasma engineers at North Carolina State University to build custom plasma instruments that will be specifically designed for treating CS powder and textiles.

In spite of our difficulty with reducing endotoxins > 1 log – we intended to achieve ≥ 3-log reduction – we were able to generate a CS hydrogel with endotoxin levels (0.02 EU/mL) that are below the regulatory threshold (0.5 EU/mL) for parenteral uses. With our mouse data demonstrating the preservation of the drug delivery properties of CS in vivo after NtNP sterilization, we believe we have a CS preparation that will be safe and effective for human use. Thus, we are currently working with the NCI on organizing a Phase I clinical trial in human bladder cancer patients.
If human trials show a positive safety profile for CS+IL-12, a large number of potential applications for CS would be enabled. For example, we have identified 3 intravesical applications in which CS would act as a sole therapeutic agent. First, based on the anti-microbial properties of CS, an intravesical injection of CS might be an effective treatment for recurrent urinary tract infections. (Chan, 2013) Second, based on the anti-oxidative properties of CS, an intravesical injection of CS before, during, and/or after pelvic irradiation might protect the bladder from the development of radiation-induced cystitis. (Crew, 2001) Third, based on the hemostatic properties of CS, an intravesical injection of CS might be an effective treatment for hemorrhagic cystitis (HC). HC is a major unsolved clinical problem with significant morbidity. (Manikandan, 2010) Beyond the bladder, mouse studies suggest CS+IL-12 might be useful for a range of other malignancies including breast, colorectal, ovarian, and pancreatic tumors. (Vo, 2014; Yang, 2013; Zaharoff, 2010; Hurteau, 2001; Lenzi, 2002) We believe human trials testing CS+IL-12 for the treatment of these malignancies are indicated and are now enabled by NtNP-sterilized CS. The tremendous pre-clinical literature on CS suggests it might be an effective delivery vehicle for a range of drugs and biologicals.

Our mouse studies (Chapter 3) showed that CS+IL-12 might be an effective vaccine for individuals at high risk of developing bladder tumors since tumor establishment rates were significantly lower in mice treated with CS+IL-12 prior to tumor cell instillation than treatment-naïve mice. On the other hand, widespread adoption of such a vaccine might be stymied by the high cost of
producing recombinant human proteins for therapeutic use. On the other hand, CS should be investigated more generally as a replacement for aluminum-based vaccine adjuvants. Numerous studies have shown the superiority of CS adjuvants over conventional adjuvants. (Li, 2015; Mori, 2012; Powell, 2015; Villiers, 2009; Vo, 2014; Yang, 2013; Zaharoff, 2007) Additionally, CS may enable non-invasive vaccines that are delivered via the nasal mucosa or even orally. (Illum, 1994; Xia, 2015)

The other clinical application of CS we sought to advance toward clinical testing in this set of studies was an implantable hemostatic agent. Our studies in a rat femoral bleeding model (Chapter 4) reinforced the exceptional hemostatic efficacy of CS. Though these studies failed to produce statistically significant findings in terms of the impact of sterilization procedures on CS, they unequivocally showed that CS is superior to standard gauze with pressure in achieving hemostasis. There was also a trend that suggested hemostatic efficacy of NtNP-sterilized CS is superior to e-beam sterilized CS. Unexpectedly, our results suggest that a combination approach to sterilization, in which CS is treated with both e-beam irradiation and NtNP, might be ideal for preserving the full hemostatic potential of CS since this combination consistently induced hemostasis at the same rate as unsterilized CS. Further studies are needed to confirm the validity of these findings and the mechanism by which this phenomenon occurs.

Our large animal hemostasis study (Chapter 5), based on porcine LPN, strengthened the non-significant trend observed in the rat study (Chapter 4) that
NtNP-treated CS has superior hemostatic properties compared to e-beam sterilized CS. More importantly, the long-term nature of this study provides preliminary evidence that NtNP-sterilized CS can be safely implanted without causing an infection or pyrogenic response. These results are in agreement with two previous porcine LPN studies that showed a similar CS hemostatic agent, the HemCon Bandage, is effective in controlling bleeding during LPN, seals the urinary collecting system, and does not cause significant adverse tissue reactivity. (Xie, 2008; Xie, 2012) This pilot study provides sufficient evidence for moving forward with developing an implantable NtNP-sterilized CS hemostatic agent for LPN and a range of other surgical operations in which hemostasis is technically challenging. However, our results also suggest that an improved physical formulation of CS, perhaps a fabric or excelsior-type form, may enhance the hemostatic efficacy and the rate of resorption after implantation. Since the CS pads used in the present study only showed minimal resorption, it might be necessary to use a CS with a lower DD or reduce the DD with a rinse after the hemostatic agent has been applied to the surgical site since CS films with lower DDs resorb significantly faster than CS films with higher DDs. (Tomihata, 1997)

**Conclusions and Future Directions**

Our primary goal currently is more clinical translation than basic science. We intend to produce enough NtNP-sterilized CS of GMP quality to enable the NCI to move forward with human trials to assess the safety and efficacy of CS+IL-12 in treating non-muscle invasive bladder cancer. Scientifically, our
future experiments will begin with characterizing the aforementioned custom-built plasma instrument(s) in terms of depyrogenation capabilities and effects on physico-chemical properties of CS. Our goal will be to achieve a minimum 3-5 log reduction in endotoxins since similar results have been shown by other groups. (Shintani, 2007; Park, 2007; Von Keudell, 2010) We also seek to reduce the sterilization dose from 15 minutes to 5 minutes or less. One of our primary goals will be to identify the key factors in achieving high depyrogenation rates with non-thermal plasmas. Finally, we plan to initiate additional animal studies assessing the hemostatic efficacy of different physical forms of NtNP-sterilized CS in other surgical operations, including those on the spleen, liver, and brain. For LPN procedures, we plan to compare CS to other currently-used hemostatic agents like FloSeal, GelFoam, and Surgicel in terms of hemostatic efficacy and tissue reactivity after implantation.
REFERENCES


Bennett BL, Littlejohn LF, Kheirabadi BS, Butler FK, Kotwal RS, Dubick MA, et al. Management of External Hemorrhage in Tactical Combat Casualty Care:


Cheever MA. Twelve immunotherapy drugs that could cure cancers. Immunol Rev. 2008;222:357-68.

Chen X, Oppenheim JJ, Howard OM. BALB/c mice have more CD4+CD25+ T regulatory cells and show greater susceptibility to suppression of their CD4+CD25- responder T cells than C57BL/6 mice. Journal of leukocyte biology. 2005;78:114-21.


Garcia-Pelayo MC, Bachy VS, Kaveh DA, Hogarth PJ. BALB/c mice display more enhanced BCG vaccine induced Th1 and Th17 response than C57BL/6 mice but have equivalent protection. Tuberculosis. 2015;95:48-53.


