ON THE PHYSICOCHEMICAL CHARACTERISTICS AND BIOLOGICAL EFFECTS OF DERIVATIZED FULLERENE AND SYNTHESIS BY-PRODUCTS

By

PAUL ANTHONY INDEGLIA

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2011
To all life adversely affected by the deficiencies of human society and to those eternally searching for the truth.
ACKNOWLEDGMENTS

I would like to express my sincerest gratitude to my advisor Dr. Jean-Claude Bonzongo for his guidance in this effort. I would like to acknowledge Dr. Angela Lindner for all that she has done and Dr. Spyros Svoronos for his unwavering support. Appreciation is extended to Dr. Brij Moudgil and Dr. Dmitry Kopelevich for spurring insight on technical, academic, and professional matters. Special thanks are extended to Dr. Gabriel Bitton, Dr. Paul Chadik, Dr. Joseph Delfino, Dr. Angelina Georgieva, Dr. Vijay Krishna, Dr. Kevin Powers, and Dr. William Wise for their encouragement and assistance. I would like to acknowledge Dr. Nancy Denslow and Dr. David Barber of the University of Florida Center for Environmental and Human Toxicology for their inspiration and intellectual stimulation. I would also like to acknowledge the University of Florida Department of Environmental Engineering Sciences, Dr. Ben Koopman, and the University of Florida Graduate School for financial support.

Appreciation is extended to University of Florida Department of Environmental Engineering Sciences staff members Ms. Amber Atteberry and Mr. Randy Switt and University of Florida Particle Engineering Research Center staff members Mr. Greg Norton and Mr. Gary Scheiffele for their assistance throughout the course of this study. I am thankful to the members of the Water Reclamation Facility, the technicians at the Interdisciplinary Center for Biological Research, especially Mr. Neal Benson and Ms. Karen Kelly, and the team at the High Performance Computer Laboratory for their help, both technical and logistical.

I also gratefully acknowledge my colleagues Dr. Natalie Magill, Dr. Julie Vernon, Mr. Sejin Youn, Mr. Jorge Gomez- Moreno, Mr. Paul Carpinone, and Ms. Casey Lamarche for their valuable contributions.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>ACKNOWLEDGMENTS</th>
<th>.................................................................</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF TABLES</td>
<td>..................................................................................</td>
<td>8</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>..................................................................................</td>
<td>9</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>..................................................................................</td>
<td>13</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>..................................................................................</td>
<td>20</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 INTRODUCTION</td>
<td>..................................................................................</td>
<td>23</td>
</tr>
<tr>
<td>Background</td>
<td>..................................................................................</td>
<td>23</td>
</tr>
<tr>
<td>Synthesis of Derivatized Fullerene</td>
<td>..................................................................................</td>
<td>25</td>
</tr>
<tr>
<td>Physicochemical Characterization of Derivatized Fullerene</td>
<td>..................................................................................</td>
<td>26</td>
</tr>
<tr>
<td>Biological Effects of Derivatized Fullerene</td>
<td>..................................................................................</td>
<td>29</td>
</tr>
<tr>
<td>Research Methodology</td>
<td>..................................................................................</td>
<td>32</td>
</tr>
<tr>
<td>2 PHYSICOCHEMICAL CHARACTERIZATION OF nC&lt;sub&gt;60&lt;/sub&gt; AND SYNTHESIS BY-PRODUCTS</td>
<td>..................................................................................</td>
<td>37</td>
</tr>
<tr>
<td>Background</td>
<td>..................................................................................</td>
<td>37</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>..................................................................................</td>
<td>38</td>
</tr>
<tr>
<td>Chemicals</td>
<td>..................................................................................</td>
<td>38</td>
</tr>
<tr>
<td>aqu-nC&lt;sub&gt;60&lt;/sub&gt; Preparation</td>
<td>..................................................................................</td>
<td>38</td>
</tr>
<tr>
<td>Concentration Determination</td>
<td>..................................................................................</td>
<td>39</td>
</tr>
<tr>
<td>Physicochemical Characterization</td>
<td>..................................................................................</td>
<td>40</td>
</tr>
<tr>
<td>Statistical Analysis of Physicochemical Characterization</td>
<td>..................................................................................</td>
<td>42</td>
</tr>
<tr>
<td>Results and Discussion</td>
<td>..................................................................................</td>
<td>43</td>
</tr>
<tr>
<td>Concentrations of nC&lt;sub&gt;60&lt;/sub&gt; in prepared suspensions</td>
<td>..................................................................................</td>
<td>43</td>
</tr>
<tr>
<td>Agglomerate Size Analysis</td>
<td>..................................................................................</td>
<td>45</td>
</tr>
<tr>
<td>pH Analysis</td>
<td>..................................................................................</td>
<td>48</td>
</tr>
<tr>
<td>Zeta Potential</td>
<td>..................................................................................</td>
<td>52</td>
</tr>
<tr>
<td>Chemical Composition Analysis</td>
<td>..................................................................................</td>
<td>54</td>
</tr>
<tr>
<td>Ultraviolet/visible light spectroscopy</td>
<td>..................................................................................</td>
<td>54</td>
</tr>
<tr>
<td>Fourier-transform infrared spectroscopy</td>
<td>..................................................................................</td>
<td>56</td>
</tr>
<tr>
<td>X-ray photoelectron spectroscopy</td>
<td>..................................................................................</td>
<td>58</td>
</tr>
<tr>
<td>Formation of derivatized nC&lt;sub&gt;60&lt;/sub&gt;</td>
<td>..................................................................................</td>
<td>61</td>
</tr>
<tr>
<td>By-products of aqu-nC&lt;sub&gt;60&lt;/sub&gt; Preparation</td>
<td>..................................................................................</td>
<td>79</td>
</tr>
<tr>
<td>Summary</td>
<td>..................................................................................</td>
<td>80</td>
</tr>
</tbody>
</table>
3 PHYSICOCHEMICAL CHARACTERIZATION OF FULLERENOL AND FULLERENOL SYNTHESIS BY-PRODUCTS ................................................................. 112

Background .................................................................................................................. 112
Materials and Methods ............................................................................................... 113
  Chemicals .................................................................................................................. 113
  Fullerol Synthesis ..................................................................................................... 114
  Physicochemical Characterization ........................................................................... 114
  Statistical Analysis of Physicochemical Characterization ........................................ 116
Results and Discussion ................................................................................................. 116
  Agglomerate Size Analysis ...................................................................................... 116
  Zeta Potential Analysis ........................................................................................... 118
  Ultraviolet/visible Light Spectroscopy Analysis ....................................................... 120
  Chemical Composition Analysis - Fullerol ............................................................... 122
  Chemical Composition Analysis – By-Products ....................................................... 127
Summary ....................................................................................................................... 132

4 BIOLOGICAL EFFECTS OF FULLERENE SPECIES ON SELECT INDICATOR SPECIES ................................................................................................................. 148

Background .................................................................................................................. 148
Materials and Methods ............................................................................................... 150
  Chemicals .................................................................................................................. 150
  Preparation of nC60 and Fullerol .............................................................................. 150
  Analytes .................................................................................................................... 151
  Treatment Concentrations ....................................................................................... 152
  Bacterial Chronic Toxicity Assays ........................................................................... 153
  Bacterial Cell Membrane Integrity Assays ............................................................... 154
  Algal Chronic Toxicity Assays ............................................................................... 155
  Invertebrate Acute Toxicity Assays ........................................................................ 156
Results .......................................................................................................................... 158
  Bacterial Chronic Toxicity Assays – Pseudomonas aeruginosa ................................ 158
  Bacterial Chronic Toxicity Assays – Staphylococcus aureus .................................... 160
  Bacterial Cell Membrane Integrity Assays ............................................................... 162
  Algal Chronic Toxicity Assays ............................................................................... 163
  Invertebrate Acute Toxicity Assays ........................................................................ 165
Discussion ...................................................................................................................... 167
  Differential Biological Responses among Fullerene Species ................................... 170
    nC60-14L ................................................................................................................. 170
    nC60-28L ................................................................................................................. 175
    Laboratory-prepared fullerol .................................................................................. 180
  Dark and Light Assays ............................................................................................. 183
    Bacterial cell membrane integrity assays ............................................................... 183
    Algal chronic toxicity assays ............................................................................... 190
    Invertebrate acute toxicity assays ........................................................................ 193
  Commercially-available and Laboratory-prepared Fullerol ...................................... 195
  Fullerol Synthesis By-products and Reactants ......................................................... 198
Summary ........................................................................................................................................199

5 CONCLUSIONS AND FUTURE RESEARCH AVENUES ..................................................218

Conclusions ..............................................................................................................................218
Future Research Avenues .......................................................................................................222

APPENDIX

A CONFIRMATION OF RAW MATERIAL PURITY .................................................................225

Manufacturer Data .................................................................................................................225
Raman and Fourier-transform Infrared Spectroscopy ............................................................226
Quantum Mechanical Simulation .........................................................................................228

B DESCRIPTION OF SURFACE GROUPS ...........................................................................234

C INTERACTIONS CONTROLLING FULLERENE AGGREGATION ..................................237

D BIOLOGICAL SPECIES .....................................................................................................243

LIST OF REFERENCES ..............................................................................................................249

BIOGRAPHICAL SKETCH ......................................................................................................278
<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>Known producers of fullerol-containing products</td>
<td>34</td>
</tr>
<tr>
<td>1-2</td>
<td>Reported toxicity results for fullerene species</td>
<td>35</td>
</tr>
<tr>
<td>2-1</td>
<td>Kinetics parameters for $nC_{60}$ formation in aqueous dispersant</td>
<td>84</td>
</tr>
<tr>
<td>2-2</td>
<td>Mass balance analysis for $nC_{60}$ synthesis</td>
<td>84</td>
</tr>
<tr>
<td>2-3</td>
<td>Numerical results from TEM image mean particle size analysis</td>
<td>84</td>
</tr>
<tr>
<td>2-4</td>
<td>Mean agglomerate size for $nC60$ determined through DLS and TEM</td>
<td>85</td>
</tr>
<tr>
<td>2-5</td>
<td>Intramolecular vibrational modes $nC_{60}$ and their symmetries</td>
<td>86</td>
</tr>
<tr>
<td>2-6</td>
<td>Quantification of $nC_{60}$ oxygenated carbon states as determined via XPS</td>
<td>87</td>
</tr>
<tr>
<td>2-7</td>
<td>Exponential parameters for $nC_{60}$ surface derivatization kinetics for water-stirred fullerene ($aqu-nC_{60}$)</td>
<td>87</td>
</tr>
<tr>
<td>3-1</td>
<td>Summary of degrees of derivatization of laboratory-prepared fullerol, commercially-available fullerol, and fullerol synthesis by-products.</td>
<td>135</td>
</tr>
<tr>
<td>4-1</td>
<td>Summary of estimated toxicity thresholds for select organisms exposed to fullerene species, synthesis by-products, and synthesis reactants.</td>
<td>202</td>
</tr>
<tr>
<td>A-1</td>
<td>Comparison of Raman ($A_g$ and $H_g$ modes) and FTIR ($T_{1u}$ modes) vibrational mode peaks from experimental and computer modeling.</td>
<td>231</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>Wire-structure diagram of fullerene</td>
<td>36</td>
</tr>
<tr>
<td>2-1</td>
<td>Electrical double-layer interfacial interaction model adapted from Stern (161) and Grahame (162)</td>
<td>88</td>
</tr>
<tr>
<td>2-2</td>
<td>Aliquots of $nC_{60}$ taken over 28-day period</td>
<td>88</td>
</tr>
<tr>
<td>2-3</td>
<td>Kinetics relationship for $nC_{60}$ formation in aqueous dispersant as a function of time</td>
<td>89</td>
</tr>
<tr>
<td>2-4</td>
<td>Number-weighted mean agglomerate size for $nC_{60}$ samples as a function of time of unfiltered material</td>
<td>89</td>
</tr>
<tr>
<td>2-5</td>
<td>Number-weighted mean agglomerate size for $nC_{60}$ samples as a function of time of filtered material</td>
<td>90</td>
</tr>
<tr>
<td>2-6</td>
<td>Number-weighted agglomerate size distribution for filtered $nC_{60}$ samples at $t = 4, 20,$ and 28 days</td>
<td>90</td>
</tr>
<tr>
<td>2-7</td>
<td>Transmission electron microscope images of $nC_{60}$</td>
<td>91</td>
</tr>
<tr>
<td>2-8</td>
<td>pH of unfiltered $nC_{60}$ stirred for 28 days in dark and light as a function of time</td>
<td>91</td>
</tr>
<tr>
<td>2-9</td>
<td>pH of unfiltered $nC_{60}$ stirred for 28 days in dark and light as a function of time</td>
<td>92</td>
</tr>
<tr>
<td>2-10</td>
<td>Zeta potential for unfiltered $nC_{60}$ samples as a function of time</td>
<td>92</td>
</tr>
<tr>
<td>2-11</td>
<td>Zeta potential for filtered $nC_{60}$ samples as a function of time</td>
<td>93</td>
</tr>
<tr>
<td>2-12</td>
<td>UV/Vis spectrum of unfiltered $nC_{60}$ stirred in dark at $t = 6, 12, 20,$ and 28 days</td>
<td>93</td>
</tr>
<tr>
<td>2-13</td>
<td>UV/Vis spectrum of filtered $nC_{60}$ stirred in dark at $t = 6, 12, 20,$ and 28 days</td>
<td>94</td>
</tr>
<tr>
<td>2-14</td>
<td>UV/Vis spectrum of unfiltered $nC_{60}$ stirred in light at $t = 6, 12, 20,$ and 28 days</td>
<td>94</td>
</tr>
<tr>
<td>2-15</td>
<td>UV/Vis spectrum of filtered $nC_{60}$ stirred in light at $t = 6, 12, 20,$ and 28 days</td>
<td>95</td>
</tr>
<tr>
<td>2-16</td>
<td>UV peak at $\lambda = 360$ nm for unfiltered $nC_{60}$ as a function of time and photo-condition</td>
<td>95</td>
</tr>
<tr>
<td>2-17</td>
<td>UV peak at $\lambda = 360$ nm for filtered $nC_{60}$ as a function of time and photo-condition</td>
<td>96</td>
</tr>
<tr>
<td>2-18</td>
<td>Ratio of UV to visible peaks for unfiltered $nC_{60}$ as a function of time and photo-condition</td>
<td>96</td>
</tr>
</tbody>
</table>
Ratio of UV to visible peaks for filtered nC₆₀ as a function of time and photo-condition .................................................................97

FTIR spectra for 7-day dark and light water-stirred fullerene samples .................................................................98

FTIR spectra for 14-day dark and light water-stirred fullerene samples ...............................................................99

FTIR spectra for 28-day dark and light water-stirred fullerene samples .............................................................100

Surface groups potentially associated with water-stirred fullerene ...............................................................101

XPS multiplex summary data on chemical composition for water-stirred fullerene samples .................................................................102

XPS spectra for 7-day dark and light water-stirred fullerene samples .................................................................103

XPS spectra for 14-day dark and light water-stirred fullerene samples .................................................................104

XPS spectra for 28-day dark and light water-stirred fullerene samples .................................................................105

Kinetics of surface group formation for aqu-nC₆₀ stirred in dark and light for 28 days ..................................................106

Simplified two-dimensional representation of aqu-nC₆₀ agglomerate in aqueous medium .................................................................107

Agglomerate of the primary agglomerate group for C₆₀ comprised of 13 molecules ..................................................108

Proposed pathway for aqu-nC₆₀ surface group formation ......................................................................................108

Alternative pathway for aqu-nC₆₀ surface group formation ..................................................................................109

An nC₆₀ agglomerate with hydrophilic, derivatized surface molecules surrounding underivatized, hydrophobic C₆₀ molecules ..............................................................................................109

FTIR spectra of by-products for 14-day light and 28-day light water-stirred fullerene samples .................................................................110

XPS spectra of by-products for 14-day light and 28-day light water-stirred fullerene samples .................................................................111

Graphical depiction of fullerenol synthesis by “modified” Kitazawa method ..............................................................136

Agglomerate size distribution of fullerenol synthesis by “modified” Kitazawa method ..............................................................136

Agglomerate size distribution of fullerenol synthesized by “modified” Kitazawa method ..............................................................137
LIST OF ABBREVIATIONS

14D  14-day, dark-stirred aqu-nC\textsubscript{60} samples
14L  14-day, light-stirred aqu-nC\textsubscript{60} samples
\( ^1 \text{C}_{60} \)  singlet (ground-state) fullerene
\( ^1 \text{O}_2 \)  singlet oxygen
28D  28-day, dark-stirred aqu-nC\textsubscript{60} samples
28L  28-day, light-stirred aqu-nC\textsubscript{60} samples
\( ^3 \text{C}_{60} \)  triplet fullerene
7D   7-day, dark-stirred aqu-nC\textsubscript{60} samples
7L   7-day, light-stirred aqu-nC\textsubscript{60} samples
\( \alpha \)  type-1 statistical error
A    absorbance, Hamaker’s constant
\( \text{aqu-nC}_{60} \)  water-stirred nC\textsubscript{60}
AOAC Association of Analytical Communities
ATCC American Type Culture Collection
\( n\text{C}_{60} \)  nano-[60]fullerene
BP #1 fullerol synthesis by-product #1
BP #2 fullerol synthesis by-product #2
BP #3 fullerol synthesis by-product #3
C    intermolecular force constant, concentration
\( \chi^2 \)  chi-square goodness-of-fit parameter
cm   centimeter
\( \Delta E \)  bonding energy differential
**LIST OF ABBREVIATIONS** (continued)

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>diffusion coefficient</td>
</tr>
<tr>
<td>D-ala</td>
<td>D-alanine</td>
</tr>
<tr>
<td>D-fuc</td>
<td>D-fucose</td>
</tr>
<tr>
<td>D-gal</td>
<td>D-galactose</td>
</tr>
<tr>
<td>D-glu</td>
<td>D-glucose</td>
</tr>
<tr>
<td>D-man</td>
<td>D-mannose</td>
</tr>
<tr>
<td>D-Neu</td>
<td>β-D-Neu5Ac, or sialic acid</td>
</tr>
<tr>
<td>D-xyl</td>
<td>β-D-xylose</td>
</tr>
<tr>
<td>d_h</td>
<td>mean particle diameter</td>
</tr>
<tr>
<td>DLS</td>
<td>dynamic light scattering</td>
</tr>
<tr>
<td>DLVO</td>
<td>Derjaguin, Landau, Verwey, and Overbeek</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>e</td>
<td>electron charge</td>
</tr>
<tr>
<td>ε_r</td>
<td>relative permittivity</td>
</tr>
<tr>
<td>ε_0</td>
<td>permittivity of free space</td>
</tr>
<tr>
<td>EC##</td>
<td>concentration resulting in adverse effect on ##% of the population</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>E_i</td>
<td>input energy</td>
</tr>
<tr>
<td>ESCA</td>
<td>electron spectroscopy for chemical analysis</td>
</tr>
<tr>
<td>eV</td>
<td>electron-volt</td>
</tr>
<tr>
<td>F_g</td>
<td>gravitational force</td>
</tr>
<tr>
<td>F_H</td>
<td>van der Waals force</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier-transform infrared</td>
</tr>
<tr>
<td>fullerenol-PAI</td>
<td>laboratory-prepared fullerenol from Nano-C stock C_{60}</td>
</tr>
<tr>
<td>fullerenol-CA</td>
<td>commercially-available fullerenol</td>
</tr>
<tr>
<td>γ</td>
<td>surface energy, surface tension</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>g</td>
<td>force due to gravity</td>
</tr>
<tr>
<td>G</td>
<td>gravitational force constant</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>ℏν</td>
<td>photon</td>
</tr>
<tr>
<td>H-NMR</td>
<td>proton nuclear magnetic resonance</td>
</tr>
<tr>
<td>H₀</td>
<td>distance separating two interacting particles</td>
</tr>
<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
</tr>
<tr>
<td>ICP</td>
<td>inductively coupled plasma</td>
</tr>
<tr>
<td>I</td>
<td>intensity, ionization potential</td>
</tr>
<tr>
<td>Iₕ</td>
<td>icosahedral symmetry</td>
</tr>
<tr>
<td>IUPAC</td>
<td>International Union of Pure and Applied Chemistry</td>
</tr>
<tr>
<td>J</td>
<td>Joule</td>
</tr>
<tr>
<td>κ</td>
<td>Debye length</td>
</tr>
<tr>
<td>k_B</td>
<td>Boltzmann’s constant</td>
</tr>
<tr>
<td>kcal</td>
<td>kilocalorie</td>
</tr>
<tr>
<td>kₑ</td>
<td>Coulomb’s proportionality constant</td>
</tr>
<tr>
<td>K_{EM}</td>
<td>eustressed mortality constant</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram</td>
</tr>
<tr>
<td>(\lambda)</td>
<td>wavelength</td>
</tr>
<tr>
<td>L</td>
<td>liter</td>
</tr>
<tr>
<td>L-ala</td>
<td>L-alanine</td>
</tr>
<tr>
<td>L-cys</td>
<td>L-cysteine</td>
</tr>
<tr>
<td>L-his</td>
<td>L-histidine</td>
</tr>
<tr>
<td>L-val</td>
<td>L-valine</td>
</tr>
<tr>
<td>LC##</td>
<td>concentration to kill ##% of the population</td>
</tr>
<tr>
<td>(\mu)</td>
<td>mean; growth rate constant</td>
</tr>
<tr>
<td>(\mu_E)</td>
<td>eustressed growth rate constant</td>
</tr>
<tr>
<td>(\mu\text{E})</td>
<td>microilluminance</td>
</tr>
<tr>
<td>(\mu\text{g})</td>
<td>micrograms</td>
</tr>
<tr>
<td>(\mu\text{m})</td>
<td>micron or micrometer</td>
</tr>
<tr>
<td>m</td>
<td>mass</td>
</tr>
<tr>
<td>M</td>
<td>mass, molar</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix-assisted laser desorption/ionization</td>
</tr>
<tr>
<td>MeOH</td>
<td>methanol</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>MIC</td>
<td>minimum inhibitory concentration</td>
</tr>
<tr>
<td>mL</td>
<td>milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>mol</td>
<td>mole</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS (continued)

mRNA  messenger ribonucleic acid
mV    millivolt
n     number of interactions
n     number of moles, number of samples
N     normal
NaCl  sodium chloride
NAG   β-D-N-acetylglucosamine
NAM   β-D-N-acetylgalactosamine
NaOH  sodium hydroxide
nm    nanometer
P     pressure
PAAP  preliminary algal assay procedure
pH    based-10 log of the activity of hydrogen ions in a solution
pm    picometer
PMDA  pyromettalic dianhydride
ppm   parts per million
PTFE  polytetrafluoroethylene
ρ     density, number of atoms within interacting molecules
r     distance from the center of interacting bodies
R     particle radius
R²    coefficient of determination
Rₜₙ UV/Vis spectrometric peak ratio
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>$\sigma_s$</td>
<td>Grahame charge</td>
</tr>
<tr>
<td>$\Psi_s$</td>
<td>Stern potential</td>
</tr>
<tr>
<td>son-$n$C$_{60}$</td>
<td>$n$C$_{60}$ synthesized through sonication in toluene</td>
</tr>
<tr>
<td>$t$</td>
<td>time</td>
</tr>
<tr>
<td>T</td>
<td>temperature</td>
</tr>
<tr>
<td>TBAH</td>
<td>tetrabutylammonium hydroxide</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>THF-$n$C$_{60}$</td>
<td>$n$C$_{60}$ synthesized using tetrahydrofuran</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer ribonucleic acid</td>
</tr>
<tr>
<td>u</td>
<td>dipole moment</td>
</tr>
<tr>
<td>$U_r$</td>
<td>total interaction energy</td>
</tr>
<tr>
<td>USEPA</td>
<td>United States Environmental Protection Agency</td>
</tr>
<tr>
<td>USPO</td>
<td>United States Patent Office</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet light</td>
</tr>
<tr>
<td>UV/Vis</td>
<td>ultraviolet/visible light</td>
</tr>
<tr>
<td>$v_i$</td>
<td>velocity</td>
</tr>
<tr>
<td>V</td>
<td>volume</td>
</tr>
<tr>
<td>W</td>
<td>watt</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS (continued)

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$W_{cr}$</td>
<td>work, measured as a function of constant charge</td>
</tr>
<tr>
<td>$W_{\Psi r}$</td>
<td>work, measured as a function of constant potential</td>
</tr>
<tr>
<td>$W_a$</td>
<td>work of attraction</td>
</tr>
<tr>
<td>$W_c$</td>
<td>work of cohesion</td>
</tr>
<tr>
<td>WET</td>
<td>Whole Effluent Toxicity</td>
</tr>
<tr>
<td>$W_r$</td>
<td>intermolecular force</td>
</tr>
<tr>
<td>WSC</td>
<td>water stable clusters</td>
</tr>
<tr>
<td>XPS</td>
<td>X-ray photoelectron spectroscopy</td>
</tr>
<tr>
<td>$\zeta$</td>
<td>zeta potential</td>
</tr>
<tr>
<td>Z</td>
<td>valency</td>
</tr>
</tbody>
</table>
ON THE PHYSICOCHEMICAL CHARACTERISTICS AND BIOLOGICAL EFFECTS OF
DERIVATIZED FULLERENE AND SYNTHESIS BY-PRODUCTS

By

Paul Anthony Indeglia

August 2011

Chair: Jean-Claude Bonzongo
Major: Environmental Engineering Sciences

This investigation examined the physicochemical characteristics and biological effects of fullerene derivatives, \( nC_{60} \) and fullerenol \( (C_{60}H_{x}O_{y}(OH)_{z}) \), and their synthesis by-products to assess mechanisms of and factors influencing biological effects. Physicochemical analyses of derivatized fullerene included molecular and agglomerate-scale characterization by dynamic light scattering (DLS) and transmission electron scattering (TEM), surface charge through electrophoretic analysis of mobility, and chemical composition analysis using ultraviolet/visible light (UV/Vis), Fourier-transform infrared (FTIR), and X-ray photoelectron spectroscopy (XPS). Detailed analysis of \( nC_{60} \) transformation over a 28-day stirring period indicated \( nC_{60} \) agglomerate concentrations can be estimated as a function of time by using a predictor model \( (R^2 > 0.99) \). Agglomerate sizes did not differ significantly over the 28-day stirring period regardless of photocondition (DLS: \( d_{\mu} = 138.2 \) nm; TEM: \( d_{\mu} = 110.0 \) nm) although distributions were more uniform as stirring time increased. The total number of surface groups identified through XPS indicated increased derivatization as a function of time with additions assigned to mono-oxygenated carbon moieties while the number of di-oxygenated moieties declined. Early phase stirring \( (t \leq 14 \) days) products were shown through FTIR analysis to contain epoxide surface
groups, which were absent in later-phase \( (t > 14 \text{ days}) \) samples, suggesting specific pathways to derivatization with preferential mono-oxygenated states.

Fullerenol was shown to persist in a molecular state at concentrations below 20 mg/L with increased concentration and sonication causing an increase in agglomerate size. Fullerenol surface group composition varied between three independent synthesis events with the number of total derivatized carbon atoms ranging from 21 to 30 and the number of mono-oxygenated groups ranging from 5 to 20. \( nC_{60} \) agglomerates displayed greater surface derivatization than fullerenol as well as a higher percentage of mono-oxygenated groups. Filter residue, a by-product of \( nC_{60} \) synthesis, demonstrated high hydrophobicity and FTIR spectra similar to underivatized material, although \( nC_{60} \) stirred for 28-days was shown to have nine mono-oxygenated carbons and no di-oxygenated groups. By-products from fullerenol synthesis contained 21 to 30 surface groups and synthesis reactants tetrabutylammonium hydroxide and sodium hydroxide were found in all by-products. Y-products generated through a methanol rinse were shown to contain 21 mono-oxygenated groups with no-di-oxygenated moieties.

Biological assays were conducted using two different bacteria (\textit{Pseudomonas aeruginosa} and \textit{Staphylococcus aureus}), one algae (\textit{Pseudokirchneriella subcapitata}), and one invertebrate species (\textit{Ceriodaphnia dubia}) as model test organisms. The bioassays assessed the effects of derivatized fullerene materials in accordance with commonly-used and widely-accepted ecotoxicity protocols. Dark-exposure bacteria population (chronic) assays were complimented with light-exposure algae population (chronic) and daphnid mortality (acute) assays. The \( LC_{50} \) for \( nC_{60} \) against the Gram-negative bacteria \textit{P. aeruginosa} was extrapolated to be 1,336 mg/L under dark-exposure conditions; however, no toxicity thresholds were calculable for the Gram-positive \textit{S. aureus}, the algae \textit{P. subcapitata}, or the invertebrate \textit{C. dubia} exposed to \( nC_{60} \) due to
insufficient biological effect with concentrations extending to 100 mg/L. The LC$_{50}$ for bacteria exposed to fullerenol was extrapolated to be 2,409 and $>$10,000 mg/L for $P$. aeruginosa and $S$. aureus, respectively, and estimated to be 299 and 31.1 mg/L for $P$. subcapitata and $C$. dubia, respectively, suggesting fullerenol exhibited greater biological impact when exposed to light.

Biological effects from fullerenol synthesis by-products on $C$. dubia were attributed to residual synthesis reactants and photoactivity of derivatized fullerene contained within the material removed, although neither by-product analyzed caused notable toxicity to unicellular organisms. Mechanisms of cell damage on bacteria populations were investigated using nucleic acid dye assays, concluding that both apoptotic and necrotic responses occurred with and without photoactivation.
CHAPTER 1
INTRODUCTION

Background

For centuries, the world has resigned itself to the knowledge that pure carbon existed in two forms: graphite and diamonds. In 1985, a third allotrope of carbon, fullerene (Figure 1-1), a closed-cage carbon molecule, was discovered and has become a prominent focus within the field of material, biomedical, and environmental science. Since publication of an arc-vaporization production method (1) enabled facile-synthesis, researchers have been studying properties of and applications for fullerene. Fullerene materials have been used in proton conductors (2), superconductors (3), photovoltaics (4, 5), electronics (6), media storage (7), and thermal insulation (8, 9). The United States Patent Office (USPO) has registered more than 2,600 fullerene patents (10) with current production in excess of 40 tons annually (11). Derivatized fullerene has shown considerable market growth in the past decade, as reflected in the number of patents and patent applications registered with the USPO for one particular fullerene species, fullerenol (Figure 1-2).

Raw, or “underivatized”, fullerene is quite insoluble in water with a solubility product ($K_{sp}$) of $1.44 \times 10^{-11}$ mg/L, as reported by Andrievsky et al. (12), resulting in the development of numerous methodologies to increase the dispersability of fullerene through derivatization of the cage surface if fullerene. Fullerene derivatization generates aqueous and solid wastes, which can be discharged to the environment, impact living organisms, and potentially be introduced to surface potable water supplies, which may lead to human exposure. Due to the stability of the carbon cage, conventional wastewater treatment technologies are unlikely to degrade fullerene and no reports reviewed indicated fullerene species would be removed from the waste stream through physical, chemical, or biological processes. Furthermore, underivatized fullerene
processed within a water treatment facility or discharged to a receiving water body can become derivatized through mixing (13), increasing mobility as well as the potential for adverse ecological impact.

Current regulations pertaining to nanoparticles presume nanoparticles of a bulk substance behave identically to the bulk substance, unless evidence has been shown to the contrary; thus, each nanoparticle receives independent investigation and assessment, and the determination for regulation is made on a case-by-case basis. As a pure carbon substance, fullerene currently is unregulated by Federal, state, or local laws in the United States, as it is considered similar to carbon black or graphite. In the absence of legislation, researchers have recognized a potential for biological effects from fullerene species and have generated a bank of information regarding fullerene; yet, additional research is required to overcome deficiencies in the understanding of the biological effects of fullerene and its synthesis waste, or by-products, to determine whether regulation is warranted.

Fullerenol, a polyhydroxylated fullerene derivative in the general form of \( \text{C}_{60}(\text{OH})_n \), has been documented for use in a number of biological applications (14) due to its increased solubility over underivatized fullerene. The biomedical industry has developed methods to facilitate delivery of pharmaceuticals to cells by attaching them to fullerenol, which enhances delivery and absorption mechanisms (15, 16). Fullerenol has been shown to inhibit human immunodeficiency virus-1 protease production (17), while the type-C hepatitis virus undergoes programmed cell death, or apoptosis, when in direct contact with fullerenol (18). Fullerenol has been shown to inhibit glutamate-induced cell death and to lower intracellular calcium concentrations, which are thought to be responsible for some cerebral conditions such as Alzheimer’s and Parkinson’s diseases (19, 20) as well as to ameliorate alcohol impacts on brain
cells in rats (21). Research has indicated the potential for fullerenol to protect against focal necrosis of hepatocytes, vascular damage, and the deposition of fatty acids on the inside of organs (22) in addition to “Type I” hypersensitivity allergic reactions (23). Multiple biomedical companies have been exploring the potential to use fullerenol-based materials for neuroprotective effects and cancer therapy (Luna Innovations, Roanoke, Virginia) as well as potential applications as anti-allergens (Tego BioSciences, Pasadena, California).

Fullerenol has been investigated as an electron scavenger, especially in terms of photo-catalytic reactions, and has been shown to increase the capacity of the anti-microbial activity of UV-activated TiO$_2$ (24, 25). The electron scavenging capacity of fullerenol has been incorporated into personal care products, specifically skin ointments and “beauty” creams.

Several additional companies in the United States and around the world have begun distribution of personal care products containing fullerene (Table 1-1). No information has been provided by the manufacturers of fullerenol-containing personal care products regarding fate, transport, or potential adverse biological effects of the material.

**Synthesis of Derivatized Fullerene**

Taylor (26) refers to more than a dozen chemical methods for the synthesis of derivatized fullerene while other, non-chemical methods also have been documented. Nano-C$_{60}$ ($n$C$_{60}$) is a dispersed fullerene species, so named as it was thought to retain its molecular integrity (i.e. C$_{60}$). Fullerene can be stirred mechanically in water from hours up to months, forming water stable clusters of nano-C$_{60}$ (aqu-nC$_{60}$), with increasing dispersed concentrations over time, yielding concentrations as high as 3.8 mg/L (27). Deguchi et al. (28) developed a method of dispersion utilizing a catalytic solvent, tetrahydrofuran (THF), a process that has been adapted widely for its facility. THF-generated $n$C$_{60}$ (THF-$n$C$_{60}$) agglomerates were reported to have a dispersability of 9.36 mg/L. A third method of $n$C$_{60}$ derivatization is through sonication in a toluene bath (son-
$nC_{60}$), which has been reported to achieve a concentration as high as 7.2 mg/L (29). While $nC_{60}$ has been studied fairly extensively for application, few have been demonstrated and $nC_{60}$ remains used primarily for bio-assessment as a surrogate for underivatized fullerene. Greater potential for application was found with fullerenol, whereby hydroxyl groups were added to the carbon cage, creating a water-soluble and surface-active fullerene. Four primary methods of hydroxyl derivatization have been identified using hydrogen peroxide, acids, bases, and halogens as intermediaries to “reserve” binding sites on the fullerene. Once activated, a limited number of binding sites become occupied with hydroxyl groups, although other molecules may be present including ether, epoxide, and hemiketal groups as well as negatively-charge oxygen ions (30). Derivatization is completed through phase separation of derivatized fullerenes from the solvent carriers and subsequent purification of material through filtration and column separation. Although there are 60 potential carbon binding sites, a maximum of 44 hydroxyl groups have been documented to be bound to fullerene (31), resulting in a fullerene derivative with a molecular diameter on the order of 1.3 nm (32) and a solubility of 80 mg/L (29). The phase separation and purification steps generated by-products that have not been characterized previously. The physicochemical and biological character of derivatized fullerenes, both $nC_{60}$ and fullerenol, and their by-products were the focus of this research effort.

**Physicochemical Characterization of Derivatized Fullerene**

Numerous properties of fullerene species are of interest to researchers in the effort to determine the capabilities of a material including size and size distribution, surface charge, and chemical composition. Previous researchers have conducted size determinations for fullerene species through the use of dynamic light scatter (DLS) technologies (13, 29, 33-42) as well as through the use of transmission electron microscopy (TEM) (44-49) with $nC_{60}$ cluster sizes ranging from 35.1 to 211.8 nm for *aqu*-nC$_{60}$, 20 to 219 nm for THF-nC$_{60}$, and 1 to 160 nm for
Although difficult to determine due to analytical limits, fullerenol solubility has been shown to be at a molecular scale (diameter = 1.3 nm) using DLS (15, 50, 51). Both size analysis techniques were employed, where applicable, during this research in effort to resolve discrepancies as to if and how agglomerate size and size distribution changed during synthesis.

Surface charge remains a parameter that has proven difficult to assess due to the lack of an adequate analytical method to measure such a minute electrical flux on such a small particle. The most accurate methodology for predicting the charge character of a particle surface is by calculating zeta potential, or the charge at the interface of the solvent layer and the liquid, through electrophoretic mobility. The stability of an agglomerate can be assessed by use of zeta potential, with the more non-zero the zeta potential measurement corresponding to greater chemical stability. Numerous studies (37, 42, 52-54) reported zeta potential values for $nC_{60}$ in the range of -52 to -21 mV, indicating a net negative charge imparted upon the surrounding environment. Fullerenol was also shown to have a negative zeta potential, ranging from -50 to -20 mV (34, 55), and suggesting little variation between the two fullerene derivatives.

Generally, fullerene derivatives have been shown to be internally stable and, therefore, useful in biomedical applications.

Characterization of derivatized fullerene chemical composition has been shown to be a developing science. Despite publications on surface groups associated with fullerene, specifically through oxygenation (56, 60-67), definitive chemical compositions were unable to be reported. Researchers have used a variety of characterization techniques without definitively assessing the formation pathway or quantifying fullerene surface derivatization moieties. Fourier-transform infrared (FTIR) spectroscopy has been used to identify some surface groups found in association with fullerene species (47, 68-72) but cannot be used reliably to quantify
these groups. X-ray photoelectron spectroscopy (XPS) studies (13, 31, 73-76) have been used to quantify carbon oxygenation states (e.g. non-oxygenated, mono-oxygenated) but limitations in resolution prohibit differentiation of specific surface groups within a band of bond energies. Due to scarcely detectable bond energy differences between the oxygenation states (i.e. \( \Delta E < 1.5 \text{ eV} \) between non-oxygenated and mono-oxygenated carbon bond energies), computer algorithms must be employed to deconvolute XPS spectra, providing an estimate of quantification, inserting assumptions, obfuscating actual compositions of surface groups, and complicating assessment of biological effects. Even if quantified results were able to differentiate functional surface groups, due to the vast number of binding site and potential configurations, complete profiling of fullerene remains outside of current technological capacity.

Proton nuclear magnetic resonance (H-NMR) has been shown to provide information on hydrogen association with C\(_{60}\) (77-86), which is useful in discussing hydroxylated groups, but the potential for non-protonated hydrogen surface groups (87-89) makes quantification difficult. Other researchers (13, 30, 53) have employed thermogravimetric analysis to obtain estimates of surface groups as they are combusted, but overlapping regions occlude accurate results and difficulty differentiating hydroxyl groups from water requires methodologies that remain to be developed. Slanina et al. (56) conducted detailed quantum mechanical simulations to assess the lowest energy state for numerous polyhydroxylated fullerene configurations in an effort to foster understanding of surface group stability. Haddon et al. (90) noted the delocalized nature of electrons within fullerene as the factor controlling binding sites; other reports have indicated electron deficiency as an impetus for fullerene surface derivatization (91-94). Despite the efforts conducted to date, the most candid representation of the chemical composition of \( n\text{C}_{60} \) and other derivatized fullerene species is a semi-quantitative result.
Biological Effects of Derivatized Fullerene

The possibility of inorganic molecules to imitate polymer molecules at the molecular and molecular-cluster scale and alter biological development has been documented (95-98). The majority of reports reviewed pertaining to the biological effects of \( nC_{60} \) was related to material synthesized with the facilitation of THF. A debate has ensued as to whether biological effects of THF-\( nC_{60} \) are caused by residual THF associated with the fullerene or the fullerene itself; \textit{Bacillus subtilis} and \textit{Pseudomonas putida} exposed to derivatized fullerene was examined, indicating reduced metabolic activity at 0.01 mg/L THF-\( nC_{60} \) (99). Others have reported reduced membrane viability in Gram-positive \textit{B. subtilis} when exposed to 5 mg/L THF-\( nC_{60} \) but not in Gram-negative \textit{E. coli} (36). Still others have demonstrated that THF degradation products have greater adverse effects to biota than THF (101, 101), bringing into question whether derivatized fullerene species inherently pose a biological threat. Additional investigations involving invertebrates indicated that the LC\(_{50}\) for \textit{Daphnia pulex} was > 5 mg/L exposed to THF-\( nC_{60} \) and > 100 mg/L exposed to \textit{aqu-nC}_{60} (102), while the copepod \textit{Hyalella azteca} demonstrated complete mortality at 35 mg/L when exposed to \textit{aqu-nC}_{60} stirred for 60 days in light (27). Data presented in Table 1-2 show increased adverse biological effects from THF-\( nC_{60} \) over \textit{aqu-nC}_{60}, though the debate persists as to the inherent toxicity of fullerene.

While much of the research on fullerenol has focused on beneficial biomedical applications, some research has indicated the potential for adverse biological effects on human endothelial cells (111), raising questions as to whether the biological impacts caused by fullerenol need to be examined further. Biological effects on human cells from various forms of fullerene have been demonstrated (110-112), elevating scientific concern for the need to assess all possible chemical and biological mechanisms resulting in toxicity. Recently, researchers
have examined the role of light in the transformation of $nC_{60}$ and fullerenol (65-67, 76, 113) and the potential for biological and ecological impacts (114).

Fullerenol has been the subject of investigation as a stable radical anion (74) and as a facilitator in reactive oxygen species (ROS) generation within aqueous solutions (77, 81, 115, 116-118). A considerable amount of fullerenol toxicity literature deals with the cytotoxic effects of ROS and their formation in the presence of photo-activated fullerene species (64, 65, 77, 115, 119). Arbogast et al. (120) and Wang et al. (121) presented the photo-physical characteristics of C$_{60}$, noting thermionic emissions. Hwang and Mauzerall (122) demonstrated a linear relationship between applied light intensity and the transfer of a photoelectric current across a lipid bilayer in the presence of underivatized fullerene. Chiang et al. (123) proposed that water-soluble fullerene species functioned as electron collectors and, subsequently, mediators of radical species induced by ultraviolet radiation while Kamat et al. (124) indicated the ability of photo-activated C$_{60}$ to degrade proteins. Multiple researchers described the optical properties of C$_{60}$ including characterization of underivatized and polymerized fullerene (125), fullerene as a thin film (126), and highly derivatized fullerene (64). Lyon et al. (36, 127) indicated that the generation of ROS in association with fullerene species required the presence of light, which was supported by the work of Lee et al. (128) and Hotze et al. (129).

Numerous investigations on mammalian cell cultures provided insight on the potential biological effects of fullerene species on more complex life forms. Markovic (38) indicated that mouse fibrosarcoma and melanoma cells experienced higher oxidation when exposed to THF-$nC_{60}$ compared to aqu-$nC_{60}$-28L at 1 mg/L and Niwa and Iwai (130) reported an LC$_{25}$ > 0.1 mg/L for Chinese hamster ovary cells in the presence of commercially-available (MER, Inc.) fullerenol. Boutorine et al. (131) noted double-stranded DNA cleavage upon irradiation and
Mroz et al. (132) indicated cleavage of single strands of rodent nucleic acid. Toxicity data related to human cell cultures included an LC$_{50} = 0.2$ mg/L for human dermal fibroblasts exposed to THF-$n$C$_{60}$ and an LC$_{50} = 50$ mg/L exposed to aqu-$n$C$_{60}$ (110), and an EC$_{50} = 15$ mg/L for human umbilical vein endothelial cells exposed to aqu-$n$C$_{60}$-14L (111). Several studies examined the effect of fullerenol on human cells indicating no definitive toxicity thresholds when impacting human dermal fibroblasts, human epidermoid-like carcinoma, or human embryonic kidney 293 cells (110, 130). As a comparison to other carbon based materials, the ubiquitous carbon allotrope, graphite, caused 80% mortality of S. aureus when applied as a coating on stainless steel (133), yet populations of P. aeruginosa placed on a graphite substrate increased 13% over a silicon substrate (134). Graphite added to fog oil decreased D. magna mortality and increased brooding over fog oil alone (135) with no significant effect to P. subcapitata. This review of the limited information available suggested only Gram-positive bacteria were impacted by graphite while Gram-negative bacteria and daphnids benefitted. No graphite toxicity threshold information was obtained from peer-review manuscripts or any regulatory body, indicating the low regard for graphite in terms of a potential toxicant.

One area that has been developing with advances in computer capacity is the field of molecular dynamic simulation, which mathematically calculates and graphically displays molecules interacting with one another. This technology has been employed to demonstrate how fullerene species interact with a lipid bilayer membrane. Qiao et al. (136) examined translocation of underivatized and derivatized fullerene noting that a single C$_{60}$ molecule moves quickly (< 4 ns) into the non-polar, center region of a lipid bilayer membrane, repelling the hydrophilic head group to form a pore, while a single, polar C$_{60}$(OH)$_{22}$ molecule stays near the hydrophilic head group of the lipid molecules without penetration. Modelers have progressed to
the point of demonstrating an agglomerate consisting of ten fullerene molecules (i.e. $nC_{60}$) penetrating a lipid bilayer membrane as an agglomerate, then dispersing inside the cell membrane to become ten individual $C_{60}$ molecules (137).

Apparent from the review of available information was the differential reports of toxicity between $nC_{60}$ and fullerenol species, with claims that the former was orders of magnitude more harmful (14). Findings suggested that there was decreased adverse biological effect with increased hydroxylation of the fullerenol (110). However, due to incomplete characterization and reporting of materials, research focused on material application rather than overall biological capacity, and due to the relative novelty of fullerene as a studied material, uncertainties have persisted. Additionally, no publications examined characterized fullerenol synthesis by-products, leaving unanswered questions about potential exposure routes and methodologies appropriate to address them. This investigation into published research on fullerene species has concluded that there is limited physicochemical and toxicological characterization information for fullerene species, which needs to be remedied through additional research.

**Research Methodology**

This investigation examined physicochemical characteristics and biological effects of derivatized fullerene, both as water-stirred material ($aqu-nC_{60}$) and as chemical-catalyzed fullerenol. Chapter 2 outlines the physicochemical characteristics of $aqu-nC_{60}$ including concentrations in aqueous suspensions, agglomerate size, surface charge, and chemical composition. As previous synthesis efforts for $nC_{60}$ provided varying parameters including stirring time and photo-condition, detailed analysis was conducted to assess optimal synthesis constraints. Chapter 3 reviews the physicochemical characteristics of fullerenol and fullerenol synthesis-by-products. Synthesized materials were examined for physicochemical properties such as suspension pH, mean agglomerate sizes and size distributions, surface potential, and
chemical composition. Attention was given to by-products from the synthesis processes, defined as material remaining from individual synthesis steps, in terms of quantities to assess production efficiencies and potential for ecological impacts in the case of environmental release.

Biological impacts were assessed in Chapter 4 through the use of short-term (acute) and long-term (chronic) toxicity assays involving unicellular organisms (bacteria and algae) as well as invertebrate species. Sensitivity to photo-condition was conducted to assess claims of photo-induced effects and the associated chemical reactivity. Proposed pathways identified in documented research into the biological effects of fullerene were balanced with mechanisms that previously were unfound in other reports to provide causal relationships and assist in the elucidation of methodologies to alleviate potential impacts. Conclusions and suggestions for future research are presented in Chapter 5. Supplemental information on the purity of raw fullerene, surface groups, interactions controlling fullerene agglomeration, and the model test organisms are provided in Appendices A through D, respectively.
Table 1-1. Known producers of fullerenol-containing products

<table>
<thead>
<tr>
<th>Personal Care Product Distributors</th>
<th>Corporate Offices</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dr. Brandt®</td>
<td>New York City, NY</td>
</tr>
<tr>
<td>EDS Services</td>
<td>Hong Kong, China</td>
</tr>
<tr>
<td>N.V. Perricone, MD Cosmeceuticals</td>
<td>Meriden, CT</td>
</tr>
<tr>
<td>PCA SKIN</td>
<td>Scottsdale, AZ</td>
</tr>
<tr>
<td>Sircuit® Skin</td>
<td>Los Angeles, CA</td>
</tr>
<tr>
<td>SkinRX MD</td>
<td>Boca Raton, FL</td>
</tr>
<tr>
<td>Vitamin C-60 BioResearch Corporation</td>
<td>Tokyo, Japan</td>
</tr>
<tr>
<td>Zelens® Skin Science</td>
<td>London, UK</td>
</tr>
</tbody>
</table>
Table 1-2. Reported toxicity results for fullerene species

<table>
<thead>
<tr>
<th>Fullerene Species</th>
<th>Fullerene sp.</th>
<th>Biological Expression</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>THF-(n) C(_{60})</td>
<td><em>Bacillus subtilis</em></td>
<td>- MIC = 0.08 mg/L</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td><em>Escherichia coli</em></td>
<td>- LC(_{50}) &lt; 1 mg/L</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td><em>Pseudokirchneriella subcapitata</em></td>
<td>- LC(_{50}) = 0.139 mg/L</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td><em>Daphnia magna</em></td>
<td>- LC(_{50}) = 0.46 mg/L</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- LC(_{50}) = 0.8 mg/L</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td><em>Ceriodaphnia dubia</em></td>
<td>- LC(_{50}) = 0.395 mg/L</td>
<td>106</td>
</tr>
<tr>
<td>son-(n) C(_{60})</td>
<td><em>Bacillus subtilis</em></td>
<td>- MIC = 0.4 mg/L</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td><em>Daphnia magna</em></td>
<td>- LC(_{50}) = 7.9 ppm</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td><em>Danio rerio</em> (larval zebrafish)</td>
<td>- &gt;50% mortality at 0.1 mg/L</td>
<td>107</td>
</tr>
<tr>
<td>aqu-(n) C(_{60})</td>
<td><em>Bacillus subtilis</em></td>
<td>- MIC = 0.4 mg/L</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td><em>Daphnia magna</em></td>
<td>- LC(_{50}) &gt; 35 mg/L</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- 40% mortality at 35 mg/L</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- no significant inhibition at 24 mg/L</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- LC(_{50}) = 10.5 mg/L</td>
<td>109</td>
</tr>
<tr>
<td>Na(^+)C(<em>{60})O(</em>{7.9})(OH)(_{12-15})</td>
<td>human dermal fibroblasts</td>
<td>- LC(_{50}) = 50 mg/L</td>
<td>110</td>
</tr>
<tr>
<td>C(<em>{60})(OH)(</em>{24})</td>
<td><em>Bacillus subtilis</em></td>
<td>- no adverse effect at 5 mg/L</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td><em>Escherichia coli</em></td>
<td>- no adverse effect at 5 mg/L</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td><em>Danio rerio</em> (larval zebrafish)</td>
<td>- &gt;50% mortality at 0.1 mg/L</td>
<td>107</td>
</tr>
<tr>
<td></td>
<td>human dermal fibroblasts</td>
<td>- LC(_{50}) &gt; 5,000 mg/L</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>HUVEC</td>
<td>- EC(_{50}) = 15 mg/L</td>
<td>111</td>
</tr>
</tbody>
</table>
Figure 1-1. Wire-structure diagram of fullerene ($C_{60}$).

Figure 1-2. Patent and patent applications for fullerenol by year. (Source: United State Patent Office, April 10, 2011)
CHAPTER 2
PHYSICOCHEMICAL CHARACTERIZATION OF $nC_{60}$ AND SYNTHESIS BY-PRODUCTS

Background

Fullerene ($C_{60}$) has been researched extensively over the past two decades for applications to exploit its unique structural, electronic, thermal, and biological characteristics (4, 78, 113, 138-141). Surface derivatized fullerene provided higher solubility than raw fullerene and, thus, had been the focus of efforts to develop biological and biomedical application (18, 55, 63, 142-146). Some researchers have pointed out potential toxicological impacts (36, 53, 112, 147) while multiple reports have questioned effects from fullerene (148, 149), leaving toxicity assessment in need of additional investigation. Underivatized $C_{60}$ is highly non-polar and water insoluble (12), but it has been shown to disperse in turbulent water over a period of days to weeks, resulting in a material termed nano-$C_{60}$ ($nC_{60}$), which was indicated to consist only of nano-scale agglomerates of $C_{60}$ with a hydrated layer permitting dispersability (45, 53). Processes have been developed using polar solvents such as toluene, benzene, and tetrahydrofuran (THF-$nC_{60}$) as well as through sonication ($son-nC_{60}$) to expedite formation of nano-sized, dispersible agglomerates (28, 36, 104, 105). It should be noted that no practical application has been reported for $nC_{60}$ formed through any preparation method and its use has been limited to that of a water-dispersible surrogate for underivatized fullerene. These $nC_{60}$ agglomerates have been used as a baseline in comparative toxicity studies with derivatized products in development, with indication that $nC_{60}$ induced greater adverse biological impact than derivatized fullerene (112).

Materials under toxicological investigation require extensive physicochemical scrutiny to ascertain size, surface characteristics, chemical composition, and formation dynamics in effort to assess the potential for specific biological responses (150-153). For example, recent reports have indicated that the suspected toxicity of THF-$nC_{60}$ may be due to residual THF degradation.
products retained within agglomerates (100, 101), obscuring the true biological effects of these materials and elucidating the need for more extensive physicochemical characterization. Additional studies indicated that C₆₀ was biologically photo-reactive (105, 128, 149), inviting investigation into the potential for light to impact the type and number of surface groups on the carbon cage, which are responsible for disturbing the non-polar character of underivatized material and enabling dispersability. Several research groups have used water-stirred fullerene (aqu-nC₆₀), the only hydroxylated fullerene derivative reported without the use of non-polar solvents during synthesis, in toxicity studies and, while limited data on size, surface character, and charge distribution have been reported (27, 105, 108, 109, 149), questions remain as to the impact of synthesis parameters such as stirring duration and photocondition on the formation of the end products. This study examined physicochemical characteristics of aqu-nC₆₀ over a stirring period extending 28 days with and without exposure to light. It was hypothesized that both stirring duration and the presence of light impacted mean agglomerate size and size distribution, surface character, and chemical composition of aqu-nC₆₀. Suspensions of aqu-nC₆₀ and Nanopure water were sampled over the stirring period and analyzed to assess how the transformation of the surface groups were affected by varied stirring times and photoconditions.

Materials and Methods

Chemicals

C₆₀ was obtained from Nano-C (Westwood, Massachusetts) with 99.5% purity as reported by the manufacturer. Magnesium perchlorate (Mg(ClO₄)₂) and potassium bromide (KBr) were purchased through Fisher Scientific (Waltham, Massachusetts).

aqu-nC₆₀ Preparation

Synthesis of aqu-nC₆₀ was conducted under both dark and light conditions in a manner used by Oberdorster et al. (147). Briefly, 100 mg of C₆₀ were added to 500 mL of de-ionized
water and stirred continuously at a mixing rate of 550 rpm to create a vortex half the height of the stirring column, a parameter maintained through the stirring duration, using a magnetic stirring plate and a polytetrafluoroethylene (PTFE) magnetic stirring bar. Dark-stirred samples were obtained by wrapping sample containers completely with aluminum foil to eliminate light exposure while light-stirred samples were maintained under natural laboratory light. Stirring was extended for 28 days and aliquots were removed over time for physicochemical characterization before and after sample filtration using Millipore™ 0.45 µm-nitrocellulose membranes (Billerica, Massachusetts). Nitrocellulose filters, which are intended for biological research, were used as opposed to PTFE (128, 154), nylon (112, 147, 148), or glass (48, 76, 99, 155) membranes, providing a methodology that eliminated introduction of additional potential contaminants, such as silica or halogens, and permitted direct comparison with other biological investigations (78, 156, 157).

**Concentration Determination**

Concentrations of nC₆₀ suspensions taken at \( t = 7, 14, \) and 28 days under both light and dark conditions were determined using spectrophotometry (absorption at \( \lambda = 336 \text{ nm} \)) as described by Fortner et al. (53). Briefly, 0.1M Mg(ClO₄)₂ was added to nC₆₀ (2:5 with sample) to destabilize surface groups, returning fullerene molecules to an underivatized and hydrophobic state, seeking energetic stability by migration from the polar solvent to the phase interface. Samples were then vortexed for 15 seconds per minute for 30 minutes followed by the addition of toluene (1:1 with sample) and supplemental vortexing, leading to the transfer of underivatized C₆₀ into the non-polar solvent phase (53, 158). The extract was analyzed using the Spectronic GENESYS 10 UV (ultraviolet) spectrophotometer (Thermo Scientific, Waltham, Massachusetts). Concentrations were determined using a calibration curve prepared with known
concentrations of $C_{60}$ in toluene. Samples were conducted in triplicate for three individually sampled aliquots from three independent synthesis events ($n = 9$).

**Physicochemical Characterization**

Aliquots of stirred samples were analyzed daily for pH at room temperature using an Accumet® Research AR-50 pH probe obtained through Fisher Scientific (Pittsburgh, Pennsylvania). Dynamic light scattering (DLS) using the NanoTrac Particle Size Analyzer (MicroTrac, Inc., York, Pennsylvania) was employed to assess mean agglomerate size and size distributions. A refractive index of 1.93 was used, based on the work of Ruoff *et al.* (159) and Makarova (125) and all DLS figures reported were based on number of particles. Aliquots of 20 mL were analyzed for 90 seconds for each sampling event and were conducted in triplicate. Dispersed agglomerates from replicate samples of $nC_{60}$ were examined using transmission electron microscopy (TEM) and digital images were taken using the JEOL TEM 200CX (Tokyo, Japan) at 75 kV to assess mean agglomerate size. An aluminum standard was used to calibrate camera length. Sample preparation for the *aqu-nC$_{60}$* suspensions entailed application directly to a Formvar TEM 200-mesh grid (PolySciences, Inc., Warrington, Pennsylvania) and vacuum-drying for 24 hours prior to introduction to the TEM vacuum chamber. Due to the high number of agglomerates expected in a sample of dispersed fullerene ($2.5 \times 10^{17} C_{60}$ molecules/mL), a minimum of 5,967 agglomerates per analysis (through convergence) was required to obtain the most statistically-defensible estimate of agglomerate size (160). The imaging software package, Image J, from the National Institutes of Health (Bethesda, Maryland) was used to count and measure agglomerates within TEM-generated images. Subsequent calculations and analyses determined number of agglomerates, average length, average width, and average diameter using an assumption that all particles were spherical (i.e. based on volume).
Since analytical techniques to measure surface charge are not available presently, use of zeta potential measurements, the charge at the shear plane of the electric double-layer (Figure 2-1), was used to characterize agglomerates. While not specifically the desired measurement (i.e. the electrical charge at the surface of the fullerene cage), knowledge of the zeta potential provides insight to the actual surface potential as well as to the stability of the agglomerate in solution. Zeta potential measurements of nC$_{60}$ samples stirred in dark and light were measured on alternate days before and after filtration using the Brookhaven ZetaPlus Zeta Potential and Particle Size Analyzer (Holtsville, New York), a DLS technique, and converting the results to zeta potential using the Smoluchowski relationship for small (nanoscale) particles:

$$\zeta = \mu_e \eta \varepsilon_r^{-1} \varepsilon_0^{-1},$$ (2-1)

where $\zeta$ is zeta potential, $\mu_e$ is electrophoretic mobility, $\eta$ is kinematic viscosity of the medium, $\varepsilon_r$ is relative permittivity of the medium, and $\varepsilon_0$ is the dielectric constant.

Samples were examined for chemical composition through UV/visible light spectroscopy (UV/Vis), Fourier-transform infrared (FTIR) spectroscopy, and X-ray photoelectron spectroscopy (XPS). The Perkin-Elmer UV/Vis Spectrophotometer Lambda 800 (Waltham, Massachusetts) was utilized to provide absorption spectra from samples at room temperature in the range of 300-800 nm at 1.0 nm intervals. Thermo Electron Magna 760 FTIR/FT-Raman/FTIR Microscope (Waltham, Massachusetts) was used to generate FTIR spectra of nC$_{60}$ samples in potassium-bromide pellets to measure the binding energy associated with each surface group attached to the fullerene resulting from Rayleigh scattered electrons, identifying the types of surface groups present (30, 31, 34, 35, 37, 39, 45, 53, 68, 69, 72, 76, 81, 87, 114, 163-167). The software package, OMNIC, was used to view spectra; the database included
provided information on the types of bonds associated with the binding energies identified, whereupon the research employed discretion to assign bond types to binding energies.

The XPS (X-ray Photoelectron Spectroscopy) Perkin-Elmer PHI 5100 ESCA (Electron Spectroscopy for Chemical Analysis) System (Waltham, Massachusetts) generated binding-energy spectra to identify the proportion of molecules associated with the surface of the carbonaceous fullerene. Samples were analyzed using an aluminum anode to augment the electronic signal. Deconvolution of the binding energy from the carbon s-level (C 1s) spectrum of the XPS output was accomplished with multiple software algorithms. AugerScan version 3.2 (RBD Instruments, Inc., Bend, Oregon) was used to subtract background from the spectrum, which was exported in a format usable by Grams/Al version 7.01 (Adept Scientific, Inc., Bethesda, Maryland), where it was subjected to the Sav-Golay smoothing function of a second degree polynomial with 17 points for a range from 280 to 300 eV. The smoothed spectra were passed with “high” sensitivity through the “Peak Fitting” algorithm, using an “offset” baseline and the “Mixed Gaussian and Lorentian” distribution parameter, which permitted flexible end conditions. Binding energies were compared to archived information from the National Institute of Standards and Technology XPS Database (Gaithersburg, Maryland).

**Statistical Analysis of Physicochemical Characterization**

Physicochemical data were analyzed for outliers using Dixon’s Q-test (168) and scrutinized for inclusion based on defensibility of results. Normality of data was verified by the Ryan-Joiner normality test using Minitab 15.1 (State College, Pennsylvania) and homogeneity of variance was determined using Bartlett’s test (United States Environmental Protection Agency, Washington, D.C.). Comparison of means over time, between sample preparations, and between preparation methods was conducted using the student’s t-test ($\alpha < 0.05$); multiple comparisons of
means were conducted using Dunnett’s test (United States Environmental Protection Agency, Washington, D.C.).

**Results and Discussion**

**Concentrations of \( nC_{60} \) in prepared suspensions**

Visual observations from the samples showed a general increase of color intensity over the stirring period (Figure 2-2). Estimated concentrations for water-stirred \( nC_{60} \) under light conditions for 7-day (7L), 14-day (14L), and 28-day (28L) were 0.027, 0.455, and 1.595 mg/L, respectively (Figure 2-3). Estimated concentrations for water-stirred \( nC_{60} \) under dark conditions for 7-day (7D), 14-day (14D), and 28-day (28D) were 0.016, 0.241, and 0.734 mg/L, respectively. Dispersion kinetics for each photo-condition were fit to the quadratic relationship:

\[
y = ax^2 + bx + c
\]

(2-2)

where \( y \) is the concentration (mg/L), \( x \) is the stirring time, and \( a, b, \) and \( c \) are coefficients, where \( c \neq 0 \) indicates an error component, although it was determined to be acceptable (\( \varepsilon < 0.2 \)) at \( t > 7 \) days (Table 2-1). Both photo-conditions show accuracy in the relationship through acceptably high coefficients of determination (\( R^2 > 0.99 \)) indicating that the development of dispersed, water-stirred, derivatized fullerene agglomerates can be predicted as a function of time.

A mass balance analysis (Table 2-2) was conducted to determine the accuracy of the methodology used; identification of the fate of all material was used to test the reliability of the results. Between 71.8 and 92.7% of the initial mass (100 mg) was identified in the dispersed mass, the filter residue, and recovery from the beaker surface, resulting in an unaccounted fraction of 7.2 to 28.7%. Several possible explanations for the unaccounted material were explored. As borosilicate glassware was shown to be dominated by electron surface charge (169), electrophilic \( C_{60} \) molecules could have accumulated on the surface of the glass beaker (90, 91, 170, 171), although KimWipes™ (Kimberly-Clark, Inc., Neenah, Wisconsin) were used to
recover mass deposited on the glassware, yet a fraction could have remained after recovery. Another possible explanation was that the Difco™ inoculating loops (BD, Franklin Lakes, New Jersey) used daily to dislodge accumulated fullerene material from the sides of the borosilicate beaker attracted and continually removed fullerenes from the system. Considered independent of other confounding elements, a mass in the range of 0.85 to 1.79 mg would need to be removed daily via use of inoculating loops. It was presumed that removal of such a large quantity was unlikely, although subsequent investigations indicate that such a mass was able to adhere to a single inoculating loop. Fortner et al. (53) reported that fullerene recovery efficiency ranged from 94% to 101% without further explanation, potentially accounting for up to 6% of material. It was possible that analytical errors in combination with the previously mentioned factors may provide explanation for some of the unaccounted for material; hence, results were determined to be acceptable.

To assess the impact of stirring on dispersion, 100 mg C_{60} was placed in 500 mL de-ionized water in the absence of light and without stirring. No transfer of the solid phase fullerene into the aqueous phase over a 28-day period was observed or detected through solvent extraction and visible light spectrometry. In a parallel experiment, 100 mg C_{60} in 500 mL water was exposed to concentrated ultraviolet radiation for a period of 28 days without stirring; again, no measurable dispersion occurred. Throughout experiments conducted as part of this research, the only varied parameters in the transfer from the solid to aqueous phase are stirring (addition of mechanical energy) and exposure to light (addition of radiation energy). As it has been shown that dispersion of fullerene in water occurred in both light and dark conditions through stirring, mechanical energy was deemed the necessary condition for the surface derivatization of undervatizated fullerene that facilitated dispersability of nC_{60}. 
**Agglomerate Size Analysis**

Minimum mean agglomerate size of unfiltered \( nC_{60} \) assessed by DLS was observed (Figure 2-4) at \( t = 2 \) days for dark samples (\( d_h = 133.2 \) nm) and at \( t = 6 \) days for light samples (\( d_h = 137.8 \) nm) while maximum mean agglomerate size was observed for both samples at \( t = 8 \) days of 231.3 nm and 224.9 nm or dark and light, respectively. The hydrodynamic diameter of both dark and light samples increased over time, as assessed by regression analysis, with a mean daily growth rate of 1.48 nm and 0.42 nm, respectively. The change in the hydrodynamic diameter of the number-weighted mean agglomerate size from \( t = 2 \) days to \( t = 28 \) days for unfiltered \( nC_{60} \) was not statistically significant for samples prepared under light or dark conditions.

Furthermore, there was no significant statistical difference between the minimum and maximum of either data set, leading to the conclusion that there was no stirring-duration effect on the agglomerate size for unfiltered samples.

Minimum mean agglomerate size of filtered \( nC_{60} \) assessed by DLS was observed (Figure 2-5) at \( t = 10 \) days for dark samples (\( d_h = 80.1 \) nm) and at \( t = 18 \) days for light samples (\( d_h = 89.5 \) nm) while maximum mean agglomerate size was observed for dark samples of 194.5 nm (\( t = 4 \) days) and for light samples of 160.0 nm (\( t = 16 \)). The hydrodynamic diameter for filtered, dark-stirred \( nC_{60} \) decreased at a rate of 0.51 nm daily, as assessed by regression analysis, while light-stirred samples increased at a rate of 0.07 nm daily. The change in the hydrodynamic diameter of the number-weighted mean agglomerate size from \( t = 2 \) days to \( t = 28 \) days for filtered \( nC_{60} \) was not statistically significant for samples prepared under light or dark conditions and there was no significant statistical difference between the minimum and maximum of either data set. Based on these data, it can be concluded that there was no stirring-duration effect on the agglomerate size for filtered samples. Mean agglomerate sizes of filtered material were
statistically smaller than for unfiltered material, as anticipated from the removal of the majority (> 50%) fraction using a size-exclusion membrane.

Figure 2-6 shows agglomerate size distributions for a select set of nC₆₀ samples at t = 4, 20, and 28 days, indicating a tighter distribution over time with standard deviations of 75.2, 46.1, and 39.0 nm for the 4-day, 20-day, and 28-day samples, respectively. A primary assumption in dynamic light scattering was that all agglomerates were spherical, which is seldom, if ever, the case. With the advent of high-resolution TEM, it was possible to view agglomerates in the nanoscale (i.e. at least one dimension of the particle being less than 100 nm) (Figure 2-7). Table 2-3 provides a summary of the agglomerate size information from TEM for the four samples nC₆₀ analyzed (14D, 14L, 28D, and 28L). The mean hydrodynamic diameter of the agglomerates was based on the cross-sectional area of the agglomerate, determined as an outline of the agglomerate, converted to a diameter of an equal-sized spherical agglomerate and ranged from 96.6 to 117.6 nm with a maximum deviation from the median of 3.0 nm or less in each case. The standard deviations for the large number of agglomerates sampled (greater than 11,000 agglomerates per sample) indicated coefficients of variation in the range of 0.025 to 0.035, suggesting that the estimated diameter was in good agreement with the actual measurement (α < 0.01). The mean height ranged from 132.8 to 158.0 nm and the width ranged from 97.3 to 122.9 nm. While the coefficients of variation of the height measurements reflect those of the diameter measurements, the standard deviation for 14D indicated a broader distribution of agglomerates for these stirring conditions. This information showed that the agglomerates stirred for 14 days in dark did not reach a height distribution consistent with agglomerates stirred in light or for 28 days, indicating that exposure to ambient light during stirring increased the rate of formation of nC₆₀ agglomerates.
Comparison of mean agglomerate size data between DLS and TEM showed consistently that the diameter of the agglomerates reported by DLS were larger (by 16% to 35%) than the aggregates measured using TEM (Table 2-4). Since DLS did not measure the actual size of the agglomerates but rather used correlations based on assigned reflective indices, measurements collected using DLS should always be scrutinized. However, as electron microscopy requires desiccation of materials prior to being introduced to the sample chamber, TEM results may not have been the most appropriate vehicle for particle size analysis in the aqueous phase, a condition under which biological interaction would be required. Due to the enhanced compaction of the samples resulting from the desiccation process, it was expected that TEM images would yield smaller dimensions than when wetted (i.e. using DLS).

Comparison of data obtained through DLS from this investigation to data from other research of similarly prepared (water-stirred and filtered) nC_{60} indicated that the mean agglomerate size range for the two photo-conditions was in agreement with previous reports (35-39, 41, 47, 49, 172), although total comparison was difficult due to lack of information within some reports on stirring time, stirring rate, and photo-condition. The mean of the diameters reported in the cited reports was 164.0 nm compared to a mean of 138.2 nm for the mean agglomerate sizes of the four DLS results presented in Table 4-5. Literature pertaining to aggregate sizes of water-stirred nC_{60} determined using an analysis of TEM images was limited to two reports: Labille et al. (47) reported a mean aggregate diameter of 14-day, dark-stirred nC_{60} as 58.6 nm and Tervonen et al. (49) reported a value of 200 nm. The mean generated from the two reports was 18% greater than the TEM data produced in this research, although caution should limit any conclusions due to the very small sample size.
**pH Analysis**

Examination of pH, a measure of hydrogen ion (hydronium) concentration, of the de-ionized water in which the C₆₀ was stirred provided insight to agglomerate formation kinetics and chemical composition dynamics. For example, if pH of a solvent increased during stirring to the point where there was an excess of hydroxyl ions, there may have been an indication of fullerene protonation. On the other hand, if the solvent became more acidic, there may have been hydroxylation of fullerene. Initial pH measurements \((n = 10)\) at \(t = 0\), when underivatized C₆₀ was added to de-ionized water were 5.20 ± 0.70. Initially, dark samples experienced a decreased in pH until \(t = 6\) days, when a minimum pH of 4.42 was reached, after which pH steadily increased to a final \((t = 28\) days) value of 6.11 (Figure 2-8). The average increase in pH over the stirring period was 0.043 per day, although from the minimum at \(t = 6\) days to the end of the experiment, the average daily increase in pH was 0.062. The pH of samples stirred in light did not decline during the first week but rather stagnated, increasing slightly at a rate of 0.053 pH units daily on average for 6 to 28 days. Controls showed initial pH measurements consistent with treated samples followed by a rapid return to equilibrium by \(t = 8\) days. It should be noted that neither dark nor light samples achieved neutrality during the 28-day stirring period, suggesting that reactions may not have been exhausted.

Two primary observations can be made regarding the pH of the treated systems: comparison of the pH of solutions with C₆₀ to controls for both light and dark samples suggested the presence of C₆₀ altered the rate at which the suspension returned to pH = 7; and, there was a substantial decline in the pH of the dark-stirred sample during the first six days of the experiment, which was not seen in the other samples (i.e. light-stirred or controls). One mechanism responsible for the system moving toward neutral conditions was infusion of carbon dioxide into the system from the atmosphere and the subsequent formation of bicarbonate.
Consumption of hydroxyl ions during bicarbonate formation altered equilibrium with hydronium, lowering the pH of the solution, and facilitated additional carbon dioxide dissolution into the aqueous phase. While carbon dioxide comprised less than 0.1% of the atmospheric gases (173), adequate concentrations of carbon dioxide dissolved into solution to foster formation of carbonic acid. Additional carbon dioxide was drawn into the system to replace mass converted to carbonic acid, driving a secondary reaction with excess carbonic acid to form additional bicarbonate, releasing hydroxyl ions achieving neutrality; in control samples, this return to neutrality occurred much more rapidly (i.e. within 8 days). Despite the effect of atmospheric CO₂ on pH of prepared suspensions during the equilibration period, the two observations noted were caused by the only system variable: the presence of C₆₀. It was observed that C₆₀ experienced transformation from a hydrophobic material to one that was, at least, partially hydrophilic. This transformation was achieved in reactions with the hydronium and hydroxyl ions, reactions with the atmospheric gases, or a combination of both, suggesting that fullerene reactions with ionic species present in aqueous solution was a factor in nC₆₀ synthesis.

The pH of dark-stirred nC₆₀ samples initially declined for the first week and then increased at a rate slower than the samples without C₆₀. The impact on the ionic concentration of the solution was an increase in hydronium, a decrease in hydroxyl, or a combination of both manifestations, which was consistent with the proposal that C₆₀ became hydroxylated and, thus, more hydrophilic, consuming hydroxyl ions, and, subsequently, reducing pH. After commencement of stirring, aggregated C₆₀ collided with other aggregates, shearing weakly affixed clusters and increasing hydroxyl binding sites. During a period of approximately one week, hydroxylation may have been the dominant force in determining pH of the suspension.
However, after approximately one week, hydroxylation slowed due to reduced binding sites and carbon dioxide equilibration controlled, increasing pH until $t = 28$, but at a rate markedly slower rate than in the absence of $C_{60}$.

The pH for light-stirred samples remained generally constant for the first week and then increased at $t > 6$ days. As seen from the concentration analysis, light-stirred samples became more rapidly dispersed than dark-stirred samples even at $t < 8$ days. Increased $nC_{60}$ concentrations in light-stirred samples suggested the hydroxylation rate was greater than in dark-stirred samples, which were shown to display an even greater drop in pH; however, no corresponding drop in pH was observed. The opposing forces of hydroxyl consumption through surface hydroxylation and effective hydroxyl generation remained equal for the first week of stirring, after which hydroxyl generation controlled the rate of pH change until the end of the stirring period.

The presence of light either facilitated the consumption of hydronium or promoted the production of hydroxyl ions. Examining the potential for hydronium consumption, Chiang et al. (123) proposed a pathway for derivatization whereby the transformation of adjacent hydroxyl surface groups into a ketone (carbonyl) surface group yielded hydronium, resulting in a net increase in pH, which was contrary to the effect observed. Alternately, hydroxyl ion generation was noted through fullerene-facilitated generation of reactive oxygen species (ROS) (36, 115, 127-129). Arbogast et al. (120) proposed a pathway for the generation of ROS using photo-excited $C_{60}$, a process replicated and expanded over the past two decades (38, 174):

$$^1C_{60} + hv \rightarrow ^1C_{60}^* \rightarrow ^3C_{60}^* + ^3O_2 \rightarrow ^1C_{60} + O_2^*.$$  \hspace{1cm} (2-3)

Irradiation of $C_{60}$ facilitated formation of excited-state singlet fullerene ($^1C_{60}^*$), after which a non-energetic transformation of the spin of the first valence electron (termed “intersystem
crossing”) occurred, generating triplet fullerene ($^3C_{60}^*$), which was capable of exciting triplet dioxygen ($^3O_2$) into a superoxide ion ($O_2^{*-}$) through a singlet oxygen intermediary ($^1O_2$).

Subsequent transformation of superoxide in the presence of $C_{60}$, an electron-deficient moiety, was proposed by Markovic et al. (38):

\[ ^1C_{60} + O_2^{*-} \rightarrow ^1C_{60}^- + O_2^- + 2H^+ \rightarrow ^1C_{60} + H_2O_2 \] (2-4)

and, subsequently, into hydroxyl species:

\[ H_2O_2 \rightarrow OH^* + OH^- \] (2-5)

which would serve to increase pH. An alternate pathway to ROS generation was presented (38) involving triplet fullerene ($^3C_{60}^*$) without irradiation but in the presence of available electrons:

\[ ^3C_{60}^* + ^3O_2 + e^- \rightarrow ^1C_{60} + ^1O_2 \rightarrow ^1C_{60} + O_2^{*-} \] (2-6)

Generation of ROS and derivatized fullerene species occurred naturally within the system either in the presence or absence of radiation, although, as seen through the multiple pathways for formation possible with the addition of radiation, transformation potential was greater when exposed to light.

Since it was proposed that $nC_{60}$ was comprised of underivatized and derivatized fullerene molecules (53) and the concentration of derivatized material was found to increase with time, ROS formation potential clearly increased after commencement of $nC_{60}$ synthesis via water-stirring. The addition of subsequent surface groups increased due to ROS generation but was tempered through a reduction in potential binding sites as derivatization proceeded. It appeared plausible that generation of radical species not only served to create hydroxyl ions in the presence of $C_{60}$ but the process promoted surface derivatization as well.

Filtered samples measured an average of 0.95 ± 0.47 pH units higher than unfiltered samples ($n = 8$). When examined between photo-condition, filtered dark samples averaged 1.11
pH units higher than unfiltered while light samples averaged 0.79 pH units higher. The coefficients of variance within the data of each set were less than 0.05, indicating no statistical significance in the difference of the measurements (Figure 2-9). The overall trend for both filtered materials, however, was for increasing pH, each posting a gain of 0.008 pH units daily throughout the 28-day stirring period with light samples being 0.18 ± 0.04 pH units on average higher than the dark samples. Filtration enabled increased interaction with atmospheric gases, allowing excess carbon dioxide to depart the suspension more rapidly than in the stirring reactor, slowing down the process of carbonic acid formation, yielding more balanced hydronium-hydroxyl concentrations and resulting in a return to neutral pH within two days.

Zeta Potential

Figure 2-10 shows zeta potential measurements before filtration for both dark and light samples with light samples generally more electro-negative, ranging from -61.50 to -18.34 mV with a final measurement of -37.76 mV, while the dark samples ranged from -48.98 to -17.78 mV with a final reading of -35.74 mV. Neither light nor dark samples changed significantly during the sampling period, with standard deviations decreasing during the final week of stirring. A control of de-ionized water only was established to examine the impact of C60 on zeta potential, indicating instability at the beginning of the stirring period (-10.71 mV at Day 6), which became more stable (-45.86 mV at Day 28). It appeared that the control gained some exigent material naturally through infusion of gases, precipitation of residual salts, or atmospheric deposition during sampling and testing; the presence of C60 overshadowed whatever additional processes may have occurred. Filtered samples (Figure 2-11) showed more variability than unfiltered samples, characterized by a less neutral charge, and light samples (-48.11 to -18.29 mV) were generally more negative and stable than dark samples (-42.14 to -4.59 mV). Dark samples provided indication during Day 10 of exacerbated destabilization with zeta
potential measurements of $-10.93 \pm 2.93$ mV, although the event appears to be anomalous given the statistical tightness of the data, attributed to equipment variability, and remained unresolved. Light samples became more negative over the final four sampling events, suggesting greater accuracy, but were matched at Day 28 by the dark samples with $-36.76$ mV and $-35.83$ mV for dark and light samples, respectively.

Removal of agglomerates larger than 450 nm through filtration increased zeta potential, nominally for light samples but significantly for dark samples. This observation suggested smaller agglomerates were less negatively charged. Based on the agglomerate size analysis, which showed tighter distributions at longer stirring times, it was suggested that smaller agglomerates were more highly derivatized. Therefore, the hydrophilic functional groups on the carbon cage proved to detract from the overall negative charge of the surface, suggesting functional groups occupied electronegative binding sites. The most probable counterpart for these negative sites would be positive ions; however, the proposal was for electronegative hydroxyl groups, nullifying this proposition.

An alternate explanation was that the act of removing an aliquot of material from the reaction chamber increased exposure to atmospheric gases, which was greatly enhanced through filtration. Dissolved oxygen levels in de-ionized water recorded during limited investigation ($n = 3$) showed that oxygen concentration increased from below $4.0 \pm 0.8$ mg/L before filtration to near fully-saturated at 20 degrees Celsius ($DO = 8.1 \pm 0.2$ mg/L) after filtration, indicating an abundance of oxygen. The presence of additional oxygen in the suspension increased the partial pressure of sizable di-oxygen molecules, which may have impeded electrophoretic mobility of the agglomerates or facilitated additional or alternate surface derivatization.
Chemical Composition Analysis

Ultraviolet/visible light spectroscopy

Ultraviolet/visible light (UV/Vis) spectroscopy was used to provide information on electronic excitations within the molecular orbitals of the dispersed \( nC_{60} \) samples. Analyzing the UV/Vis spectra of \( nC_{60} \) samples prepared over time, stirred under two photo-conditions, and before and after filtration was designed to provide useful information on chemical composition during the synthesis process as well as to provide insight into potential for a cost-effective chemical composition screening methodology. Energy in the form of ultraviolet (\( \lambda < 400 \text{ nm} \)) and visible (\( \lambda = 400 - 800 \text{ nm} \)) light excited electrons to higher molecular orbital energy levels. The intensity of the energy emitted during relaxation was plotted versus wavelength, forming UV/Vis spectra. Figures 2-12 and 2-13 depict UV/Vis spectra (\( \lambda = 300-800 \text{ nm} \)) for dark-stirred material before and after filtration, respectively. Figures 2-14 and 2-15 provide UV/Vis spectra for light-stirred material before and after filtration, respectively. While the samples were analyzed on alternate days, mean data were shown only for four select days (Day 6, Day 12, Day 20, and Day 28) to facilitate presentation (\( n = 3 \)).

The intensity of emitted energy, represented as absorbance in concert with the Beer-Lambert law:

\[
A = -\log \left( \frac{I}{I_0} \right)
\]  

(2-7)

where \( A = \) absorbance, \( I = \) intensity of detected wavelength, and \( I_0 = \) intensity of the incident wavelength, increased as the wavelength decreased with few exception. Notable was the repetitive presence of two peaks in all samples (\( n = 168 \)): 366 ± 7 nm and 527 ± 5 nm, corresponding to UV and visible peaks, respectively. A shallow shoulder was detectable in the visible light range near \( \lambda = 445 \text{ nm} \) for the dark-stirred filtered material and was more
pronounced as a distinct peak in the light-stirred filtered material; however, this peak was absent from the unfiltered dark-stirred samples. Absorption was one magnitude higher in unfiltered samples than filtered samples, as would be expected from the removal of 71% to 92% mass by filtration.

Time-series plots of peaks in the UV range at $\lambda = 360$ nm were generated for dark and light samples before and after filtration. Both unfiltered dark and light samples increased during $t \leq 16$ days, after which dark samples declined in absorbance while light samples continued to increase, generating a two-fold disparity between photo-conditions at the conclusion of the synthesis period (Figure 2-16), which was consistent with the concentration differential at $t = 28$ days, but not $t = 14$ days. Absorbance of filtered samples increased throughout the synthesis period (Figure 2-17), although variation in data provided no statistical difference.

An analysis of the ratio of absorbances in UV and visible light peaks, termed the “UV/Vis peak ratio ($R_{\lambda}$)”, was conducted to assess the potential to use this parameter to screen for material chemical composition. The range of UV/Vis peak ratios for the unfiltered material was 0.985 to 1.036 ($\sigma = 0.012$) for dark samples and 0.999 to 1.108 ($\sigma = 0.025$) for light samples. As noted in Figure 2-18, the change in $R_{\lambda}$ over the stirring period for unfiltered $nC_{60}$ was negligible and statistically indifferentiable. The range of $R_{\lambda}$ for filtered material was 1.359 to 2.758 ($\sigma = 0.186$) for dark samples and 1.532 to 2.338 ($\sigma = 0.394$) for light samples (Figure 2-19). While $R_{\lambda}$ for both dark and light filtered samples trended downward over the stirring period, the oscillating variation in the data, more prominent in the dark samples, was possibly a result of analytical error, and no significance was determinable. Comparison of the UV/Vis peak ratio between samples was intended to provide a method for facile differentiation of the material during the synthesis process; however, there was no discernible trend to facilitate differentiation
of distinct $nC_{60}$ species as they formed. Additional sensitivity analyses were conducted at a
variety of wavelength combinations ($R_{\lambda} = 375/445$, $R_{\lambda} = 375/535$, and $R_{\lambda} = 535/445$) with
similar results (data not shown). As seen in consistent return of near unity for the ratio in dark
samples over time and the variability in light samples, the data did not allow for the
establishment of a statistically reliable predictor.

However, the difference between the UV/Vis peak ratios before and after filtration was
statistically significant, which indicated that filtration disproportionately removed material in the
visible light range. It was hypothesized that the larger material removed, comprised of more-
hydrophobic, less-derivatized $C_{60}$, was more active in visible light and the more-hydrophilic and
more-derivatized dispersed material was more active in the UV range. The material that passed
through the filter, by virtue of its dispersability, was derivatized through stirring and, for some
samples, through exposure to light, to become more hydrophilic. The hydrophilic moieties
associated with the carbon cage of $C_{60}$ were assigned to the UV peak near 360 nm. The
underivatized $C_{60}$, contained within both the residue and filtrate, was assigned to the visible light
peak near 525 nm.

**Fourier-transform infrared spectroscopy**

The methodology available to determine chemical composition of the surface of $nC_{60}$ was
to quantify oxygenation states, as determined using XPS, which quantifies the presence of
surface group types, which are determined specifically through FTIR, following the process
utilized by Krishna *et al.* (25). FTIR spectra were generated for $nC_{60}$ samples stirred in dark and
light for 7, 14, and 28 days (Figures 2-20, 2-21, and 2-22). All samples showed vibrational
peaks in seven distinct ranges (1001-1006 cm$^{-1}$, 1129-1183 cm$^{-1}$, 1383-1384 cm$^{-1}$, 1428-1460
cm⁻¹, 1636-1689 cm⁻¹, 2364-2368 cm⁻¹ and 3172-3466 cm⁻¹). Additional peaks were noted in selected samples in ranges of 803-880 cm⁻¹, 940-979 cm⁻¹, and 1538-1577 cm⁻¹.

Determination of chemical composition began with an assessment of the available chemicals that could comprise the product, which were limited to hydrogen, carbon, and oxygen. Figure 2-23 provides schematic illustrations of a number of possible surface conditions associated with nC₆₀ including hydroxyl, epoxide, ether, oxygen ion, hemiketal, carbonyl, and carboxylic acid groups; Appendix B provides additional information on each group and issues related to fullerene derivatization. Hydroxyl, epoxide, and ether surface groups as well as negatively charged, single-bonded oxygen ions represented mono-oxygenated carbon moieties while hemiketal, carbonyl, and carboxylic acid represented di-oxygenated carbon moieties.

Assessment of peaks from FTIR spectra for aqu-nC₆₀ was presented in the context of previously published data (Table 2-5). Non-oxygenated carbon (C=C, C=≡C) was identified at peaks occurring in the range of 1129-1183 cm⁻¹ and 1383-1384 cm⁻¹. The 1001-1066 cm⁻¹ and 1428-1460 cm⁻¹ peaks were identified as mono-oxygenated carbon (C-O) and were assigned to ether and hydroxyl, respectively. Epoxide groups were attributed to the peaks at 943-975 cm⁻¹. The peaks ranging from 1636-1689 cm⁻¹ were attributed to the presence of hemiketal surface groups. Peaks at 1538-1577 cm⁻¹, 2364-2368 cm⁻¹, and 3172-3466 cm⁻¹ were identified as clathrated oxygen, clathrated carbon dioxide, and water, respectively, which were not unexpected given the atmospheric conditions to which samples were exposed during synthesis. Several samples showed peaks in the range of 803-880 cm⁻¹, which was identified as hydrogenated carbon (C-H), suggesting protonation of the carbon surface or possible sample contamination.
Carbonyl groups, which have been reported to have a peak between 1710-1770 cm\(^{-1}\) (88, 114, 175-177), were absent from the spectra of all samples. The absence of carbonyl suggested these surface groups may have formed at the beginning of the stirring and then transformed to other species prior to the first sampling event \((t = 7\) days\), were present in such small quantities that they were undetectable to the equipment, or did not form at all. Additionally, carboxylic acid groups, which have been reported to provide peaks between 1700-1800 cm\(^{-1}\) (178), were absent, as was suspected since carboxylic acid surface groups would result in structural deterioration of the fullerene cage.

**X-ray photoelectron spectroscopy**

XPS multiplex data (Figure 2-24) indicated between 73% and 89% of the bonds were associated with carbon and oxygen. Nitrogen (0.4 to 3.2%), sodium (1.1 to 3.4%), and silicon (14.3 to 29.4%) were detected in all the samples. The presence of nitrogen was proposed to be a result of atmospheric gases present in the aqueous environment or through non-reactive deposition during laboratory handling of the samples. As a result of the presence of atmospherically-deposited nitrogen, samples were suspected of atmospheric deposition of carbonaceous material as well, and the data were analyzed for the appearance of adventitious carbon. The presence of adventitious carbon was noted in all samples, presenting minor peaks in the range of 283.0 and 284.0 eV, accounting for a maximum of 4.3% of the carbon bonds present. This quantity was subtracted from the calculations and omitted from the spectra so as not to confound the determination of fullerene-related carbon species. The appearance of silicon in the spectra was expected as X-rays penetrated samples to a depth greater than the thickness of the material, hence recording the silicon substrate. The presence of sodium could be an indication of equipment contamination, as other fullerene samples containing sodium were handled simultaneously by the XPS operator. A percentage of the oxygen was expected to be
associated with silicon as an oxide. Therefore, to preclude misrepresenting the oxygenation of material detected other than carbon, the range of binding energies associated with the 1s region for carbon was the focus of the analysis, and analysis the 1s region of oxygen was omitted.

XPS analysis was expected to provide peaks for adventitious carbon at 283.6 eV (179), non-oxygenated carbon at 284.60 eV based on graphite (180), mono-oxygenated carbon between 286.30 eV based on ethanol (181) and 286.45 eV based on polyethylene glycol (182), di-oxygenated carbon between 288.50 eV based on pyromellitic dianhydride (PMDA) (183) and 288.90 eV based on polyglycolide (182), and tri-oxygenated carbon at 289.50 eV based on sodium carbonate (181). An underlying assumption of the methodology was manifest in assigning of the adventitious carbon peak to 283.60 eV and shifting the spectrum accordingly. XPS spectra were generated and deconvoluted for dark and light samples at \( t = 7, 14, \) and 28 days (Figures 2-25, 2-26, and 2-27), and were depicted as the concentration of each carbon oxygenation state, represented as counts per second against the binding energy (in eV). The area under each deconvoluted curve was quantified to represent the electronic effect of that carbon oxygenation state (e.g. non-oxygenated, mono-oxygenated, etc.) on the \( nC_{60} \) molecule.

Grams/AI software calculated and continually monitored both the chi-squared (\( \chi^2 \)) “goodness-of-fit” parameter and the coefficient of determination (\( R^2 \)) to optimize the fit of the deconvoluted curves. The chi-squared parameter sought was a minimum with a target threshold of \( \chi^2 < 10 \). The threshold was achieved prior to convergence for only one sample (14-day light); however, all samples had final \( \chi^2 \) below 40 after initial values of \( \chi^2 > 1000 \). The coefficient of determination sought was a maximum and, for all samples analyzed, the range was from 0.993 to 0.999. Despite less than optimal, but permissible, \( \chi^2 \) values, \( R^2 \) showed highly acceptable agreement with experimental data; hence, deconvolution curves were deemed acceptable.
The non-oxygenated carbon peak represented between 45% and 65% of the samples, indicating that between 27 and 39 of the 60 carbons in the $C_{60}$ were not bound to oxygen. Using the generic formula for a derivatized fullerene:

$$C_{60}H_z + H_2O + E_i \rightarrow C_{60}H_x(OH)y$$

where $E_i$ = input energy, either in the form of mechanical (e.g. stirring, sonication) or radiation energy, and $x + y$ = total number of derivatized carbon atoms, a representation of the derivatized chemical composition in terms of the number of oxygenated carbons was estimated. As presented in Figure 2-28, it was determined that between 21 and 33 carbons were oxygenated ($x + y$) for the $nC_{60}$ samples with the more derivatized samples occurring after greater stirring times and when exposed to light.

At $t = 7$ days, the dark sample was nearly balanced between mono-oxygenated and di-oxygenated carbons with 10 and 11 groups, respectively (Table 2-7). The 7-day light sample had four additional oxygenated carbons but twice as many of the oxygenated carbons were mono-oxygenated compared to di-oxygenated. At the midpoint in stirring, the number of carbons with surface groups in the dark sample increased 33% and three-quarters of the oxygenated carbons were mono-oxygenated. In the light sample, total oxygenated carbons increased 16% and the percent of mono-oxygenated carbons increased to 79%. Of particular interest was that between $t = 7$ days and $t = 14$ days, the absolute number of di-oxygenated (hemiketal) groups declined 37% for the dark sample and 25% for the light sample. During the final two weeks of stirring, dark and light samples gained one and four additional mono-oxygenated carbon, respectively, while the number of di-oxygenated carbons remained unchanged.

The kinetics of surface group formation can be described through an exponential relationship:
\[ y_i = a \ln(x) + b \] (2-9)

with \( y \) = number of surface groups of type \( i \), \( x \) = stirring time, and parameters \( a \) and \( b \) as described in Table 2-8. The coefficients of determination \( (R^2) \) range from 0.9808 (dark) and 0.09910 (light) for the combined total number of surface groups to 0.7136 (dark) and 0.8357 (light) for the di-oxygenated (hemiketal) surface groups. Di-oxygenated groups achieved a maximum at \( t = 7 \) days for both dark and light samples, after which they decreased over the subsequent week and remained unchanged for the final two weeks of stirring. The occurrence of mono-oxygenated groups increased rapidly during the first week of stirring and then displayed more moderate growth at \( t > 7 \) days. Dark-stirred samples appeared to have completed derivatization near \( t = 14 \) days, with only one mono-oxygenated group being added during the final two weeks of stirring. The transformation of light-stirred samples did not appear to have completed during the 28-day synthesis period, as the number of mono-oxygenated groups continued to increase.

**Formation of derivatized \( nC_{60} \)**

The method used to quantify the carbon oxygenation states assumed that the only influence on spectra was derivatized \( nC_{60} \) with surface functional groups. One potential shortfall of this approach was the possibility that not all of the molecules scanned by the XPS system were derivatized. Assuming the XPS scanned to a depth of 5.0 nm (184) and diameters of \( nC_{60} \) and underivatized fullerene were 1.3 nm and 0.7 nm, respectively, dispersed in agglomerates with a single derivatized layer, a scan 5.0 nm deep would encounter four to seven underivatized molecules for each derivatized molecule, suggesting that as little as 14% of the signal was from the outer surface molecules. The data indicated that between 45% and 65% of the carbon atoms scanned were oxygenated, suggesting that the aggregate surface was comprised of several concentric layers of derivatized \( C_{60} \) surrounding underivatized molecules. Such an agglomerate
configuration would indicate that the degree of derivatization of the surface layers followed a gradient, with the most derivatized and, hence, hydrophilic, molecules closer to the interface with the aqueous medium and the less-derivatized molecules were located closer to the center of the agglomerate.

Agglomerate size analyses indicated that the material analyzed was not singular derivatized C\textsubscript{60} molecules but rather a colloidal suspension of agglomerates. The colloidal phenomena was facilitated by attractive (hydrophobic, van der Waals, and depletion) and repulsive (steric, solvation, and electrostatic) forces within both solvent and solid phases. In the case of derivatized C\textsubscript{60}, colloidal system formation was initiated by the polarity difference between water and fullerene. In theory, underivatized fullerene molecules are neither polar nor charged. Dried fullerene, synthesized by the method described by Kratschmer \textit{et al.} (I), formed aggregates held together by van der Waals forces within a face-centered cubic (fcc) crystalline structure at atmospheric temperatures (185). Dried crystals displayed a Miller’s index of $<111>$ with the diffraction pattern clearly visible from transmission electron microscopy (45). The structure formed through alignment of electron-rich olefinic regions (the $\pi$-bond at the intersection of hexagons) and electron-poor regions (pentagonal faces), yet were orientationally disordered at a regional scale, indicating potential for a high degree of structural interlocking, which was partly responsible for holding the aggregate together at the macro-molecular level (30). For underivatized C\textsubscript{60}, attractive (van der Waals) forces proved to be stronger than repulsive forces caused by steric interference, which were minimal due to the spherical nature of the molecule, and electrostatic repulsion, which was reported to occur locally (186). However, crystalline alignment served to minimize surface energy, as the aggregates proved to be stable, requiring pulverization with a mortar and pestle to reduce the size of larger aggregates. Pure
water, on the other hand, formed a network of hydrogen bonds that was highly organized so as to minimize surface energy of the system and, although the system was dynamic in terms of hydrogen-bond transfer, displayed little disruption to localized energy profiles (12, 53, 187-189).

The mere addition of 100 mg of C$_{60}$ to 500 mL of de-ionized water did not cause dispersion of the aggregates; upon introduction to the aqueous environment, underivatized fullerene remained suspended at the liquid-gas interface. Unlike solubilized fullerene derivatives, which are dispersed readily in water, non-polar C$_{60}$ and polar solvent displayed highly disparate surface energies, causing fullerene to further aggregate on the water surface (189, 190). The addition of mechanical energy through stirring, in the case of water-stirred aqua-nC$_{60}$, propagated interaction between the two systems, causing aggregated C$_{60}$ to commence dispersion in water after an average of 34.2 ± 4.8 minutes ($n = 9$) of stirring at 550 rpm, as determined by visual observation of the reaction chambers.

The introduction of C$_{60}$ within the aqueous system disrupted the entropic equilibrium of the solvent, which isolated the non-polar material within the aqueous medium, compartmentalizing the energy increase caused by the non-polar material (Figure 2-29), thus lowering system entropy to a local minimum under conditions posed by the introduction of non-polar fullerene. Several researchers have described a hydrated layer forming around clusters of underivatized fullerene (12, 53, 187-189), suggesting that reorganization of water molecules lent to fullerene dispersability. In addition, fullerene molecules facilitated colloid formation by reducing their contribution to interfacial tension and the associated entropy increase through the formation of clusters, minimizing its own surface area and, consequently, the interfacial surface, which further reduced the energy differential. "Hydrophobic forces", which are the tendency for a system to reduce an increase in entropy caused by a polar/non-polar disturbance, were the
driving force behind agglomeration of C\textsubscript{60} molecules. Force interactions for fullerene aggregation are described further in Appendix C.

Aravand and Semsarzadeh (191) showed that stirring velocity impacted the size of epoxy emulsions formed at the submicron-scale, suggesting a similar relationship with other materials such as fullerene. Measurements of agglomerate size for the nC\textsubscript{60} samples prepared after stirring in water at 550 rpm associated with this study were in the range of 96.6 to 117.6 nm for 14 days and 108.2 to 117.0 nm after 28 days (Table 2-3). Despite the fact that aspect ratios (height:weight) did not change significantly over time either, shaping of the clusters did occur, as was observed in the TEM images (Figure 2-7). Clusters shown in images taken at $t$ =14 days appeared with greater irregularity of form than those at $t$ = 28 days; smaller primary groups were visibly adhered to the bulk solid in 14-day samples. The aggregates viewed after 28 days of stirring did not decrease significantly in size but rather appeared with smoother edges, suggesting that primary groups adhered with forces unable to withstand the shear caused by stirring and, while the agglomerate size did not change significantly over time, the range of sizes narrowed, as seen in Figure 2-6.

Supporting the deduction that stirring velocity impacted the agglomerate size in the early stages of nC\textsubscript{60} synthesis, Ma and Bouchard (41) reported that extending mechanical stirring to 100 days did not significantly alter agglomerate size, yet size differences rearranged considerably. On the other hand, Li et al. (192) stirred C\textsubscript{60} at 500 rpm for 29 days in light and reported that the agglomerate sizes changed dramatically during the first two days, noting hydrodynamic radii of 32, 16, and 6 nm for stirring durations of 5, 24, and 48 hours, respectively. This finding suggests that a critical period for analysis of agglomerate size dynamics occurred prior to the first sampling period ($t$ = 2 days). Some research examining the
formation of $nC_{60}$ presented observations about the shape of aggregates but included limited information on the parameters for stirring (e.g. photo-condition, stirring duration, stirring velocity, etc.) making comparison of the results of this investigation with previously published reports difficult. Many studies reported a spherical shape of $nC_{60}$ agglomerates (12, 14, 15, 46, 193). Brant et al. (37) indicated the presence of hexagonal and pentagonal $nC_{60}$ agglomerates while Dhawan et al. (35) described them as “elongated”. Further complicating comparison, Fortner et al. (53) reported rounded edges in $nC_{60}$ associated with smaller agglomerates and more angular edges were associated with larger agglomerates while Labille et al. (47), Brant et al. (37) and Baun et al. (194) reported the opposite. Lyon et al. (36) indicated that water-stirred $nC_{60}$ had more rounded edges than either sonicated or THF-assisted $nC_{60}$. None of these investigations, however, addressed the dynamics of agglomerate shape or size as related to stirring time or velocity. From the information reviewed, no correlation between stirring velocity and particle size or shape was established. This data void would benefit from additional research on the relationship between stirring velocity and particle size and shape; subsequent investigations into $C_{60}$ agglomeration should closely examine early formation mechanisms in the time frame of $t < 2$ days, although examination of size and morphology during first few hours of stirring may also provide meaningful insight on water-stirred fullerene surface derivatization. All experiments should be designed and supported so as to be statistically defensible.

The mechanisms of agglomeration were expected to include both the conversion of larger, aggregated material shorn to nano-scale agglomerates and the nucleation of smaller, extricated groups into agglomerates able to withstand shear forces. Andrievsky et al. (45) proposed that the primary agglomerate group for $C_{60}$ maintained a size of 3.4 nm, consisting of 13 individual molecules (Figure 2-30), which represented a typical group dislodged during smoothing. These
residual groups were proposed to nucleate and form new agglomerates with size governed by the relationship between nucleation (attractive van der Waals) forces and shear forces. Brant et al. (37) suggested that the initial formation of nC\textsubscript{60} agglomerates was through nucleation, as was demonstrated by Zhai and Efrima (195) and Burshtain et al. (196) with silver nanoparticles, supporting dislodgement as a potential mechanism for agglomerate formation. The shearing of smaller groups from larger clusters provided opportunities for surface derivatization, as once meta-stable binding sites became exposed to the aqueous medium, comprised of hydronium and hydroxyl groups, dissolved gases (e.g. nitrogen, oxygen, carbon dioxide) and ROS when in the presence of light. Based on the surface groups identified through FTIR spectra, hydrogenated, hydroxylated, and oxygenated groups were expected to be associated with the formation of nC\textsubscript{60}.

It was determined through FTIR analysis that some carbon sites may have been protonated, which required modification to the fundamental nC\textsubscript{60} chemical formula to include the presence of hydrogen:

\[
C_{60} + H_2O + E_i \rightarrow C_{60}H_xO_x(OH)_y
\]

where \(x\) and \(y\) were unchanged, determined through XPS, and the molecular subscript \(z\) was unquantifiable through the FTIR/XPS methodology. Ajie et al. (197) postulated C\textsubscript{60}\textsuperscript{2+}, determined by mass spectroscopy, as a hydrogenated form of fullerene, although these species only appeared at elevated activation energies. Potential exists for analysis of hydrogen atoms associated with fullerene through proton nuclear magnetic resonance (H-NMR), although complications persist as methodologies to differentiate hydrogen bound within complex molecules, as in the case of a hydrogenated/hydroxylated fullerene, and definitive quantification of surface group remains elusive (195). Quantification of hydrogenated sites associated with the nC\textsubscript{60} samples through H-NMR was not undertaken as part of this research, although future
research endeavors may seek to incorporate NMR to investigate the surface configuration. Additionally, use of electron-spin resonance (ESR) may provide insight into the presence of unpaired electrons within the carbon cage.

The source of hydrogen for protonation was assessed to be through hydrolysis, residing in hydronium, occurring at the liquid/solid interface. As shown in the pH analysis, the system was initially acidic ($\text{pH} \approx 5.2$), indicating an excess of hydrogen ions caused by the removal of free hydroxyl ions through reaction with carbon dioxide, driving the system toward an equilibrium of greater hydrogenated products. Early research in fullerene derivatization reflected the limited understanding of the material as high-pressure chemical ionization with a variety of reagent gases was used to induce hydrogenation \cite{198, 199}. Haufler \textit{et al.} \cite{87} and Kroto \textit{et al.} \cite{94} facilitated protonation of $C_{60}$ in highly acidic media using \textit{tert}-butyllithium, indicating surface derivatization with both hydrogen and $t$-Bu. Brant \textit{et al.} \cite{43} indicated the presence of hydrogen surface groups through sodium-chloride potentiometric dissociation analyses, although no claim was made as to the potential for quantification of such ions using this methodology. Chiang \textit{et al.} \cite{30} proposed that protonation occurred within a previously oxygenated surface group, namely carbonyl, to form hemiketal structures; however, no evidence of carbonyl groups was detected in the $nC_{60}$ samples during this investigation despite the presence of hemiketal surface groups. Based on results from molecular dynamic simulation \cite{200}, a highly dynamic system was proposed whereby water molecules were organized loosely around $C_{60}$ with the electronegative oxygen facing the electrophilic, hydrophobic molecule presenting the hydrogen end to the aqueous medium, which facilitated dispersion. The protonated species were reported to be less hydrophilic than the parent material, suggesting the propensity for agglomeration was elevated with hydrogen bound to the surface of the fullerene due to increased hydrophobic
forces. Protonated fullerene was shown to be more effective in forming a hydrated layer with less fleeting interactions as water molecules formed hydrogen bonds with C$_{60}$H$_x$.

Protonation and formation of a hydrated layer were proposed as the initial occurrences in synthesis of nC$_{60}$ agglomerates. Continued infusion of mechanical energy through stirring facilitated further interaction between solvent and molecules at the agglomerate surface, which promoted derivatization with oxygenated surface groups. Previous studies have proposed that the first functional state in nC$_{60}$ transformation was hydroxylated with the reduction of the π-bonded carbons located at the intersection of hexagonal faces and the attachment of two hydroxyl groups (30, 67), resulting in a 1,2-dihydroxylated [C$_{60}$] fullerene, or vic-diol fullerene. Several reports have indicated that the FTIR peak range of 1390-1412 cm$^{-1}$ represented hydroxylated C$_{60}$ (31, 74, 115) while others have identified a mono-oxygenated structure in that range without specifically assigning it to hydroxylation (25, 60, 70, 83, 177).

Vileno et al. (115) proposed two mechanisms for fullerene derivatization with hydroxyl groups through photo-activation including surface group formation involving radical species (O$_2^-$, H*, OH*), resulting from charge-transfer mechanisms, as well as from collisions between molecules, resulting in resonant energy transfer. Brant et al. (37) discussed two derivatization mechanisms specific to hydroxyl derivatization including chemical adsorption of available hydroxyl groups from the aqueous medium and hydrolysis reactions occurring at the surface instigated through energy from molecular collisions. Researchers also have demonstrated increased hydroxylation through the introduction of ultraviolet radiation to previously dispersed nC$_{60}$ (13, 76, 114).

The inclusion of other oxygenated adducts to C$_{60}$ has been documented by numerous researchers in multiple bonding configurations, on underivatized and derivatized C$_{60}$, and with
and without the association of hydroxyl groups. Creegan et al. (164) heralded achievement of the controlled synthesis of fullerene epoxide through photolysis in a benzene substrate, indicating evidence supporting the predominance of epoxides over an etherized configuration through the lack of NMR peak in the sample. Bensasson et al. (201), however, indicated that no epoxide was present in a mixture of C\textsubscript{60} and toluene, citing the lack of a UV/Vis peak in optical density at 424 nm. Schuster et al. (202) and Birkett (203) synthesized C\textsubscript{60}H\textsubscript{2}O through infusion of singlet oxygen (\textsuperscript{1}\text{O\textsubscript{2}}\textsuperscript{*}) while Cataldo et al. (204) generated epoxide surface groups through ozonation, documenting an FTIR peak at 931 cm\textsuperscript{-1} as symmetrical C-O stretching.

The formation of ether groups was indicated initially by Chiang et al. (30) as the result of no carbonyl FTIR peak, an argument that was later amended, indicating the presence of the ether formation as part of the hemiketal configuration with an associated hydroxyl group (205). Kamaras et al. (68) proposed that ether formation resulted from the presence of clathrated oxygen and carbon dioxide gases, which showed peaks at 1538 and 2327 cm\textsuperscript{-1}, respectively. Other researchers have since confirmed the presence of ether groups in a variety of derivatized fullerenes using FTIR spectra, especially for the case of nC\textsubscript{60} (59, 75, 114, 204).

Chiang et al. (30) and Kong et al. (67) proposed pathways of C\textsubscript{60} surface transformation involving oxygenated and hydroxylated functional group with initial surface group attachment consisting of two hydroxyl groups affixed to adjacent \pi-bonded carbons. The hydroxylated groups, vic-diol moieties, were proposed to be transformed to a single carbonyl through hydration and desorption of one of the hydroxyl groups. Hydration of the bond with hydronium yielded a hydroxyl ion/hydroxyl complex and, after protonation, the two carbon sites were occupied by a carbonyl and a hydroxyl group. Through subsequent loss and gain of a proton, the
resulting surface group was di-oxygenated hemiketal, which was able to be protonated, returning to the form of the hydroxyl ion/hydroxyl system in cyclic fashion.

Several observations from the nC\textsubscript{60} chemical composition analysis provided insight to the pathways and mechanisms potentially involved in the dispersion of fullerene. XPS data showed the number of surface groups increased when stirring occurred in light, indicating photo-active fullerene-facilitated derivatization. Dark samples had more hemiketal groups but fewer mono-oxygenated groups than light samples stirred for the same duration, which suggested that exposure to light caused surface groups to favor the mono-oxygenated carbon state. The number of total and mono-oxygenated surface groups increased while the number of hemiketal groups decreased over time for both dark and light samples, demonstrating that total mechanical energy impacted the type of surface groups and, to a lesser degree the number of surface groups, formed. It was determined through FTIR that epoxide groups decreased over time, from being measureable at \( t = 7 \) and 14 days to not being detected at \( t = 28 \) days in either photocondition.

A pathway of nC\textsubscript{60} surface derivatization based on the FTIR and XPS analyses was proposed (Figure 2-31). The initial derivatization process of C\textsubscript{60} (1) was protonation of the molecules (not shown) with hydrogen provided by hydronium present in the liquid medium, readily available from the acidic conditions noted though the pH analysis (pH \( \approx 5.2 \) at \( t = 0 \)).

\[
C_{60} + zH_3O^+ \leftrightarrow C_{60}H_z + zH_2O
\]  

(2-11)

Protonation serves to facilitate the conversion of dried aggregates to wetted agglomerates, fostering interaction of constituents through collision-induced shear and chemical processes such as hydrolysis. Newly exposed carbon binding sites were expected to be the location of surface derivatization, unshielded by other fullerene molecules, non-hydrogenated, and in direct contact with the aqueous medium, enabling chemical reactivity. Hydroxylation was proposed to occur in
connection with π-bonded carbons resulting in the attachment of two hydroxyl groups (2), as was previously proposed, with the source of the hydroxyl groups being the liquid medium:

\[ C_{60}H_2 + 2OH^{-} \leftrightarrow C_{60}H_2(OH)_2 \]  

(2-12)

It was anticipated that previously protonated sites were not substituted with oxygenated groups as hydrogenated sites were apparent even after 28 days of stirring, although no conclusions were able to be drawn and its relevancy to the overall transformation process, outside of facilitating cluster formation, remained unknown. The process may have been repeated at other locations on the same molecule simultaneously, as a minimum of 21 surface groups were shown in the XPS analysis; however, attachment likely occurred slowly based on the 28-day derivatization kinetics. The hydroxyl moieties, present in a mechanically energized system, displayed high potential for steric interaction, as observed through graphical depiction, which promoted the transformation of the hydroxyl groups to a single epoxide moiety (3) at the intersection of the two hexagonal faces:

\[ C_{60}H_2(OH)_2 \leftrightarrow C_{60}H_2O_{epoxide} + H_2O \]  

(2-13)

The epoxide transformed to an ether group (4) through a redistribution of the electrons comprising the σ-bond of associated carbon atoms:

\[ C_{60}H_2O_{epoxide} \leftrightarrow C_{60}H_2O_{ether} \]  

(2-14)

Through subsequent hydroxylation on one of the involved carbon sites, the hemiketal structure (5) proposed by Chiang et al. (30) was formed:

\[ C_{60}H_2O_{ether} + OH^- \leftrightarrow C_{60}H_2O(OH) \]  

(2-15)

drawing on the liquid medium for ions.

The pathway was determined to be reversible as was demonstrated by the instability of some species, specifically epoxide and hemiketal groups, which appeared in greater numbers at
earlier sampling events. Numerous researchers have documented the formation of isolated oxygen species on the carbon cage (30, 31, 58, 93), mainly under the presumption that these groups were transformed to or from ether, hemiketal, carbonyl, or epoxide groups. Epoxidation to form fullerene oxide ($C_{60}H_2O$) without intermediate species has been proposed previously (59), whereby exposure of exited, triplet fullerene ($^3C_{60}$) in the presence of singlet oxygen (203) yielded epoxide surface groups. The carbon-oxygen bond dissociation energy within the proposed epoxide was reported to be less than for the bonding of the hydroxyl surface group (204) and, in the presence of both singlet oxygen and hydroxyl ions, may signify a preferential transformation pathway. Lee et al. (13) indicated that derivatization of $C_{60}$ occurred more slowly in the absence of oxygen, which suggested the transformation of $C_{60}$ was a function of dissolved oxygen concentration and potentially resulted in the subsequent generation of exited-state oxygen. While no experiments were engaged to determine the role of dissolved oxygen in surface group formation, it seems plausible that both hydroxyl and epoxide species formed directly from material present in the aqueous media as depicted in an alternate transformation pathway (Figure 2-32).

Molecular transformation on the fullerene surface during synthesis could be attributed to mechanical processes such as resonance transfer energy through collisions, chemical processes such as hydrolysis or from a pH-shift caused by the formation of carbonic acid, or photochemical processes with ROS in the presence of light. The impacts of mechanical energy infused into the system over time were dynamic in nature due to maintenance of a vortex at half-height throughout the stirring duration. It was proposed that the frequency of collisions increased initially over time as more material was brought into the agglomerate state. Furthermore, as agglomerates collided, nominally-sized primary fullerene groups were shorn, adding to the total
number of agglomerates, which increased the probability of two agglomerates interacting. The mechanical energy enabled derivatization but only until diameters were achieved with adhesive forces adequate to withstand shear forces associated with stirring. Given an adequate amount of time, however, fewer primary fullerene groups were able to be dislodged due to cohesive forces, resulting in a decline of the interaction rate. Additionally, given that each fullerene molecule has a limited number of π-bonds and that the number of π-bonds decreases with each attached surface group and the stirring rate decreased over time due to vortex conditions, a decrease in the rate derivatization was anticipated.

Several researchers have examined aspects of the interaction of C₆₀ and photoelectric energy, though none of the literature reviewed specifically discussed the impact of light on the type of surface groups associated with nC₆₀. Lee et al. (13) indicated the potential for ultraviolet radiation to mineralize derivatized fullerene, presumably from cage degradation, which can be caused by excessive derivatization, while Kong et al. (67) reported that derivatized fullerene was degraded physically when exposed to ultraviolet light primary through the reduction of hemiketal groups to carbonyl groups, which initiated cleavage of the carbon cage. Hwang and Li (76) demonstrated photo-transformation of nC₆₀ when irradiated both in terms of degree of oxygenation as well as degradation, which was enhanced in the presence of di-oxygen, suggesting again that dissolved oxygen affects the chemical reactions. Lee et al. (207), on the other hand, indicated that nC₆₀ was generally recalcitrant to photo-activated radical hydroxyl. The rate of derivatization decreased for both dark and light samples over the stirring period and surface derivatization appeared to stagnate in the dark-stirred samples at t > 14 days. However, for light-stirred samples, a notable derivatization rate (two surface groups weekly) was maintained until the end of synthesis. It is interesting to note that only mono-oxygenated groups
were added during the final two weeks of stirring under both phot condi-
tions, suggesting that conditions for di-oxygenated group formation and ma-
tenance were only ideal in the first two weeks of stirring. As photo-
condition was the primary difference in the experimental conditions,
exposing \( C_{60} \) stirred to light was determined to prolong the active derivatization period and to favor mono-oxygenated surface groups over di-oxygenated moieties.

Hydrolysis is a ubiquitous phenomenon in aqueous solution and the probability of spontaneous hydrolysis was assumed to be equal throughout the aqueous medium. As hydrolysis is a function of hydronium and hydroxyl ion concentration, investigation was conducted into the impact of pH on rates of hydrolysis. Vos (208) reported that the rate of hydrolysis generally was augmented with an increase in hydroxyl ion concentration, suggesting that conditions for hydrolysis were enhanced as pH increased over the synthesis period. However, given the heightened surface energy at the interface between the non- to moderately-polar fullerene systems and highly polar aqueous medium, a reduced probability of hydrolysis occurring at the interface as compared to within the bulk aqueous medium was anticipated. Despite reduced probabilities, omnipresent hydrolysis suggested adequate spontaneous reactions occurred at the interface to enable not only hydroxylation of the underivatized fullerene but also transformation of existing surface groups, especially the transformation of epoxide surface groups, which were shown to be eliminated by \( t = 28 \) days, likely to \( \text{vic-diol groups} \):

\[ H_2O \leftrightarrow H^+ + OH^- + C_{60}H_2O_{epoxide} \leftrightarrow C_{60}H_2(OH) \]  

(2-16)

Similarly, hydrolysis at the location of an ether formation could result in a hemiketal group:

\[ H_2O \leftrightarrow H^+ + OH^- + C_{60}H_2O_{ether} \leftrightarrow C_{60}H_2O(OH) \]  

(2-17)

An analysis of pH as it related to \( nC_{60} \) agglomerate formation was undertaken to assess the impact of environmental chemistry on fullerene derivatization. Numerous reports indicated
stability of $nC_{60}$ in the pH range of 1 to 12 (45, 189, 209). Several studies have shown through surface chemistry the dependence of fullerene on pH in the context of ion dissociation (34, 43), zeta potential (137), ROS formation (113, 116, 119, 202), and agglomerate size (53, 74). It was proposed that hemiketal moieties were reduced in polyhydroxylated fullerene through protonation, transforming to carbonyl moieties, when pH was decreased (53, 74). Vileno et al. (64) suggested the number of surface groups remained the same while the type of the surface groups was related to the availability of acidic protons, suggesting two mechanisms for ROS formation: hydrolysis/electron transport; and, resonant energy transfer.

The pH of the suspensions either decreased or remained unchanged statistically during the first week (dark: $pH_\mu = 4.81 \pm 0.27$ and $\Delta pH = -0.52$; light: $pH_\mu = 5.19 \pm 0.10$ and $\Delta pH = +0.06$), which corresponded to the period marked by the appearance of 72% of the ultimate number of surface groups in dark samples and 76% of the ultimate number surface groups in light samples. The pH increased consistently from the first chemical composition sample ($t = 7$ days) to the end of the synthesis period ($t = 28$ days). During Week 2, when the pH of dark samples recovered ($\Delta pH = +0.58$) and pH of light samples rose to a mean of $5.96 \pm 0.69$ ($\Delta pH = +0.70$), surface derivatization increased with 24% and 12% of the ultimate number of surface groups added for dark and light samples, respectively. However, during Weeks 3 and 4, dark samples added only 3% of the total number of surface groups ($\Delta pH = +0.42/week$) while light samples formed the remaining 12% of its surface groups ($\Delta pH = +0.32/week$), indicating a disparity in growth rates in relationship to change in acidity. Therefore, it appeared that pH was not a factor in determining the quantity of surface groups formed.

Equilibrium chemistry suggested that a suspension with higher pH would be more favorable to the formation of hydroxylated groups, either mono-oxygenated vic-diol or di-
oxygenated hemiketal configuration. The rate of pH change during the Week 2, when a large number of mono-oxygenated surface groups was added (dark: 11; light: 6), was not significantly different than the rate of pH change for during the remaining two weeks; in fact, there were fewer relative hydroxyl ions available earlier in the process. It was observed that the quantity of di-oxygenated hemiketal groups initially decreased in both dark and light samples between 7 and 14 days, and then remained unchanged for the duration of the test, suggesting late-stage surface group enhancement through the addition of mono-oxygenated hydroxyl groups only. Hemiketal groups may have formed during periods when hydronium was above a certain concentration, transforming to a mono-oxygenated species prior to sampling and resulting in fewer di-oxygenated hemiketal groups recorded for light sample at \( t = 7 \) (pH = 5.3) compared to dark sample (pH = 4.7). The number of hemiketal groups decreased during Week 2; however, there was no significant difference in the rate of pH change during the remaining synthesis period, fortifying the assertion that pH did not affect surface group quantities. Hemiketal moieties may have experienced steric strains that favor an ether configuration with dissociation of a hydroxyl ion:

\[
C_{60}H_2O(OH) \leftrightarrow C_{60}H_2O_{ether} + OH^- \tag{2-18}
\]

As the number of di-oxygenated hemiketal groups decreased after \( t = 7 \) days, it was demonstrated that all post-Week 1 growth in surface derivatization days occurred in the form of mono-oxygenated groups.

Epoxide moieties were present at \( t = 7 \) days and \( t = 14 \) days, which were marked by stagnant (light: Week 1), declining (dark: Week 1), and increasing (dark and light: Week 2) changes in pH. The pH range for all samples during the first two weeks, when epoxide groups were noted, was 4.42 to 5.96, indicating that fullerene oxides were possible in a more-acidic
range. Epoxide appeared to be the least stable oxygenated moiety, evidenced by its disappearance over the stirring period while the total number of mono-oxygenated groups increased. The carbon-carbon-oxygen bond angles measure 61.5 degrees each, indicating a heightened physical strain energy (+25 kcal/mol) within epoxide and a tendency to exist as an unstable intermediate species (210), hydrolyzing to the vic-diol state (2) or forming an ether structure (4), which supported the proposal that hydroxyl and ether groups were the predominant mono-oxygenated groups.

The disappearance of epoxide groups occurred during Weeks 3 or 4, when the pH increased at the same rate as for Week 2, suggesting a critical pH, rather than a linear relationship between acidity and surface groups, may have been present, impacting the preferential configuration for the oxygenated groups. The pH range selected was between 5.9 and 6.1, as the former represented the light sample at $t = 14$ days, the highest recorded pH with the presence of epoxide, while the latter represented the dark sample at $t = 28$ days, the lowest recorded pH without the presence of epoxide. At this critical pH, the concentration of hydroxyl ions in the aqueous medium was adequate to transform the epoxide to the vic-diol configuration of hydroxyl groups:

$$\text{C}_{60}\text{H}_{12}\text{O}_{\text{epoxide}} + \text{OH}^- \rightarrow \text{C}_{60}\text{H}_{12}(\text{OH})(2)$$

Conversion to the di-oxygenated hemiketal configuration was not a predicted transformation route, as such a process would entail modification to the more stable ether conformation and then a secondary transformation to the hemiketal configuration. Furthermore, the number of hemiketal groups did not increase during this period for either dark or light samples, providing support for the postulate that epoxide groups did not transform to hemiketal groups after $t = 7$ days. Assignment of the critical pH was estimated based on the available information from a
limited number of samples and sampling events. A more detailed pH-chemical composition analysis may serve to validate the estimate.

XPS was unable to provide distinction in the signal generated by ether and hydroxyl groups. A feasible scenario as to which species were present in relative frequency was that the increasingly alkaline environment was adequate to favor a hydroxylated mono-oxygenated state while an equally valid view was that, while increasingly alkaline, the suspension was, on the whole, acidic (pH < 7) for the entire experiment and the preferential mono-oxygenated state should be reflective of the aqueous medium. It could be posited that the ratio of hydroxyl groups to ether groups was increasing at \( t = 28 \) days, given the increasing pH. The preferential order, based on this investigation placed hydroxyl more prevalent than ether moieties, followed by hemiketal groups and then epoxide groups. Due to potential connections to biological activity, future research endeavors on fullerene derivatives may choose to focus on determination of the preferential state of mono-oxygenated surface groups.

An \( nC_{60} \) agglomerate was defined as a collection of surface-derivatized, nominally polar \( nC_{60} \) molecules surrounding a core of hydrophilic yet electrophilic \( C_{60} \) molecules in a polar solvent in a configuration suggestive of a micellular structure (Figure 2-33). As only surface molecules of a cluster are derivatized, the ratio of derivatized molecules to underivatized molecules is extremely low. Assuming a fractal coefficient of 2.1 (211), and a spherical agglomerate diameter of 100 nm, 0.4% of all \( C_{60} \) molecules comprised the surface, indicating that only a small fraction of the molecules were derivatized sufficiently to be to persist as a hydrophilic entity at the solid-liquid interface. The underivatized \( C_{60} \) within the core of the agglomerate maintained its highly electrophilic nature, seeking to satisfy its electron deficiency from any available source, which, in this case, was from the aqueous medium. A charge was
acquired opportunistically using unoccupied electron-acceptor sites on the surface of the derivatized \( nC_{60} \) molecules and, utilizing the delocalized electron characteristics of fullerene, charge was transferred to underivatized \( C_{60} \) within the agglomerate core \((37)\). The agglomerate increased charging until a collision occurred and the energy was transferred both to the colliding agglomerate as well as back to the aqueous medium, returning the agglomerate to a less unstable, more neutrally-charged state.

**By-products of \( aqu-nC_{60} \) Preparation**

Synthesis by-products generated during the preparation of \( aqu-nC_{60} \) were analyzed for chemical composition using FTIR spectroscopy (Figure 2-34) and XPS (Figure 2-35). The analysis of \( aqu-nC_{60} \) preparation by-products was limited to the 14-day light-stirred (WASTE-\( nC_{60}-14L \)) and 28-day light-stirred (WASTE- \( nC_{60}-28L \)) samples, as these materials were selected for biological investigation. It was hypothesized that filter residues were comprised solely of underivatized \( C_{60} \). FTIR spectra for residues of both stirring durations indicated solid consistency with underivatized \( C_{60} \), the only exception being the peak at 3434 cm\(^{-1}\), which is typical of the hydroxyl bond of entrained water molecules, indicating that the material was not completely dried prior to analysis. While FTIR spectra for underivatized \( C_{60} \) showed very little background throughout, the by-products show several minor peaks, which can be attributed also to the material having been previously wetted, whereas the raw material was analyzed as it was received from the manufacturer, that is, after industrial-grade vacuum drying. The background could also be attributed to the formation of a hydrated layer, discussed by Andrievsky *et al.* \((45)\) and Fortner *et al.* \((53)\).

XPS spectrum for WASTE-\( nC_{60}-14L \) indicated that the material was not entirely devoid of derivatized surface groups. This material was shown to have one surface group, a mono-oxygenated moiety. Recalling that potential mono-oxygenated carbons can possess either a
hydroxyl, epoxide, or ether group, and noting that the FTIR spectrum was void of the epoxide peak (range from 940 to 945 cm\(^{-1}\)), it was proposed that this surface group must have been either a hydroxyl or ether group. Since the ether group requires two carbon molecules (i.e. \(-\text{C-O-C}-\)), it was proposed that the material filtered was \(\text{C}_{60}\text{H}_z(\text{OH})\). Slanina \textit{et al.} (56) studied the energetics of various hydroxylated fullerene molecules, noting that hydroxyl groups only were stable on the surface of the carbon cage as pairs, binding to adjacent, double-bonded carbon molecules, suggesting that the actual chemical composition of the by-product was \(\text{C}_{60}\text{H}_z(\text{OH})_2\), which was diluted to appear as a single moiety through mixture with completely underivatized material. Based on this assumption, however, an ether group was equally valid, resulting in \(\text{C}_{60}\text{H}_z\text{O}\). The XPS spectrum for WASTE-\textit{nC}_{60}-28L indicated the presence of nine mono-oxygenated groups, suggesting a chemical composition of \(\text{C}_{60}\text{H}_z\text{O}_x(\text{OH})_y\), \(x + y = 9\), where these groups could be any combination of hydroxyl and ether groups. Perhaps more accurately, the XPS spectra for the by-products reflected some partially mono-oxygenated groups, either hydroxyl or ether moieties, and some underivatized \(\text{C}_{60}\).

**Summary**

Stirring time and photocondition were found to be factors effecting \(\text{aqu-nC}_{60}\) synthesis. Water-stirred fullerene, \(\text{aqu-nC}_{60}\), was prepared over a 28-day synthesis period, showing strong correlation \((R^2 > 0.99)\) between concentration and stirring time with samples prepared under light, yielding twice the mass of dispersed fullerene. Stirring duration did not affect agglomerate size in either dark or light samples, although agglomerate size distributions showed indication of becoming more uniform over time, consistent with formation pathways that include nucleation and shearing through agglomerate collision infused through mechanical stirring. Analysis of suspension pH indicated that stirring for 28 days was not adequate to achieve equilibrium in either dark or light stirring conditions, suggesting a kinetically slow chemical transformation.
process. Zeta potential analysis before and after filtration led to the deduction that smaller agglomerates were less negatively charged as a result of being more highly derivatized, which follows from reduced binding sites being present on the increasingly derivatized fullerene molecules.

While a UV/visible light chemical characterization screening method was tested and failed to establish a functional relationship, it was proposed that derivatized fullerene molecules absorb light more in the UV region while underivatized material removed during filtration was more absorbent in the visible light region. From FTIR analysis, it was determined that all $nC_{60}$ samples contained mono-oxygenated, hydroxyl and/or ether, surface groups and di-oxygenated hemiketal surface groups. Additionally, 7-day and 14-day stirred dark and light samples contained epoxide surface groups and no carbonyl or carboxylic acid surface groups were recorded in any samples, as had been previously reported. Surface groups were quantified either as mono-oxygenated (hydroxyl, ether, or epoxide) or di-oxygenated (hemiketal), with light samples consistently having more total and mono-oxygenated groups than dark samples. Mono-oxygenated moieties exceeded di-oxygenated moieties in all samples except for 7-day dark-stirred samples, indicating light and stirring duration increased both total derivatization and mono-oxygenation. It was proposed the presence of epoxide occurred only at solution pH < 6.1 with epoxide moieties being transformed to a vic-diol conformation as a result of increasing hydroxyl ion concentrations. Furthermore, di-oxygenated hemiketal groups decreased from Day 7 to Day 14 but remained constant for the final two weeks of synthesis, suggesting traceable pathways whereby di-oxygenation and epoxidation were less stable conditions than hydroxylation or etherization. A mathematical relationship was proposed to describe the kinetics of $aqu-nC_{60}$ surface group formation, indicating stronger correlation between stirring and total
surface groups ($R^2 > 0.98$) than with either mono- or di-oxygenated groups individually. Water-stirred fullerene filter residue, the only waste material generated from the process, was shown to consist of increasing mono-oxygenated surface groups over time, with a maximum of nine groups identified at $t = 28$ days, despite being non-dispersible, indicating that the minimum number of surface groups required for dispersability was greater than nine, but less than the 21 found in 7-day stirred material.

Through this research, it was determined that $aqu-nC_{60}$ cannot be considered a substitute for $C_{60}$ as the presence of multiple types of surface groups (hydroxyl, epoxide, hydroxyl, and ether) were documented. Further, the confirmation of derivative groups elucidated the need for providing physicochemical analysis on all fullerene materials and emphasized the imperative for omitting assumptions as to chemical composition. Many aspects regarding the physical properties of $aqu-nC_{60}$ remained unresolved including the mechanisms by which researchers formed $aqu-nC_{60}$ agglomerates with sizes and shapes that differ from those presented here; possible explanations include quality of raw water, stirring velocity, and source of raw, underivatized material. Additional investigation on the kinetics of surface derivatization may choose to include more detailed investigations of early-stage derivatization ($t < 7$ days) as well as the period after 28 days (until chemical equilibrium has been achieved), and provide repetitive analysis of independently prepared samples to refine the expressions for statistically acceptability. Additionally, mechanisms of fullerene interaction with dissolved atmospheric gases such as oxygen and carbon dioxide may provide a greater level of insight on formation mechanisms and pathways. Subsequent investigations into the influence of dissolved oxygen on the surface group formation of derivatized fullerene would benefit from developing experiments that control and continuously monitor the dissolved oxygen concentrations of the reaction vessel.
Additional experiments could increase or decrease the dissolved oxygen level for all or part of the stirring duration, creating mathematical models of the dynamic kinetics of fullerene derivatization. The results from such investigations can be used to provide a foundation for understanding how biological systems respond to derivatized fullerene. All experiments should be designed and supported so as to be statistically defensible.
### Table 2-1. Kinetics parameters for $nC_60$ formation in aqueous dispersant

<table>
<thead>
<tr>
<th>Photocondition</th>
<th>$a$</th>
<th>$b$</th>
<th>$c$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light</td>
<td>0.0020</td>
<td>0.0032</td>
<td>-0.025</td>
<td>0.9954</td>
</tr>
<tr>
<td>Dark</td>
<td>0.0008</td>
<td>0.0052</td>
<td>-0.016</td>
<td>0.9911</td>
</tr>
</tbody>
</table>

### Table 2-2. Mass balance analysis for $nC_60$ synthesis$^a$

<table>
<thead>
<tr>
<th>Fullerene</th>
<th>7D</th>
<th>7L</th>
<th>14D</th>
<th>14L</th>
<th>28D</th>
<th>28L</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_60$</td>
<td>100.000</td>
<td>100.000</td>
<td>100.000</td>
<td>100.000</td>
<td>100.000</td>
<td>100.000</td>
</tr>
<tr>
<td>Dispersed</td>
<td>0.003</td>
<td>0.005</td>
<td>0.039</td>
<td>0.091</td>
<td>0.147</td>
<td>0.319</td>
</tr>
<tr>
<td>Filter Residue</td>
<td>92.700</td>
<td>87.400</td>
<td>80.700</td>
<td>79.100</td>
<td>75.800</td>
<td>71.500</td>
</tr>
</tbody>
</table>

$^a$All weights in mg.

### Table 2-3. Numerical results from TEM image mean particle size analysis

<table>
<thead>
<tr>
<th>Fullerene Species$^a$</th>
<th>Diameter$^{b,c}$</th>
<th>Width$^c$</th>
<th>Height$^c$</th>
<th>Aspect Ratio$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$nC_60$-14D</td>
<td>96.6 (10.6)</td>
<td>132.8 (13.7)</td>
<td>97.3 (24.6)</td>
<td>1.35 (0.00)</td>
</tr>
<tr>
<td>$nC_60$-14L</td>
<td>117.6 (9.0)</td>
<td>158.0 (11.9)</td>
<td>122.9 (9.5)</td>
<td>1.34 (0.00)</td>
</tr>
<tr>
<td>$nC_60$-28D</td>
<td>117.0 (12.9)</td>
<td>155.9 (15.5)</td>
<td>122.2 (13.2)</td>
<td>1.32 (0.00)</td>
</tr>
<tr>
<td>$nC_60$-28L</td>
<td>108.2 (8.8)</td>
<td>143.6 (11.2)</td>
<td>111.7 (8.9)</td>
<td>1.35 (0.00)</td>
</tr>
</tbody>
</table>

$^a$nC6014D, nC6014L, nC6028D, and nC6028L indicate nC60 stirred for 14 days in dark, 14 days in light, 28 days in dark, and 28 days in light, respectively.

$^b$Diameters obtained from images using Image J software.

$^c$Diameter, width, and height are in nm.

$^d$Aspect ratio is unitless.
Table 2-4. Mean agglomerate size for nC60 determined through DLS and TEM

<table>
<thead>
<tr>
<th>Fullerene Species</th>
<th>DLS&lt;sup&gt;b, c&lt;/sup&gt;</th>
<th>TEM&lt;sup&gt;c&lt;/sup&gt;</th>
<th>%Δ&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>n C&lt;sub&gt;60&lt;/sub&gt;-14D</td>
<td>126.2</td>
<td>96.6</td>
<td>23.5%</td>
</tr>
<tr>
<td>n C&lt;sub&gt;60&lt;/sub&gt;-14L</td>
<td>158.4</td>
<td>117.6</td>
<td>25.8%</td>
</tr>
<tr>
<td>n C&lt;sub&gt;60&lt;/sub&gt;-28D</td>
<td>142.3</td>
<td>117.0</td>
<td>17.8%</td>
</tr>
<tr>
<td>n C&lt;sub&gt;60&lt;/sub&gt;-28L</td>
<td>125.8</td>
<td>108.2</td>
<td>14.0%</td>
</tr>
</tbody>
</table>

DLS = dynamic light scattering
TEM = transmission electron microscopy
%Δ = [(DLS - TEM) / DLS] x 100

<sup>a</sup> nC<sub>60</sub>-14D, nC<sub>60</sub>-14L, nC<sub>60</sub>-28D, and nC<sub>60</sub>-28L indicate nC<sub>60</sub> stirred for 14 days in dark, 14 days in light, 28 days in dark, and 28 days in light, respectively.

<sup>b</sup> DLS measurements based on number-weighted statistics.

<sup>c</sup> All sizes in nm.

<sup>d</sup> Percent difference (%Δ) defined between TEM/DLS.
Table 2-5. Intramolecular vibrational modes $nC_{60}$ and their symmetries

<table>
<thead>
<tr>
<th>Experimental Values</th>
<th>Reported Values</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>7D 7L 14D 14L 28D 28L</td>
<td>Vibrational Mode</td>
<td>Reference</td>
</tr>
<tr>
<td>803 805 850 880</td>
<td>C-H (out of plane bending)</td>
<td>126</td>
</tr>
<tr>
<td>940 945 979 943</td>
<td>C-O (epoxide)</td>
<td>40, 94</td>
</tr>
<tr>
<td>1066 1062 1063 1001 1034 1050</td>
<td>C-O</td>
<td>11, 74, 75, 80, 94, 96, 97, 101, 102, 104, 105, 110, 124, 127</td>
</tr>
<tr>
<td>1183 1185 1129 1129 1183 1183</td>
<td>C-C</td>
<td>29, 32, 101, 102</td>
</tr>
<tr>
<td>1384 1384 1384 1384 1384 1383</td>
<td>C-OH</td>
<td>75, 97, 114</td>
</tr>
<tr>
<td>1428 1429 1453 1460 1431 1428</td>
<td>C-C</td>
<td>11, 74, 105</td>
</tr>
<tr>
<td>1538 1577</td>
<td>O$_2$</td>
<td>128</td>
</tr>
<tr>
<td>1669 1670 1679 1636 1689 1636</td>
<td>hemiketal</td>
<td>105, 114</td>
</tr>
<tr>
<td>2364 2364 2364 2364 2364 2364</td>
<td>CO$_2$</td>
<td>128</td>
</tr>
<tr>
<td>2919 2963 2920 2921 2920 2973</td>
<td>CH$_2$ (asymmetrical stretching)</td>
<td>32, 101, 129</td>
</tr>
<tr>
<td>3175 3172</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3466 3431 3443 3317</td>
<td>O-H stretching</td>
<td>11, 74, 80, 89, 104, 105, 110, 124</td>
</tr>
<tr>
<td>3627</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2-6. Quantification of $nC_{60}$ oxygenated carbon states as determined via XPS

<table>
<thead>
<tr>
<th>Fullerene Species</th>
<th>Mono-oxygenated</th>
<th>Di-oxygenated</th>
</tr>
</thead>
<tbody>
<tr>
<td>$nC_{60}$-7D</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>$nC_{60}$-7L</td>
<td>17</td>
<td>8</td>
</tr>
<tr>
<td>$nC_{60}$-14D</td>
<td>21</td>
<td>7</td>
</tr>
<tr>
<td>$nC_{60}$-14L</td>
<td>23</td>
<td>6</td>
</tr>
<tr>
<td>$nC_{60}$-28D</td>
<td>22</td>
<td>7</td>
</tr>
<tr>
<td>$nC_{60}$-28L</td>
<td>27</td>
<td>6</td>
</tr>
</tbody>
</table>

XPS = X-ray photoelectron spectroscopy

Table 2-7. Exponential parameters for $nC_{60}$ surface derivatization kinetics for water-stirred fullerene ($aqu-nC_{60}$)

<table>
<thead>
<tr>
<th>Surface Group Type</th>
<th>$a$</th>
<th>$b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$nC_{60}$-dark-stirred</td>
<td>3.64</td>
<td>16.49</td>
</tr>
<tr>
<td>$nC_{60}$-light-stirred</td>
<td>4.23</td>
<td>19.50</td>
</tr>
<tr>
<td>Mono-oxygenated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$nC_{60}$-dark-stirred</td>
<td>2.59</td>
<td>11.11</td>
</tr>
<tr>
<td>$nC_{60}$-dark-stirred</td>
<td>3.18</td>
<td>14.12</td>
</tr>
<tr>
<td>Di-Oxygenated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$nC_{60}$-dark-stirred</td>
<td>1.05</td>
<td>5.38</td>
</tr>
<tr>
<td>$nC_{60}$-dark-stirred</td>
<td>0.09</td>
<td>4.29</td>
</tr>
</tbody>
</table>
Figure 2-1. Electrical double-layer interfacial interaction model adapted from Stern (161) and Grahame (162).

Figure 2-2. Aliquots of $nC_{60}$ taken over 28-day period. Starting from the upper left ($t = 2$ days) to the upper right ($t = 14$ days) and continuing to the lower left ($t = 16$ days), terminating at the lower right ($t = 28$ days), depicting increasing material in dispersed state.
Figure 2-3. Kinetics relationship for $nC_{60}$ formation in aqueous dispersant as a function of time.

Figure 2-4. Number-weighted mean agglomerate size for $nC_{60}$ samples as a function of time of unfiltered material (with standard deviation).
Figure 2-5. Number-weighted mean agglomerate size for nC_{60} samples as a function of time of filtered material (with standard deviation).

Figure 2-6. Number-weighted agglomerate size distribution for filtered nC_{60} samples at \( t = 4, 20, \) and 28 days.
Figure 2-7. Transmission electron microscope (TEM) images of $nC_{60}$. JEOL TEM 200CX (Tokyo, Japan) at 75 kV with an aluminum standard.

Figure 2-8. pH of unfiltered $nC_{60}$ stirred for 28 days in dark and light as a function of time ($n = 3$) (with standard deviation). Unfiltered control samples ($n = 1$) are shown in light-weighted lines.
Figure 2-9. pH of unfiltered nC₆₀ stirred for 28 days in dark and light as a function of time ($n = 3$) (with standard deviation).

Figure 2-10. Zeta potential ($\zeta$) for unfiltered nC₆₀ samples as a function of time ($n = 3$) (with standard deviation). Control is shown ($n = 3$) (with standard deviation).
Figure 2-11. Zeta potential ($\zeta$) for filtered $nC_{60}$ samples as a function of time ($n = 3$) (with standard deviation).

Figure 2-12. UV/Vis spectrum of unfiltered $nC_{60}$ stirred in dark at $t = 6, 12, 20,$ and $28$ days ($n = 3$) (error bars were omitted for clarity).
Figure 2-13. UV/Vis spectrum of filtered $nC_{60}$ stirred in dark at $t = 6, 12, 20, \text{ and } 28 \text{ days (} n = 3 \text{)}$ (error bars were omitted for clarity).

Figure 2-14. UV/Vis spectrum of unfiltered $nC60$ stirred in light at $t = 6, 12, 20, \text{ and } 28 \text{ days (} n = 3 \text{)}$ (error bars were omitted for clarity).
Figure 2-15. UV/Vis spectrum of filtered nC₆₀ stirred in light at t = 6, 12, 20, and 28 days (n = 3) (error bars were omitted for clarity).

Figure 2-16. UV peak at λ = 360 nm for unfiltered nC₆₀ as a function of time and photo-condition (n = 3) (error bars were omitted for clarity).
Figure 2-17. UV peak at $\lambda = 360$ nm for filtered $nC_{60}$ as a function of time and photo-condition ($n = 3$) (error bars were omitted for clarity).

Figure 2-18. Ratio of UV ($\lambda = 360$ nm) to visible ($\lambda = 525$ nm) peaks ($R_\lambda$) for unfiltered $nC_{60}$ as a function of time and photo-condition ($n = 3$) (error bars were omitted for clarity).
Figure 2-19. Ratio of UV ($\lambda = 360$ nm) to visible ($\lambda = 525$ nm) peaks ($R_{\lambda}$) for filtered $nC_{60}$ as a function of time and photo-condition ($n = 3$) (error bars were omitted for clarity).
Figure 2-20. FTIR spectra for 7-day dark and light water-stirred fullerene (\textit{aq}u-\textit{n}C_{60}) samples. A) 7-day dark water-stirred fullerene sample. B) 7-day light water-stirred fullerene sample.
Figure 2-21. FTIR spectra for 14-day dark and light water-stirred fullerene (aqu-nC₆₀) samples. A) 14-day dark water-stirred fullerene sample. B) 14-day light water-stirred fullerene sample.
Figure 2-22. FTIR spectra for 28-day dark and light water-stirred fullerene (aqu-\(n\)C\(_{60}\)) samples. 
A) 7-day dark water-stirred fullerene sample. B) 7-day light water-stirred fullerene sample.
Figure 2-23. Surface groups potentially associated with water-stirred fullerene (\textit{aqu-nC}_{60}).
Figure 2-24. XPS multiplex summary data on chemical composition for water-stirred fullerene (\textit{aqu-nC}_{60}) samples.
Figure 2-25. XPS spectra for 7-day dark and light water-stirred fullerene (aqu-$nC_{60}$) samples. A) 7-day dark water-stirred fullerene sample. B) 7-day light water-stirred fullerene sample.
Figure 2-26. XPS spectra for 14-day dark and light water-stirred fullerene (\textit{aqu}-nC_{60}) samples. A) 14-day dark water-stirred fullerene sample. B) 14-day light water-stirred fullerene sample.
Figure 2-27. XPS spectra for 28-day dark and light water-stirred fullerene (aqu-\(nC_{60}\)) samples. A) 28-day dark water-stirred fullerene sample. B) 28-day light water-stirred fullerene sample.
Figure 2-28. Kinetics of surface group formation for aqu-nC$_{60}$ stirred in dark and light for 28 days.
Figure 2-29. Simplified two-dimensional representation of $aqu-nC_{60}$ agglomerate in aqueous medium (including hydronium and hydroxyl ions).
Figure 2-30. Agglomerate of the primary agglomerate group for C$_{60}$ comprised of 13 molecules, as proposed by Andrievsky et al. (2002).

Figure 2-31. Proposed pathway for aqu-$n$C$_{60}$ surface group formation. Due to non-affiliation with subsequent chemical transformations, the hydrogenated state of fullerene (C$_{60}$H$_x$) was omitted.
Figure 2-32. Alternative pathway for aqu-nC₆₀ surface group formation. Due to non-affiliation with subsequent chemical transformations, the hydrogenated state of fullerene (C₆₀Hₓ) was omitted.

Figure 2-33. An nC₆₀ agglomerate with hydrophilic, derivatized surface molecules surrounding underivatized, hydrophobic C₆₀ molecules. Representation is analogous to a micelle, where polar (hydrophilic) head groups protect the non-polar (hydrophobic) carbon chains. nC₆₀ molecule shown contains approximately 100 molecules and is 6.4 nm in diameter.
Figure 2-34. FTIR spectra of by-products for 14-day light and 28-day light water-stirred fullerene (aqu-nC₆₀) samples. A) 14-day light water-stirred fullerene sample. B) 28-day light water-stirred fullerene sample.
Figure 2-35. XPS spectra of by-products for 14-day light (top) and 28-day light (bottom) water-stirred fullerene (aqu-\(nC_{60}\)) samples. A) 14-day light water-stirred fullerene sample. B) 28-day light water-stirred fullerene sample.
CHAPTER 3
PHYSICOCHEMICAL CHARACTERIZATION OF FULLERENOL AND FULLERENOL SYNTHESIS BY-PRODUCTS

Background

Fullerenol is a hydroxylated, water-soluble derivative of fullerene (C\textsubscript{60}) that has found numerous biomedical applications as a carrier in drug-delivery systems (15, 16), an inhibitor of human immunodeficiency virus-1 protease production (17), type-C hepatitis virus (18), and “Type I” hypersensitivity allergic reactions (23) as well as being shown to prevent precursors to Alzheimer’s and Parkinson’s diseases (19, 20). Fullereneol can be synthesized through several procedures to derivatize the surface of the carbon cage including reactions with acids, bases, hydrogen peroxide, and bromide (26). Fullereneol also can be obtained commercially through several manufacturers, who have indicated chemical compositions of C\textsubscript{60}(OH)\textsubscript{n} synthesized using the sodium hydroxide (NaOH) facilitated “Kitazawa method” (214) and with the stated number of hydroxyl surface groups, \(n\), ranging from 18 to 26 (212, 213). Fullereneol generated using the Kitazawa method was investigated due to its potential for proliferation supported by its many documented applications including augmentation of semiconductor activity (24, 25), near infrared radiation-induced combustion (215), and detection and treatment of malignancies (216), illustrating a potential for biological exposure. Despite having beneficial applications, fullereneol has been studied in terms of toxicological effects with results varying considerably from no adverse effect on bacteria species at 5 mg/L (53) or on human dermal fibroblasts at 5,000 mg/L (112) to greater than 50% mortality of larval zebrafish at 0.01 mg/L (107) and an EC\textsubscript{50} = 15 mg/L acting on human umbilical vein endothelial cells (111).

Reports have shown that the chemical composition analysis of laboratory-synthesized fullereneol using the Kitazawa method produced structures exceeded the complexity claimed by commercially-available material including protonated (74, 205, 217), non-hydroxylated mono-
oxygenated (31, 74, 83, 205, 218-220), and di-oxygenated (30, 74, 83) surface groups. As
fullerene materials are currently unregulated in the United States, this material and by-products
generated from the synthesis process may be discharged to the environment, representing a
critical pathway for fullerene exposure and in determining the overall potential for biological
impact. However, no information was presented in reviewed literature on the physicochemical
properties of fullerenol synthesis by-products, presenting a critical deficiency in fullerenol
characterization. It was found that determination of the physicochemical properties of fullerenol
materials remained inconclusive specifically in terms of solubility and capacity for
agglomeration in aqueous solution (74, 83, 119, 205, 218-219) as well as the impact
concentration has on surface charge (34, 55, 101, 221, 222). The solubility of nanoparticles was
shown to impact strongly the cytotoxic response of mammalian cells (223), suggesting that the
more insoluble a material is and, in a related manner, the greater the degree of agglomeration, the
lesser the potential for biological impact, elucidating the importance of greater understanding of
fullerenol physicochemical properties. The physicochemical characteristics of fullerene were
explored with the hypothesis that mean fullerenol agglomerate size, a factor in assessing
solubility, and surface charge, as represented by zeta potential, were impacted by concentration
of aqueous fullerenol. This investigation examines physicochemical properties of fullerenol
synthesized in the laboratory from underivatized fullerene (C\textsubscript{60}) as well as commercially-
available fullerenol in an effort to provide a foundation for understanding the biological systems
associated with fullerenol and fullerenol synthesis by-products.

Materials and Methods

Chemicals

Underivatized fullerene C\textsubscript{60} was purchased from Nano-C (Westwood, Massachusetts).
Toluene, tetrabutylammonium hydroxide (TBAH), sodium hydroxide (NaOH), methanol
(MeOH), hydrochloric acid (HCl), and potassium bromide (KBr) were purchased from Fisher Scientific (Waltham, Massachusetts). Derivatized fullerene samples to be used for comparison were purchased from Nano-C (Westwood, Massachusetts), labeled by the manufacturer as “Hydroxylated C_{60}(OH)_{22/24}”, and from BuckyUSA (Houston, Texas), labeled by the manufacture as “Polyhydroxy-C_{60}(OH)_n, n \approx 18-22”.

**Fullerenol Synthesis**

Fullerenol was synthesized by a “modified” Kitaoka Method, shown in Figure 3-1. Briefly, 80 mg of C_{60} was stirred at 300 rpm with 60 mL of toluene, used in place of the more carcinogenic benzene (37-39, 74, 79, 155, 167, 203) presented by Li et al. (214), at 22 ± 2 degrees Celsius for 21 ± 1 min. Once C_{60} aggregates were no longer visible in the stirring vessel and the solution attained a purple color, 0.2 mL of 0.1N TBAH and 2 mL of concentrated NaOH (25M in de-ionized water) was added and stirred for 3 minutes at 300 rpm, until a brown precipitant formed at the bottom of the stirring vessel. The clear toluene layer was decanted and the precipitant was dried for 24 hours in a vacuum chamber. After drying, the material was bathed for 10 hours in 10 mL of de-ionized water then vortexed with an additional 20 mL of de-ionized water. The mixture was filtered using a MilliporeTM 0.45 µm-nitrocellulose membrane (Billerica, Massachusetts) and rinsed with 50 mL MeOH, vortexed, and centrifuged for 20 minutes at 10,000 rpm, a process that was repeated three times. The final residue was vacuum-dried for 24 hours, yielding a mean mass of 188.1 ± 30.0 mg for the three batches used in subsequent biological investigations.

**Physicochemical Characterization**

Aliquots of fullerenol samples were analyzed in triplicate for agglomerate size distribution through dynamic light scattering (DLS) with a NanoTrac Particle Size Analyzer (MicroTrac, Inc., York, Pennsylvania) at 90-second sample runs (n = 3), using a refractive index of 1.93 (125,
Samples of various concentrations were analyzed for pH, using an Accumet© Research AR-50 obtained through Fisher Scientific (Pittsburgh, Pennsylvania), and zeta potential by means of measuring electrophoretic mobility using a Brookhaven ZetaPlus Zeta Potential and Particle Size Analyzer (Holtsville, New York), which relies on DLS technology and converts mobility to zeta potential using the Smoluchowski relationship:

\[ \zeta = \mu_e \eta \varepsilon_r^{-1} \varepsilon_0^{-1} \]  

(3-1)

where \( \zeta \) is zeta potential, \( \mu_e \) is electrophoretic mobility, \( \eta \) is kinematic viscosity of the medium, \( \varepsilon_r \) is relative permittivity of the medium, and \( \varepsilon_0 \) is the dielectric constant.

Fullerenol samples were analyzed by UV/visible light (UV/Vis) spectroscopy, X-ray photoelectron spectroscopy (XPS), Fourier-transform infrared (FTIR) spectroscopy to assess chemical composition and to validate data against known results from previous investigations. UV/Vis absorption spectra were generated at room temperature in the range of 300 to 800 nm at 1.0 nm intervals using a Perkin-Elmer UV/Vis Spectrophotometer Lambda 800 (Waltham, Massachusetts). Binding energy spectra for surface moiety identification was obtained from Rayleigh-scattered electrons using a Thermo Electron Magna 760 FTIR/FT-Raman/FTIR Microscope (Waltham, Massachusetts) in a KBr carrier pellet and analyzed using the software OMNIC package (ALS, Berkeley, California). Binding energy spectra for molecular quantification were obtained using a Perkin-Elmer PHI 5100 ESCA (Electron Spectroscopy for Chemical Analysis) System (Waltham, Massachusetts) excited with monochromatic aluminum K\( \alpha \) X-rays and deconvoluted with the computer software Grams/AI version 7.01 software (Adept Scientific, Inc., Bethesda, Maryland). Binding energies were identified through comparison with archived information maintained by the National Institute of Standards and Technology XPS Database (Gaithersburg, Maryland).
Statistical Analysis of Physicochemical Characterization

Data generated on the physicochemical characteristics of fullerol and fullerol synthesis by-products were scrutinized for outliers using Dixon’s Q-test (168) and analyzed for inclusion based on defensibility of results. The Ryan-Joiner normality test, an application in the software program Minitab 15.1 (State College, Pennsylvania), was used to test the normality of the data and Bartlett’s test, an application in the software program SPSS (SYSTAT, Chicago, Illinois), was used to test homogeneity of variance. Means were compared for difference using the student’s \( t \)-test \((\alpha < 0.05)\) while multiple groups had means tested using Dunnett’s program version 1.5 (United States Environmental Protection Agency, Washington, D.C.).

Results and Discussion

Agglomerate Size Analysis

Results from DLS indicated that fullerol displayed different agglomerate characteristics depending on the concentration in solution. At 2 mg/L, primary agglomerate size was shown to be 1.34 nm with all agglomerates registered between 1.04 to 6.39 nm (Figure 3-2). At 20 mg/L, 92.6% of the material was shown to be at 1.34 nm and a discernable, secondary peak at 2.79 nm accounted for an additional 4.9% of the material with some agglomerates shown to be as large as 11.7 nm, roughly nine molecules in diameter. At 100 mg/L, the primary agglomerate size was shown to be 10.7 nm, representing an agglomerate eight molecules in diameter, with a range from 8.28 to 21.5 nm. At 200 mg/L, the peak spanned two size bins, from 27.9 nm to 30.4 nm with an overall range of agglomerates from 23.4 to 486 nm. At 2,000 mg/L, the agglomerate size distribution was clearly bimodal, with one peak at 36.1 nm and a second, slightly less august peak at 102 nm with a range of agglomerates from 33.1 to 315 nm. While dilute concentrations were shown to be soluble in water as individual fullerol molecules (i.e. 1.34 nm in diameter), solutions greater than 2 mg/L displayed increasing agglomeration with increasing concentration.
Determination of the precise concentration at which agglomeration commenced requires sensitivity analyses of concentration as well as other factors such as temperature, degree of derivatization, total volume, and exposure to atmospheric conditions. Brant (29) showed a distribution peak at 100 nm with a fullerenol concentration of 44 mg/L noting pH independence and indicating forces stronger than hydrogen bonding, such as hydrophobic forces, were driving agglomeration. Kokubo (83) noted fullerenol agglomerate sizes ranging from molecular to greater than 100 nm with concentrations in excess of 1,000 mg/L. Assemi (222) indicated a mean fullerenol agglomerate size at a concentration of 1.3 mg/L was 2.3 nm and Zhang (224) noted 100-nm diameter agglomerates of C$_{60}$(OH)$_8$ fullerenol at concentrations as low as 12.8 mg/L. This affinity for clustering at higher concentrations suggested that the amphiphilic surface of fullerenol persists even at high degrees of derivatization, enabling clustering to minimize polarity differences.

Select samples were sonicated to assess the impact of an alternate mechanical energy infusion on the material as several studies have shown that sonication of fullerene agglomerates reduces mean agglomerate size (29, 44, 46, 48). After sonication, the primary agglomerate size of the 20 mg/L solution increased from 1.34 to 10.7 nm with a range from 8.28 to 11.7 that encompassed 86% of all material, indicating that individual molecules amassed upon sonication (Figure 3-3). Similarly, the primary agglomerate size of the 100 mg/L solution increased from 10.7 to 102 nm with a range from 85.9 to 133 that encompassed 84% of all material. Both concentrations displayed bimodal agglomerate sizes prior to sonication but more normalized agglomerate distributions were seen after the addition of mechanical energy. Given that the 20 mg/L mixture, which was mostly molecular prior to sonication, increased in diameter to that of the 100 mg/L sample without sonication (i.e. 10.7 nm), interest was piqued, resulting in the
proposition that such a phenomenon was more than mere coincidence. The pre- and post-sonication data were amalgamated and three primary size peaks were identified at 1.34, 10.7, and 102 nm (Figure 3-4), suggesting preferential agglomeration sizes based on factors such as charge, steric conformation, and supplemental derivatization. Due to the limited material sampled, future studies may seek to expand available data and examine factors such as electrolytic concentration and complexation with organic compounds to determine characteristics including physical stability, chemical reactivity, and capacity for photo-activation.

**Zeta Potential Analysis**

The zeta potential of laboratory-prepared fullerol was -34.8 ± 6.9 at the lowest examined concentration (0.2 mg/L). As concentration was increased, zeta potential increased (Figure 3-5), capped at -13.9 ± 1.7 at 2,000 mg/L, indicating decreased stability of the agglomerates. Lecoanet *et al.* (221) indicated that the zeta potential of fullerol was not detectable, but others have reported fullerol zeta potential ranging from -50 mV (34) to -19.6 mV (55) with range validation provided by still other investigators (101, 222). None of the reports reviewed discussed the effect of fullerene concentration on zeta potential, although researchers have described the impact of electrolyte concentrations and preparation method on fullerene agglomeration (34, 37, 43, 46). Medrzycka (225) noted that zeta potential was a function of concentrations of particles in dilute solution while Kitabara *et al.* (226) indicated that zeta potential of surfactants in non-aqueous solutions varied with modified concentration. Busscher *et al.* (227) described the interdependence of zeta potential and concentration in hydrocarbons. Pertaining to biological systems, Haydon (228) discussed indiscriminant adhesion of anionic particles, which fullerene derivatives have been shown to be, to the negatively charged surface of cell membranes due to charge density and the multiplicity of cell surface binding sites.
As agglomerate size was shown to increase with concentration, it was logical to expect that the larger agglomerates would be less stable. As each individual fullerenol molecule, represented in the 0.2 mg/L concentration (diameter = 1.3 nm), was shown to be have a negative zeta potential, the clustering of these negatively charged molecules into an agglomerate creates internal instability as molecules are forced together through hydrophobic forces but forced apart by electrostatic repulsion. It was noted in the fullerenol size analysis that fullerenol migrated preferred agglomerate sizes (10.7, 30.4, and 102 nm) at concentrations between 20 and 2,000 mg/L, suggesting that the stability of these sizes was greater than other sizes between these marks. However, the structural stability suggested in the size analysis did not hold when the charge of these agglomerates was inspected. It was possible agglomerates initially formed outside of the peak ranges had higher zeta potential than those within the peak ranges and either attracted other smaller fullerenol clusters to attain a more stable configuration or disaggregated to a smaller, more stable size.

The pH of several prepared fullerenol solutions of low concentration (2 mg/L) was modified through the addition of HCl or NaOH, ranging from pH = 3 to 9.5 (Figure 3-6). As shown in other studies (29, 222, 229), the zeta potential of fullerenol was pH-dependent, with a overall minimum occurring at slightly alkaline conditions, and with decreasing negative charge as the hydrogen ion concentration deviated from the point of minimum charge (pH = 8.0). Technically, the addition of NaOH, used to increase the pH to 9.5, did not modify the hydrogen ion content directly but, rather, through the addition of hydroxyl ions to the solution, two events were possible. First, the available hydrogen ion (hydronium) concentration was reduced, as newly available hydroxyl ions from the NaOH were able to associate with the excess hydronium, increasing pH. Alternately, the potential for additional hydroxyl ions to become bound to the
carbon cage as surface groups existed. As hydroxyl ions are negatively charge and expected to associate with an electron deficient portion (high electrophilic region) of the carbon cage, the anticipated result was that the surface would become increasingly negative with the addition of hydroxyl groups. It was possible that such an increase in negative charge might have occurred during an increase in pH from 3 to 8; however, the original solution was at pH = 7 and ions, either from HCl or NaOH, were added accordingly until the designated ion concentrations was achieved. The initial addition of NaOH to achieve a pH of 8 reduced zeta potential 26.1% but subsequent addition increased the surface charge to a measurement less negative than that of the neutral solution. Addition of HCl may have caused hydroxyl groups to dissociate from the surface, reducing zeta potential. Alternately, excess hydrogen ions may have become bound to the fullerenol surface, lowering the overall charge of the material. Evidence of protonation of the fullerene cage has been noted (29, 30), which offered an equally plausible explanation for the low-pH charge modifications.

**Ultraviolet/visible Light Spectroscopy Analysis**

Ultraviolet/visible light absorption spectra for laboratory-prepared fullerenol were analyzed to assess differences in optical density based on chemical structure. It should be noted that analyses of fullerenol species performed using the Perkin-Elmer UV-Vis Spectrophotometer Lambda 800 (Waltham, Massachusetts) were done without the assistance of standards and, while this deficiency limited the ability of absolute comparison, all measurements were relative, based on arbitrary absorbance. Three independently prepared fullerenol samples (fullerenol-PAI #1, fullerenol-PAI #2, and fullerenol-PAI #3) were examined (Figure 3-7) after determination of a concentration (20 mg/L) that offered the distinct fullerenol spectrum morphology (24, 67, 83, 155, 167, 218), that is, a smooth curve with less intensity at lower energy wavelengths, increasing with energy (Figure 3-8) without generating absorbance values outside the detection
limit. Samples with concentrations > 20 mg/L proved too dense optically at higher energy wavelengths and, thus, were omitted from this element of investigation. As can be discerned from the inset (Figure 3-7), very little relative difference was noted in the independent preparations, although the long-wavelength visible light range (> 500 nm) appeared to be less intense for PAI #1 than for either of the latter synthesis products.

The mean of laboratory-prepared fullerenol samples \((n = 3)\) was compared to commercially-available fullerenol obtained from Nano-C and BuckyUSA, two materials prepared using the same synthesis procedure (i.e. “the Kitazawa Method”) \((214)\). Fullerenol-\(\mu_{PAI}\) (the mean, \(\mu\), of the three independently prepared fullerenol samples) and fullerenol-Nano-C traced almost identically \((\Delta A = 0.099 \pm 0.062)\), despite being statistically different, with a sum-of-error-squared (PAI to Nano-C) of 7.54, while the sum-of-error-squared for PAI\(\mu\) compared to BuckyUSA was 31.47 \((\Delta A = 0.176 \pm 0.161)\). Fullerenol-BuckyUSA exhibited a shallower increase in absorbance than the other two materials in the wavelength range from 300 nm to 500 nm but a notably sharper increase in the ultraviolet range (< 270 nm) while the rates of increase for fullerenol-PAI and fullerenol-Nano-C began to decrease over the same sector of the spectrum. These qualitative observations were made visually from the spectrum and verified by examination of individual data sets.

The differences in spectra, both among the independent, laboratory-prepared fullerenol samples as well as among the various commercially-available materials, suggested differences in material composition. Differential composition of fullerene entailed not only the number of surface groups, but also possibly the type (mono-oxygenated or di-oxygenated), location, and distribution of surface groups on the carbon cage. Fullerenol-PAI #1 absorbed relatively less visible light but more UV light than the other two laboratory-synthesized preparations. As was
surmised from the absorbance study on \textit{aqu-nC}_{60}, material capable of greater visible light absorbance was removed from the water-stirred fullerene samples upon filtration with a Millipore\textsuperscript{TM} 0.45 \textmu m-nitrocellulose membrane, which was deduced to be underderivatized fullerene and was consistent with the data generated in spectrophotometric analysis. Conversely, it was theorized that the more soluble, more hydrophilic, and more derivatized material was able to pass through the filter, thus providing greater absorbance in the ultraviolet range. Applying these findings to fullerenol, it was hypothesized that fullerenol-PAI #1, with lower absorbance in the visible light range, had fewer hydrophobic functional groups than other preparations. Similarly, it was hypothesized that BuckyUSA contained fewer hydrophilic functional groups than either fullerenol-PAI\textsubscript{m} or fullerenol-Nano-C.

**Chemical Composition Analysis - Fullerenol**

This research has presented two methodologies for the derivatization of fullerene: extended duration water-stirring (\textit{aqu-nC}_{60}) and using the Kitazawa method (214), which yields fullerenol (30, 31, 57, 73, 79, 82). The transformation of fullerene to \textit{aqu-nC}_{60} included several parameters such as stirring time and photocondition, which were shown to generate materials with differing chemical composition. Conversely, synthesis of fullerenol followed a prescribed formula, which should have yielded products with a consistent chemical composition. Fullerenol prepared in the laboratory through the Kitazawa method (fullerenol-PAI) was synthesized in triplicate for statistical purposes. The only difference in the synthesis process from that which was presented in the literature was the use of toluene for dispersing fullerene (C\textsubscript{60}) as an alternate solvent for the more hazardous benzene, a substitution that was not expected to yield variable products as it was used consistently in each preparation. However, it was seen from the UV/Vis spectra that
the materials, within groups and between groups, provided varied responses to the range of wavelengths, suggesting differentiation within the independently-generated products.

These materials were analyzed for chemical composition utilizing FTIR spectroscopy to assess specific surface moieties, and XPS to quantify functional groups, leading to the proposition of the following generalized chemical formula assigned to represent the material:

$$C_{60}H_xO_y(OH)_z$$

(3-2)

where $x + y$ represents the total number of oxygenated surface groups, indistinguishable due to the analytical limitations described previously, and $z$ represents the number of protonated surface groups. Surface groups, which bind directly to the 60 carbon molecules that comprise the fullerene cage, were identified by FTIR and quantified as either mono-oxygenated or di-oxygenated moieties by XPS. Mono-oxygenated groups could be hydroxyl, epoxide, or ether groups or negatively charged, single-bonded oxygen ions, perhaps associated with sodium ions remaining in solution from the reactant NaOH, while di-oxygenated groups indicated the presence of carbonyl, carboxylic acid, or hemiketal groups. Additional information pertaining to the specific surface groups is available in Appendix B.

Epoxide groups provided a distinct FTIR peak as did di-oxygenated carbonyl and hemiketal groups and tri-oxygenated carboxylic acid. Mono-oxygenated groups required a single bond to carbon, which enabled the fullerene cage to remain completely closed. Di-oxygenated bonds, such as those formed with carbonyl or hemiketal groups, necessitated that the associated carbon relinquish its closed configuration, vacating its bond with an adjacent carbon, and initiating the degeneration of the carbon cage. Tri-oxygenated groups, which were generally limited to carboxylic acid and carbonates, were not considered associated with a closed, carbon cage as the carbon would only be connected to the rest of the system by a single bond. More
than one such moiety in a region could cause the cleavage of a section of the cage with further tri-oxygenation resulting in total degradation of the fullerene molecular structure. Since such a scenario has not been documented as occurring during the synthesis of fullerenol, tri-oxygenated groups were considered remnant sodium carbonate, disassociated in solution but precipitated during the drying process required for XPS equipment.

The chemical composition of fullerenol-PAI was compared to commercially-available fullerenol prepared using the Kitazawa method (214) and purchased from two manufacturers, labeled fullerenol-Nano-C and fullerenol-BuckyUSA. The three FTIR spectra for fullerenol-PAI (Figure 3-9) indicated similar peaks at 1061-1069 cm\(^{-1}\), 1452-1468 cm\(^{-1}\), 1692-1696 cm\(^{-1}\), and 2919-2979 cm\(^{-1}\), representative of C-O stretching (70, 74, 214), C-O-H bending (31, 64, 214), hemiketal groups (31, 205), and C-H stretching (70, 230), respectively, as well as common peaks noted at 1574-1596 cm\(^{-1}\) and 1692-1694 cm\(^{-1}\), indicating carbon-carbon double bonds (31, 64, 74, 177, 205, 214). Additional common peaks were noted in the spectra for PAI #1 and PAI #2 at 866-868 cm\(^{-1}\), which indicated C-O bonding (70), and 1412-1414 cm\(^{-1}\), another carbon-carbon double bond peak, as well as an unidentified peak at 686 cm\(^{-1}\). The spectrum for PAI #3 indicated another C-O-H peak at 1377 cm\(^{-1}\) (64, 175). The PAI #1 spectrum had two additional unassigned peaks at 778 cm\(^{-1}\) and 903 cm\(^{-1}\) while the spectra for PAI #2 and PAI #3 offered one additional unassigned peak each, at 882 cm\(^{-1}\) and 803 cm\(^{-1}\), respectively.

The spectral peaks for commercially-available fullerenol samples (Figure 3-10) showed consistency with those of laboratory-prepared material including peaks at 1063-1067 cm\(^{-1}\) (C-O-H), 1599 cm\(^{-1}\) (C=C), and 1659-1666 cm\(^{-1}\) (hemiketal). Additional peaks were noted in fullerenol-Nano-C at 776 cm\(^{-1}\), 1377 cm\(^{-1}\), and 3231 cm\(^{-1}\), corresponding to unassigned, C-O-H bending (74, 205), and O-H bending of water (74, 83, 214), respectively, whereas additional
peaks for fullerenol-BuckyUSA were observed at 686 and 866 cm$^{-1}$ (previously assigned), and water at 3233 cm$^{-1}$. It was shown through FTIR analysis that no fullerenol samples contained epoxide surface groups, which was reminiscent of 28-day stirred samples of $nC_{60}$. Hence, hydroxyl and ether groups and oxygen ions were possible mono-oxygenated functionalities, yet no procedure has been developed to differentiate conclusively between specific mono-oxygenated groups. In summary, functional groups found in all fullerenol samples included C-O, which could indicate hydroxyl or ether groups or, alternately, oxygen ions, and hemiketal groups as well as typical peaks seen in carbon-based nanomaterials (i.e. C-C, C=C). XPS spectra for the three fullerenol-PAI samples (Figure 3-11) indicated 21, 30, and 25 surface groups, respectively, with PAI #1 having five mono-oxygenated groups, PAI #2 having 13 mono-oxygenated groups, and PAI #3 having 15 mono-oxygenated groups. The XPS spectrum for fullerenol-Nano-C indicated 29 surface groups, 20 of which were mono-oxygenated, while fullerenol-BuckyUSA indicated 25 surface groups, 12 being mono-oxygenated (Figure 3-12).

The disparity among the laboratory-prepared fullerenol indicated that not all variables in the system were kept consistent. Uncontrolled factors that may have led to such variability included purity and stability of reactants ($C_{60}$, toluene, NaOH, TBAH, or MeOH), quality of de-ionized water, temperature, ambient laboratory light, and exposure to atmosphere. de-ionized water quality was monitored throughout the duration of the research with periodic maintenance to the equipment as prescribed by the manufacturer. Laboratory temperatures varied daily due to inadequate environmental control equipment and ranged from 16 to 27 degrees Celsius despite efforts to maintain constant temperature. Recorded laboratory temperatures on the days synthesis was initiated were 24, 19, and 26 degrees Celsius for PAI #1, PAI #2, and PAI #3, respectively. Interestingly, the total number of surface groups changed corresponding to the
inverse of temperature, although with the limited number of samples, statistically significant correlation could not be determined ($R^2 = 0.5438$). The methodology followed did not specify ambient lighting conditions; however, ambient light throughout the synthesis events varied beyond control due to shared laboratory facilities. Likewise, exposure to ambient air was not controlled and variability in the content of atmospheric oxygen, which has been shown to be a factor in the formation of oxygenated surface groups (114), was possible. While atmospheric air was not considered prior to synthesis, recommendations for further study include the use of a controlled environment including the use of composition-controlled compressed air or pure nitrogen. Furthermore, the impacts of environmental conditions on the chemical composition and rate of transformation by monitoring and altering the atmospheric temperature and recording the resulting surface group compositions.

In addition to the above described parameters, experimental error may have attributed to chemical composition variability in regard to measurement of materials, duration of stirring, and potential for contamination, despite the best efforts to avoid such occurrences. Operator error may be been encountered during outsourcing of samples for XPS analysis, which was unavoidable due to restrictions placed upon the equipment. Furthermore, material sampling error may have contributed to variability as a result of not taking representative samples and the limitation of only one XPS spectrum generated from each sample. Subsequent investigations should seek environmental laboratory conditions that are more controlled to ensure consistent synthesis products and resources that permit repetitive sampling as well as developing and supporting research plans that ensure statistical defensibility.

The importance of the ratio of di-oxygenated to mono-oxygenated surface group (R) relates to the hypothesis that the reactivity of fullerenol was a function of the degree of
hydroxylation (31). While the number of mono-oxygenated groups did not directly relate to hydroxyl groups and while there was no definitive, published method by which to ascertain the number of hydroxyl groups, the assumption that mono-oxygenated groups were critical to fullerene reactivity has been widely promoted in the literature (25, 70, 110, 111, 117, 167, 193) and, until conclusive discovery of the specific mechanisms of biological activity are presented, use of material with a low R (proportionately more mono-oxygenated groups than di-oxygenated groups) remains desired. Comparing mono-oxygenated to di-oxygenated groups in the fullerene samples, only PAI #3 and Nano-C displayed a ratio less than one, while the remaining samples indicated R > 1. In fact, PAI #1 showed R = 3.2, suggesting that it possessed less functionality as a biological agent than PAI #3 (R = 0.4). The only material that provided a lower ratio was fullerene-Nano-C (R = 0.31).

**Chemical Composition Analysis – By-Products**

The three by-products generated through the alkali synthesis of fullerene (214), labeled BP #1, BP #2, and BP #3, were qualified for surface groups through FTIR spectroscopy (Figure 3-13). Due to the disparity of material comprising each by-product only two spectral peaks were common among the samples, which occurred at 835-869 cm\(^{-1}\), representative of C-H out-of-plane bending (175), and at 1666-1673 cm\(^{-1}\), indicating hemiketal moieties (31, 205). Additional peaks identified with commonality were found at 1032-1033 cm\(^{-1}\) (BP #1 and BP #2), representing ether groups, 1365-1384 cm\(^{-1}\) (BP #1 and BP #2) and 1453-1461 cm\(^{-1}\) (BP #2 and BP #3), representing hydroxyl groups, 2960 cm\(^{-1}\) (BP #2 and BP #3), indicative of C-H stretching, and 3300-3400 (BP #1 and BP #1), suggesting the presence of water within the samples and indicating incomplete desiccation during vacuum-drying.

Additional spectral peaks were noted at 706 cm\(^{-1}\) (BP #1), 707 cm\(^{-1}\) (BP #2), 903 cm\(^{-1}\) (BP #3), and 999 cm\(^{-1}\) (BP #3), all of which were previously unassigned as being associated with
derivatized fullerene. The synthesis reactants toluene, TBAH, and NaOH all presented FTIR spectral peaks in the range of 702-738 cm\(^{-1}\) (Figure 3-14), suggesting at least one of the reactants were present in each of these by-products. Subsequent FTIR spectra from mixtures of C\(_{60}\)-TBAH as well as toluene-TBAH-NaOH (Figure 3-15) indicated a 702-704 nm peak. While peaks from pure NaOH samples and C\(_{60}\)-TBAH most closely resided in the range of these by-products, BP #1 was known to be primarily toluene, suggesting that all reactants (toluene, TBAH, and NaOH) were present in BP #1. The inclusion of the 702-704 cm\(^{-1}\) peak in BP #2, which consisted of the filter residues, also suggested the presence of toluene. The BP #3 spectrum was absent of this peak, but did contain an unassigned peak in common with fullerenol-PAI #1 and PAI #2, suggesting that the methanol washes that generated BP #3 removed some derivatized fullerenol during purification.

The unassigned peak seen in BP #3 at 903 cm\(^{-1}\) was also observed in PAI #1. A similar peak was found in the unadulterated TBAH samples (922 cm\(^{-1}\)) as well as in the C\(_{60}\)-TBAH mixture (928 cm\(^{-1}\)), providing strong evidence for the presence of the reactant TBAH in at least one of the end products (i.e. PAI #3). The inclusion of a catalytic reactant in the final product suggests incomplete purification, which was possibly caused by compromised purification chemicals (e.g. water, MeOH). To ensure the removal of reactants from the synthesis stream, additional purification steps may wish to be included during future experiments including additional methanol washes or the use of size-exclusion separator such as the one offered by Sephadex, a dextrose gel manufactured by Pharmacia (Stockholm, Sweden) and distributed by Fisher Scientific (Waltham, Massachusetts).

Perhaps most interestingly, additional peaks were observed in two of by-products (BP #1: 1622 cm\(^{-1}\); BP #2: 1632 cm\(^{-1}\); BP #2: 1736 cm\(^{-1}\)) that were attributable to carbonyl (C=O)
moieties, the first observation of carbonyl in this investigation. The presence of carbonyl in association with fullerol has been mentioned extensively by others (30, 31, 67, 74, 83, 124, 177, 205, 230), but since no carbonyl bonds were noted in the final products by FTIR or in any of the reactants, it was hypothesized that the formation of a carbonyl moiety resulted from the interaction of one or more of the reactants. Subsequent FTIR spectra from a mixture of C\textsubscript{60} and TBAH indicated a peak at 1655 cm\textsuperscript{-1}, which was reminiscent of the 1622-1632 cm\textsuperscript{-1} carbonyl peaks, suggesting the presence of TBAH in the carrier toluene (i.e. BP #1) as well as in the filter residue (BP #2). The FTIR spectrum for the toluene-TBAH-NaOH mixture indicated a peak at 1744 cm\textsuperscript{-1}, very near to the 1736 cm\textsuperscript{-1} peak in BP #2, reinforcing the presence of TBAH in BP #2. However, the toluene-TBAH-NaOH mixture was void of fullerene, suggesting two or more reactants interacted and resulted in a double-bonded oxygen surface carbonyl moiety.

XPS spectra for fullerol synthesis by-products (Figure 3-16) indicated that derivatized fullerene passed through the purification processes, suggesting that the Kitazawa method did not present maximum material recovery. As preparation for analysis of the phase-separated toluene extract (BP #1), toluene was permitted to evaporate \textit{en vacuo} at ambient laboratory temperatures (< 25 degrees Celsius), leaving a residual with a mass << 1 mg; due to limitations of gravimetric techniques, the exact mass was indeterminable. Despite being available in quantities that proved inadequate for complete physicochemical assessment in concert with the other material, characterization of the material in terms of chemical composition was conducted. The spectrum for the (BP #1) showed 17 surface groups attached to carbon, 11 of which were mono-oxygenated (hydroxyl, ether, or oxygen ions) and six were di-oxygenated hemiketal groups. These molecules were adequately derivatized (> 25% of the carbon atoms were associated with oxygen), but it was suspected that the degree to which they were derivatized did not permit
residence in the aqueous phase, instead preferring the non-polar toluene solvent phase. It should be noted that BP #1 also contained TBAH and NaOH and it was suspected that there was trace toluene entrained with the residual material. Recovery of this fullerene material could be attained through the use of selective ion exchange in highly dilute aqueous solution to extract the sodium and tetrabutylammonium ions. While vacuum-drying was expected to remove toluene, more hygroscopic desiccants, lower pressure, or higher temperatures could prove more efficient.

The XPS spectrum for the filter residue (BP #2) displayed a total of 30 surface groups, evenly split between mono-oxygenated and di-oxygenated moieties. Precipitants captured by the Millipore™ 0.45 µm-nitrocellulose membrane included agglomerates larger than 450 nm as well as those slightly smaller, entrained by filtered residues. Based on XPS analyses, water-soluble material, determined as such by persistence in the aqueous phase and by having more functional groups (both mono-oxygenated and di-oxygenated) than the commercially-available fullerol-BuckyUSA, was identified in the filter residual. Due to the high concentration of material filtered, estimated to be 188.1 ± 30.0 mg of fullerol plus an additional 251.7 ± 14.8 mg residue per 32 mL (C_total = 15,143 mg/L), a considerable degree of agglomeration was likely to occur, resulting in 57.2% of the material being removed during filtration. Future synthesis of fullerol using the Kitazawa method (2/4) may choose to dilute the suspension prior to filtration to a concentration below that which agglomeration occurs (e.g. < 20 mg/L). While such a process may prove to be more resource intensive, the commercial value of raw material ($25/g for C_{60}$) and end product ($1,375 to $2,500/g) may warrant improvement in synthesis efficient. This material was shown through FTIR to contain toluene, TBAH, and NaOH, which would need to be removed from the final residual through purification prior to recycling of material for reuse.
The spectrum for the MeOH rinse (BP #3) indicated that fullerene residual contained 22 surface groups, all of which were mono-oxygenated. As methanol was the most water miscible of all alcohol species (log $K_{ow} = -0.69$), it was assumed that fullerenol was equally soluble in MeOH as with water. Each bath occurred in 50 mL of MeOH, resulting in a mean concentration of 3,762 mg BP #3/L MeOH, still adequately high to promote agglomeration and inhibit complete molecular contact with the MeOH intended to remove reactants. Further endeavors into the synthesis of fullerenol by the Kitazawa method may seek to use a larger volume of MeOH (i.e. $>> 50$ mL) to promote solubilization and ensure efficient contact with fullerenol, potentially leading to a higher quality end product. The absence of any di-oxygenated groups in BP #3 was unique for the fullerenol materials analyzed, suggesting unique processes occurred during purification. First, it was possible that all fullerenol material with di-oxygen surface groups was extracted during the filtration process. The likelihood of such an occurrence was determined to beyond realization as di-oxygenated groups were identified in the final fullerenol present after filtration. Another possibility was that MeOH reacted with fullerenol, causing the di-oxygenated groups to relinquish their bonds with the carbon cage or to transform to mono-oxygenated moieties. Also likely was that the single sample of BP #3 analyzed was not representative of the overall material, indicating experimental error influenced the measurement. It was determined that a potential for contamination of the samples existed during operator handling.

Assuming that the integrity of the results were not compromised, BP #3 provided the first mono-oxygenated fullerenol sample generated by the Kitazawa method during this research including those synthesized by commercial manufacturers (Nano-C and BuckyUSA) and by numerous other researchers within the research team working on fullerene and fullerenol through
the Particle Engineering Research Center at the University of Florida. Fortunately, the mass of BP #3 material recovered was 60.2 ± 6.1 mg per batch, indicating adequate material per synthesis event to conduct not only physicochemical analyses but also, as will be seen in the following chapter, biological characterization.

As fullerenol-BuckyUSA contained only one more mono-oxygenated surface group than insoluble BP #1 yet persisted in the aqueous phase, it is suggested that the number of mono-oxygenated groups does not adumbrate solubility. It was possible the determining factor in aqueous solubility of fullerene species was the total number of surface moieties; there were 71% more total surface groups in fullerenol-BuckyUSA than in BP #1. The fullerenol with the fewest number of surface groups was fullerenol-PAI #1, which was determined to have 5 mono-oxygenated and 16 di-oxygenated groups, only 23% more total surface groups than BP #1, and, yet, was water soluble. As a comparison, the waste material generated from the synthesis of \textit{aqun}C_{60}-28L (WASTE-\textit{n}C_{60}-28L) was estimated to have 9 surface groups, all mono-oxygenated and was decidedly insoluble in water.

\textbf{Summary}

Fullerenol products, laboratory-synthesized and commercially-available, were shown to have high variability in chemical composition, both in terms of total and mono-oxygenated surface groups. Uncontrolled ambient laboratory temperature may have contributed to differences in chemical composition, although the number of samples analyzed was inadequate to provide statistical significance. Neither temperature nor composition of atmospheric air was considered factors prior to synthesis; hence, it is recommended that future research include these parameters to assess impacts on chemical composition and conversion efficiency. While dilute concentrations of fullerenol-PAI were shown to be soluble in water as individual molecules (i.e. 1.34 nm in diameter), solutions greater than 2 mg/L displayed increasing agglomeration with
increasing concentration. Since the greatest number of surface groups in the insoluble by-products was less than the lowest number of soluble fullerenol, it was proposed that solubility of fullerene species occurs at a threshold between 16 and 21 surface groups. Additional investigation into factors that influence aqueous solubility of fullerene species has been shown to be warranted. Sonication of fullerenol solutions increased agglomeration and caused fullerenol agglomerates to settle in either 10.7 nm or 102 nm sizes, suggesting agglomerates of these sizes were more stable and, hence, energetically more favored, which was supported by zeta potential measurements. Determination of the precise concentration at which agglomeration commences requires sensitivity analyses of concentration as well as other factors such as temperature, degree of derivatization, total volume, and exposure to light. Future efforts may wish to explore mechanisms controlling preferential fullerenol agglomerate sizes using variables such as electrolytic concentration and organic compound complexation to determine characteristics, which may include physical stability, chemical reactivity, and capacity for photo-activation. While this report presented the first known data on the relationship between fullerenol concentration and zeta potential, more in-depth sensitivity analysis is warranted to assess how higher concentrations, which may be necessary to affect desired biological responses, impart effect.

Carbonyl surface moieties appeared in by-products of fullerenol synthesis (BP #1 and BP #2) but not in the end-products of any of the independent synthesis trials, supporting previous reports that such surface groups were present; phase separation and filtration may serve to distinguish them, although to what end was not apparent. Investigators may seek to determine how these purification methods selectively removed carbonyl-containing molecules or whether, in fact, the presence of carbonyl was independent of fullerene and a result of toluene, TBAH,
and/or NaOH interaction. Reactants were present in all three by-products generated from the synthesis process, as would be expected, but also appeared in at least one of the end products (PAI #3), suggesting that the triplicate rinsing in MeOH prescribed by the Kitazawa method did not ensure purification. The only purely mono-oxygenated fullerenol material generated was the by-product from the MeOH washes (BP #3), indicating additional investigation into the by-products may yield material with additional applications. This synthesis process was shown to warrant additional attention in terms of hazard, material conversion, and final product recovery; improvements to the method could include use of alternate non-polar solvents, pre-filtration dilution, and supplemental purification.
Table 3-1. Summary of degrees of derivatization of laboratory-prepared fullerol, commercially-available fullerol, and fullerol synthesis by-products.

<table>
<thead>
<tr>
<th>Fullerene Species</th>
<th>Mono-oxygenated Carbon</th>
<th>Di-oxygenated Carbon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waste: nC60 - 14L</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Waste: nC60 - 28L</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Fullerenol - PAI #1</td>
<td>5</td>
<td>16</td>
</tr>
<tr>
<td>Fullerenol - PAI #2</td>
<td>13</td>
<td>17</td>
</tr>
<tr>
<td>Fullerenol - PAI #3</td>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td>Fullerenol - Nano-C</td>
<td>20</td>
<td>9</td>
</tr>
<tr>
<td>Fullerenol - BuckyUSA</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>Fullerenol - BP #1</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>Fullerenol - BP #2</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Fullerenol - BP #3</td>
<td>22</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 3-1. Graphical depiction of fullerenol synthesis by “modified” Kitazawa method (214).

Figure 3-2. Agglomerate size distribution of fullerenol synthesis by “modified” Kitazawa method (214). Various concentrations determined by dynamic light scattering.
Figure 3-3. Agglomerate size distribution of fullerenol synthesized by “modified” Kitazawa method (214) before and after sonication. Various concentrations determined by dynamic light scattering.

Figure 3-4. Amalgamated agglomerate size distribution of fullerenol synthesized by “modified” Kitazawa method (214). Data consists of results from samples of various concentrations determined by dynamic light scattering.
Figure 3-5. Zeta potential of fullerenol synthesized by “modified” Kitazawa method (214) as a function of concentration.

Figure 3-6. Zeta potential of fullerenol synthesized by “modified” Kitazawa method (214) as a function of pH.
Figure 3-7. Ultraviolet/visible light spectrum for fullerenol of varying concentrations. Samples synthesized by “modified” Kitazawa method (214.) Inset indicates individual aliquots of fullerenol.

Figure 3-8. Ultraviolet/visible light spectrum for fullerenol. Fullerenol samples synthesized by “modified” Kitazawa method (214) with commercially-available samples.
Figure 3-9. FTIR spectra for laboratory-prepared fullerenol. Fullerenol samples by “modified” Kitazawa method (214). A) Fullerenol-PAI #1. B) Fullerenol-PAI #2. C) Fullerenol-PAI #3.
Figure 3-10. FTIR spectra for commercially-available fullerenol. A) FTIR spectrum for fullerenol-Nano-C. B) FTIR spectrum for fullerenol-BuckyUSA.
Figure 3-11. XPS spectra for laboratory-prepared fullerol. Samples by “modified” Kitazawa method (214).  A) Fullerol-PAI #1.  B) Fullerol-PAI #2.  C) Fullerol-PAI #3 (bottom).
Figure 3-12. XPS spectra for commercially-available fullerenol.  A) Sample obtained from Nano-C, Inc. (Westwood, Massachusetts).  B) Sample obtained from BuckyUSA, Inc. (Houston, Texas).
Figure 3-13. FTIR spectra for by-product of fullerol synthesized by “modified” Kitazawa method (214). A) By-product #1 (BP #1). B) By-product #2 (BP #2). C) By-product #3 (BP #3).
Figure 3-14. FTIR spectra for toluene, tetrabutylammonium hydroxide (TBAH), and sodium hydroxide (NaOH). A) FTIR spectrum for toluene. B) FTIR spectrum for TBAH. C) FTIR spectrum for NaOH.
Figure 3-15. FTIR spectra for toluene-tetrabutylammonium hydroxide-sodium hydroxide mixture and for Nano-C C_{60}-tetrabutylammonium mixture. A) FTIR spectrum for toluene-tetrabutylammonium hydroxide-sodium hydroxide mixture. B) FTIR spectrum for Nano-C C_{60}-tetrabutylammonium mixture.
Figure 3-16. XPS spectra for by-products of fullerenol. Synthesized by “modified” Kitazawa method (214). A) By-product #1 (BP #1). B) By-product #2 (BP #2). C) By-product #3 (BP #3) (bottom).
CHAPTER 4
BIOLOGICAL EFFECTS OF FULLERENE SPECIES ON SELECT INDICATOR SPECIES

Background

Fullerene derivatives have been the subject of considerable research over the past two decades due to novel biological properties, leading to their use as an antimicrobial (117), a radical scavenger (51), a therapeutic drug (146), a neuroprotective agent (139), and a contrast agent enhancer for magnetic resonance imaging (14) among other uses. This research has shown that by-products from the synthesis process for fullerenol, a hydroxylated fullerene species that can be produced through an alkaline reaction, and aqu-nC$_{60}$, a water-stirred fullerene, contain underivatized and derivatized fullerene species that can be released to the environmental and become assimilated in biological species (27, 85, 194). Fullerene and its derivatives are currently unregulated as are their synthesis by-products, with the exception of the reactants, which, once removed, enable discharge of fullerene species from municipal and industrial sources to receiving water bodies, presenting concern for potential ecological impacts (35, 36, 53, 127).

The biological effects of fullerene species have been reported on a variety of organisms with a range of results. For example, Sayes et al. (110) indicated that nC$_{60}$ imparted toxicological impacts on cells at concentrations four orders of magnitude less than fullerenol; however, subsequent research (100, 101) has demonstrated that toxicity from nC$_{60}$ synthesized with the facilitation of non-polar solvents may be due to residual solvent degradation products, not from fullerene. Few researchers have reported on the biological effects of aqu-nC$_{60}$ (27, 36, 105, 108, 109) or fullerenol (53, 107, 110, 111), with conflicting results ranging from no biological impact at 24 mg/L (108) to a minimum inhibitory concentration at 0.4 mg/L (36) for aqu-nC$_{60}$ and no biological impact at 5 mg/L (53) to an LC$_{50} = 50$ mg/L (110) for fullerenol on
bacteria, invertebrates, fish larvae, and human cells. To complicate conclusions regarding the biological effects of fullerene species further, multiple mechanisms of biological impact have been proposed (36, 122-124, 127, 132, 136, 137) including photoactivation (24, 25, 64, 65, 77, 115, 119-129), yet no consensus has been achieved within the scientific or medical communities as to the exact pathway leading to impacts. No comprehensive investigation has been presented that analyzed biological effects from acute and chronic toxicological assays on a range of biological organisms including unicellular bacteria and photosynthetic algae as well as higher order species such as invertebrates exposed to physicochemically characterized fullerene material (i.e. aqu-\textit{nC}_{60} and alkaline-synthesized fullerenol), which would enable legitimate comparison. Furthermore, no reports reviewed compared biological impacts of aqu-\textit{nC}_{60} prepared under different conditions (stirring duration and photocondition) or fullerenol prepared by different manufacturers (commercial and from underivatized material synthesized in the laboratory).

The biological impacts of aqu-\textit{nC}_{60} and fullerenol were studied using two distinct bacteria (Gram-negative \textit{Pseudomonas aeruginosa} and Gram-positive \textit{Staphylococcus aureus}), an algae (\textit{Pseudokirchneriella subcapitata}), and an invertebrate daphnid (\textit{Ceriodaphnia dubia}). Organisms were exposed to \textit{nC}_{60} stirred for 14 and 28 days in light, which were shown to possess different surface groups, and five fullerenol samples, three of which were prepared in the laboratory from the same batch of underivatized fullerene and two were commercially-acquired fullerenol prepared using the same alkaline synthesis process. Detailed descriptions of each biological species are provided in Appendix D. Chronic and acute toxicity assays were conducted, encompassing both dark-phase and light-phase exposure to capture potential impacts caused by fullerene species photo-reactivity. Nucleic acid dye assays were conducted on
bacteria under light and dark exposure to assess cell membrane damage and determine whether impacts were necrotic or apoptotic.

Materials and Methods

Chemicals

Fullerene (C$_{60}$) was obtained from Nano-C, Inc. (Westwood, Massachusetts) with 99.5% purity and commercially-available fullerenol was obtained from Nano-C, Inc. (Westwood, Massachusetts) and BuckyUSA, Inc. (Houston, Texas). Toluene, tetrabutylammonium hydroxide (TBAH), sodium hydroxide (NaOH), methanol (MeOH), tryptic soy broth, tryptic soy agar, phosphate buffered solution, sodium chloride (NaCl), isopropyl alcohol, magnesium perchlorate (Mg(ClO$_4$)$_2$), magnesium chloride heptahydrate, calcium chloride dihydrate, sodium nitrate, magnesium sulfate septahydrate, potassium phosphate, sodium bicarbonate, boric acid, manganese chloride tetrahydrate, zinc chloride, ferric chloride heptahydrate, cobalt chloride heptahydrate, sodium molybdate dihydrate, cupric chloride dihydrate, sodium ethylenediaminetetraacetic acid dihydrate, sodium selenate, calcium sulfate, magnesium sulfate, and potassium chloride were obtained from Fisher Scientific, Inc. (Waltham, Massachusetts). Wheat germ and a yeast-cereal-tetramin mixture were obtained from Aquatropics, Inc. (Gainesville, Florida).

Preparation of $n$C$_{60}$ and Fullerenol

Water-stirred fullerene, or aqu-nC$_{60}$, was synthesized by adding 100 mg of 99.5% purity C$_{60}$ to 500 mL of de-ionized water, and stirring at 550 rpm, the rate required to create a vortex equal to one-half of the water-column, a condition maintained throughout the stirring period, as proposed by Oberdorster et al. (27). The reactions were conducted in both the absence and presence of ambient laboratory light, although the yield from dark-stirred samples was inadequate to conduct toxicological assays; hence, only light-stirred samples were used for
biological investigation. Synthesis of fullerenol was conducted through the Kitazawa Method (214) with a modification in the solvent used for dispersion (Figure 4-1). Briefly, 80 mg underivatized C\textsubscript{60} was stirred at 22 ± 2 degrees Celsius with 60 mL of toluene at 300 rpm for 21 ± 1 minutes, until the solution obtained the characteristic purple color (6, 28, 79, 138) and no aggregates remained in the bottom of the reactor, indicating solubilization of the fullerene.

Toluene was used in lieu of benzene due to its ability to dissolve twice the mass of C\textsubscript{60} and has lower expected incidence of carcinogenicity (37-39, 74, 79, 155, 167, 203). Concentrated NaOH (2 mL of 25M in de-ionized water) was added along with 0.2 mL of 0.1N TBAH and the mixture was stirred at 300 rpm for an additional 3 minutes upon the formation of a brown precipitant. The toluene layer, which became clear, was removed through decantation and stored for physicochemical analysis. The precipitant was vacuum-dried for 24 hours, after which 10 mL of de-ionized water was added and stirred at 300 rpm for 10 hours. An additional 20 mL of de-ionized water was added, vortexed for 15 seconds, and filtered using a Millipore\textsuperscript{TM} 0.45 μm-nitrocellulose membrane. The residue was stored for subsequent analysis and the filtrate was purified three times with a rinse of 50 mL MeOH, each time vortex agitated for 1 minute, and centrifuged at 10,000 rpm for 20 minutes to separate fullerenol from entrained toluene, TBAH, NaOH, and MeOH.

**Analytes**

Each biological organism was exposed to a variety of fullerene species, which included 14-day water-stirred nC\textsubscript{60} in light (aqu-nC\textsubscript{60}-14L, or 14L), 28-day water-stirred nC\textsubscript{60} in light (aqu-nC\textsubscript{60}-28L, or 28L), laboratory-synthesized fullerenol, labeled fullerenol-PAI, the initials of the author, and commercially-available fullerenol, obtained from Nano-C, Inc. (Westwood, Massachusetts), labeled fullerenol-Nano-C, and BuckyUSA, Inc. (Houston, Texas), labeled fullerenol-BuckyUSA, collectively referred to as “fullerenol-CA”, as well as the materials
utilized in the synthesis of fullerenol, which included the surfactant TBAH, NaOH, and MeOH. Additional assays were attempted for the by-products from synthesis processes for both \( nC_{60} \) and fullerenol. By-products of \( aqu-nC_{60} \) synthesis were omitted from comprehensive analysis due to demonstrated insolubility of the material. Of the three distinct by-products generated from fullerenol synthesis, the first encountered sequentially (BP #1) consisted primarily of toluene, which yielded a residual mass below measurable limits when evaporated and was of inadequate mass to use as an analyte. The second by-product (BP #2) consisted primarily of NaOH, as was evidenced by the characteristic white powder remaining after evaporation, which was generated in quantities sufficient to be used in the biological assays. The third by-product (BP #3) was generated from repetitive rinses with MeOH and yielded adequate residual mass to justify toxicological investigation. Physicochemical characterization of these materials including agglomerate size, surface charge, and chemical composition analysis has been reported previously.

**Treatment Concentrations**

Preliminary population studies were conducted to find appropriate ranges of analyte concentrations for each of the four biological indicator species exposed to the analytes. Previously published reports (27, 36, 53, 102, 104-111, 194) were reviewed to assess expected ranges for concentrations to obtain an ideal concentrations that showed no impact to an upper limit encompassing at least 50% mortality, corresponding to an LC\(_{50}\) (concentration to kill 50% of the population compared to control samples). Five concentrations and a control were recommended by US EPA Methods 1002.0 and 1003.0 for algal and daphnid toxicity assays, which was extended to bacterial assays for consistency. Difficulty was encountered during preliminary population studies in assessing concentrations of some analytes that would cause 50% mortality due to an apparent lack of adverse effect; thus, practical limitations were
instituted. Due to the low concentration generated during synthesis, aqu-
C$_{60}$ samples were prepared in serial dilutions of 0.01, 0.1, 1.0, 10, and 100 mg/L while more abundant fullerenol, fullerenol synthesis by-products, and fullerenol synthesis reactants (except BP #1) were exposed to concentrations one order of magnitude higher (0.1, 1.0, 10, 100, and 1000 mg/L).

**Bacterial Chronic Toxicity Assays**

The ability of Gram-negative *P. aeruginosa* (ATCC 15442) and Gram-positive *S. aureus* (ATCC 6538) to grow in the presence of fullerene species was studied at pH = 7 using tryptic soy agar, which contained 17.0 g/L casein pancreatic peptone, 3.0 g/L soya papainic peptone, 5.0 g/L sodium chloride, 2.5 g/L dipotassium phosphate, and 2.5 g/L dextrose as carbon and electron sources. Culture purity was determined monthly through examination of cells through Gram-staining and observation using visible light microscopy. Specimens of *P. aeruginosa* (ATCC 15442) were obtained from Microbiologics, Inc. (Saint Cloud, Minnesota) in the form of a lyophilized pellet. Pellets were crushed in autoclaved borosilicate culture tubes, dissolved in tryptic soy broth, and incubated at 37 degrees Celsius in the absence of light while being agitated at 150 rpm on a reciprocating table-top shaker for 24 hours. Cultures were triply rinsed in de-ionized water, separated by centrifugation at 1,000 rpm for 5 minutes, and dispersed using a vortex shaker. Purified samples were plated on tryptic soy agar and incubated at 37 degrees Celsius in the absence of light for 24 hours. Bacteria were transferred by inoculating loop to autoclaved borosilicate culture tubes containing 4 mL tryptic soy broth, vortexed, and incubated in triplicate at 37 degrees Celsius in the absence of light while being agitated at 150 rpm for 24 hours. Specimens of *S. aureus* (ATCC 6538) were obtained from the Department of Microbiology at Shands Hospital at the University of Florida in cultured Petri dishes. Bacteria were transferred to borosilicate culture tubes with 4 mL tryptic soy broth and incubated for 24 hours at 37 degrees Celsius in the absence of light.
Serial dilutions of analytes were prepared and 3.6 mL of each sample was placed in autoclaved borosilicate culture tubes, to which 0.4 mL of cultured bacteria was added, vortexed, and agitated during incubation, as previously described. Negative control samples were prepared identically to the treated samples with the exclusion of analytes. De-ionized water was used as dilution media for controls as phosphate buffered solution was found to increase *P. aeruginosa* CFU propagation 38 times more than de-ionized water (data not shown). Cultures were triply rinsed as previously described and purified samples were plated on tryptic soy agar in triplicate and incubated at 37 degrees Celsius in the absence of light for 20 hours to obtain enumerable colonies. Colony-forming units (CFUs) were counted manually, multiplied by the dilution factor, and tallied. Determination of biological effect of the samples was assessed by comparing the mean CFU/mL of the treated samples to that of negative controls and recorded as “Population – Percent Change”. For each experiment, nine plates were prepared at each concentration as well as for controls. Tests were performed in triplicate for each sample and in triplicate for separately prepared analytes except for NaOH and MeOH, which were tested in duplicate for separately prepared material, and Nano-C and BuckyUSA fullerene, which were sampled once each. Statistical significance was determined using Dunnett’s program version 1.5 (United States Environmental Protection Agency, Washington, D.C.). LC$_{50}$ was determined, when applicable, using linear regression analysis.

**Bacterial Cell Membrane Integrity Assays**

The integrity of bacteria cell membranes was examined independently using the fluorescence-based LIVE/DEAD BacLight Bacterial Viability Assay (Molecular Probes, Carlsbad, California). This assay consisted of the addition of two nucleic acid dyes, SYTO 9 dye and propidium iodide, to treated bacterial samples and quantification of dye intensity using flow cytometry. The unencapsulated deoxyribonucleic acid (DNA) of healthy bacteria was dyed
green by membrane-permeable SYTO 9 dye while DNA of membrane-compromised cells were dyed red by membrane-impermeable propidium iodide. The use of propidium iodide has been shown as an effective indicator of cell-membrane damage in *P. aeruginosa* (231, 232) and *S. aureus* (231, 233). SYTO 9 dye is a proprietary material, but reports have documented its use in determining cell viability in *P. aeruginosa* (234, 235) and *S. aureus* (236, 237).

Briefly, bacteria suspensions treated with 100 mg/L fullerenol-PAI were prepared through centrifugation of 1 mL samples at 10,000 x g for 2 minutes to form bacterial pellets. One pellet was resuspended in 0.85% NaCl solution as a “live” cell sample and a complementary pellet was resuspended in 70% isopropyl alcohol as a “dead” cell sample, both incubated at 37 degrees Celsius for one hour, agitating every 15 minutes. Samples were pelletized via the same centrifugation process, rinsed in 1 mL 0.85% NaCl solution, centrifuged as before, and resuspended in 1 mL 0.85% NaCl solution. Bacterial samples (10 μL) were added to 987 μL 0.85% NaCl solution, 1.5 μL 30 mM propidium iodide, and 1.5 μL 3.34 mM SYTO 9 dye, incubated for 15 minutes at 20 ± 2 degrees Celsius, resuspended through vortex, and sonicated in a water bath for 5 minutes. Dyed bacteria were introduced to the LSR II Flow Cytometer (BD Biosciences, San Jose, California), excited at 488 nm, and fluorescence was collected using the green (fluorescein) and red (Texas Red) filters with logarithmic signal amplification.

**Algal Chronic Toxicity Assays**

Algal stock cultures (*P. subcapitata*) were obtained from Hydrosphere Research Bioassay Laboratory (Alachua, Florida). Algal cultures were maintained and utilized for assessing biological effect using EPA Method 1003.0, also known as the Wastewater Effluent Toxicity (WET) test (US EPA, 2002). Briefly, algae were cultured from dormant concentrated stock in Preliminary Algal Assay Procedure (PAAP) medium in an environmental chamber maintained at
25 ± 2 degrees Celsius under continuous "cool-white" fluorescent lighting of intensity 86 ± 8.6 μE/m2/s for six days. Stock cultures were grown in a 2-L Erlenmeyer flask receiving continual aeration and shaken by hand twice daily to avoid mass deposition and culture clustering at the solid-fluid interface. Algal samples were measured using a Multisizer 3 Coulter Counter with a 100-μm aperture and averaged over triplicate measurements. Culture purity was determined monthly through examination of algal cells using visible light microscopy. Replicate samples of algae were incubated with 50-mL of pre-determined concentrations of C60 species, fullerene synthesis materials (NaOH, MeOH, and TBAH), and a negative control diluted with PAAP medium under continuous "cool-white" fluorescent lighting of intensity 86 ± 8.6 μE/m2/s within an Erlenmeyer flask, hand-shaken twice daily for 96 hours. Initial algal populations were established at 10,000 cells/mL. Populations were measured, multiplied by dilution factors, and tallied.

Determination of biological effect of the analytes was achieved by comparing the mean algal concentration (cells/mL) of the treated samples to that of a control and recorded as “Population – Percent Change”. Tests were performed in triplicate for each sample and in triplicate for separately prepared sample materials with each sample measured three times (n = 27). Homogeneity of variance was determined using Bartlett’s Test and statistical significance was determined using Dunnett’s program version 1.5 (United States Environmental Protection Agency, Washington, D.C.). LC50 was determined, when applicable, using linear regression analysis.

Invertebrate Acute Toxicity Assays

Invertebrates (C. dubia) were obtained from Hydrosphere Research Bioassay Laboratory (Alachua, Florida). Daphnids cultures were maintained and utilized for assessing biological effect using EPA Method 1002.0, also known as the Wastewater Effluent Toxicity (WET) test.
Briefly, daphnids were cultured in reconstituted moderately hard water in an environmental chamber maintained at 25 ± 2 degrees Celsius under controlled lighting of intensity 20 ± 2 μE/m²/s with a 16-hour light/8-hour dark photo-period. Cultures were nurtured on P. subcapitata, wheat germ, and a yeast-cereal-tetramin mixture, 1 mL each per 250 mL of medium, provided twice daily. Culture purity was ensured by continually culling populations, purging storage water, and avoiding contamination. Samples were examined monthly by visible light microscopy to confirm culture uniformity (238). Replicate samples of daphnids were incubated with 15-mL of pre-determined concentrations of C60 species, fullerene1 synthesis materials (NaOH, MeOH, and TBAH), and a negative control consisting of only reconstituted moderately hard water. Each test consisted of five neonates (≤ 24 hours old) in 30-mL dilution cups in an environmental chamber maintained at 25 ± 2 degrees Celsius under controlled lighting of intensity 20 ± 2 μE/m²/s with a 16-hour light/8-hour dark photo-period for 48 hours without nourishment. The number of surviving daphnids was tallied.

Determination of biological effect of the samples was assessed by comparing the total number of survivors in the treated samples to that of the negative control. Tests were performed in triplicate for each sample and in triplicate for separately prepared sample materials except for NaOH and MeOH, which were tested in duplicate for separately prepared material, and Nano-C and BuckyUSA fullerene1, which were sampled once each. Homogeneity of variance was determined using Bartlett’s Test and statistical significance was determined using Dunnett’s program version 1.5 (United States Environmental Protection Agency, Washington, D.C.). LC50 was determined using the probit method (US EPA Probit Analysis Program, version 1.5, Environmental Monitoring Systems Laboratory, Cincinnati, Ohio).
Results

Bacterial Chronic Toxicity Assays – *Pseudomonas aeruginosa*

Mean population changes for *P. aeruginosa* exposed to nC\textsubscript{60}-14L ranged between +1.1% and +27.9% with the largest growth seen at the 1.0 mg/L concentration and the smallest seen at the 0.1 mg/L concentration (Figure 4-2). None of the means of nC\textsubscript{60}-14L treated samples were significantly different than control or one another, suggesting no relationship between population and nC\textsubscript{60}-14L suspensions. Mean population changes for *P. aeruginosa* exposed to nC\textsubscript{60}-28L ranged between -32.4% and -10.7% with the smallest decline seen at the 0.01 mg/L concentration and the largest seen at the 100 mg/L concentration, suggesting a relationship between population and nC\textsubscript{60}-28L suspensions (Figure 4-2). Results for nC\textsubscript{60}-28L treated samples were not significantly different from one another; therefore, it was not determined conclusively that nC\textsubscript{60} stirred for 28 days exhibited a dose-response effect on *P. aeruginosa*.

Mean population changes for *P. aeruginosa* exposed to fullerenol-PAI ranged between -38.4% and -18.9% with the smallest decline seen at the 10 mg/L concentration and the largest seen at the 1,000 mg/L concentration (Figure 4-3). This fullerenol caused a statistically significant population reduction as compared to control at 1.0, 100, and 1,000 mg/L, not a 0.1 and 10 mg/L, suggesting higher concentrations (≥ 100 mg/L) imparted greater impact than lower concentrations (≤ 10 mg/L), yet differences in mean values for treated samples were not statistically significant from one another, prohibiting any conclusions about a dose-response relationship. Regression analysis was used to estimate an LC\textsubscript{50} of 2,409 mg/L; however, caution must be used in data interpretation when projecting outside the range of the input data upon which the regression relationship was based as extrapolation can yield figures that may prove to be unrealistic. Mean population changes for *P. aeruginosa* exposed to fullerenol-CA ranged between –60.1% and +6.0% with the largest increase seen at the 1.0 mg/L concentration and the
largest decrease seen at the 1,000 mg/L concentration (Figure 4-3). This fullerol caused a statistically significant reduction of the population as compared to control at the highest two concentrations (100 and 1,000 mg/L) but not at the lower concentrations (≤ 10 mg/L). Differences in mean values for the treated samples at 100 and 1,000 mg/L were statistically significant from the 0.1 mg/L sample, suggesting that there was a relationship between fullerol-CA dose and *P. aeruginosa* response.

Mean population changes for *P. aeruginosa* exposed to TBAH ranged between -69.8% and -1.8%, indicating the population declined with concentration and statistically significant differences were demonstrated between concentrations ≥ 100 mg/L and control (Figure 4-4). Furthermore, mean populations of the lower three concentrations (0.1, 1.0, and 10 mg/L) were significantly different from the higher concentrations (100 and 1,000 mg/L), suggesting a dose-response relationship. The LC$_{50}$ for TBAH determined through regression analysis was estimated to be 45.5 mg/L. Mean population changes for *P. aeruginosa* exposed to NaOH ranged between -62.7% and -26.1% with the population declining with concentration (Figure 4-4). Statistically significance difference was found between control and samples with concentrations ≥ 10 mg/L but not among treated samples, indicating that a dose-response relationship was unable to be confirmed. The LC$_{50}$ for NaOH determined through regression analysis was estimated to be 36.8 mg/L. Mean population changes for *P. aeruginosa* exposed to MeOH ranged between -63.0% and -11.5% with the population declining with concentration (Figure 4-4). The only statistically significant difference was found between the highest concentrations (≥ 100 mg/L) and control, indicating that a dose-response relationship was unable to be established. The LC$_{50}$ for MeOH determined through regression analysis was estimated to be 51.0 mg/L.
Mean population changes for *P. aeruginosa* exposed to by-product #2 (BP #2) ranged between -5.6% and +24.9% with > 15% growth at 0.1, 1.0, and 100 mg/L and near zero values at 10 and 1,000 mg/L with no value being significantly different from control or one another (Figure 4-5). While the population trend decreased with increasing concentration, no reliable threshold concentrations were able to be determined. Mean population changes for *P. aeruginosa* exposed to by-product #3 (BP #3) ranged between +40.0% and +77.7% (Figure 4-5). Statistical difference was found between all concentrations and control except the 1,000 mg/L samples, yet no differences were found between individual concentrations, indicating that BP #3 stimulated growth but no dose-response relationship could be established and no reliable threshold concentrations were able to be determined.

**Bacterial Chronic Toxicity Assays – *Staphylococcus aureus***

Mean population changes for *S. aureus* exposed to nC$_{60}$-14L ranged between -10.9% and +61.2% with the largest growth seen at the 10 mg/L concentration and the largest decrease at the 1.0 mg/L concentration (Figure 4-2). Statistically significant difference with control was observed at the lowest concentration (0.01 mg/L) and at the highest concentrations (10 and 100 mg/L) as growth stimulation. The only other significant difference was between the 0.01, 10, 100 mg/L samples and the 1.0 mg/L suspension, suggesting a global minimum in the response at 1.0 mg/L with no growth, yet growth at higher and lower concentrations. Mean population changes for *S. aureus* exposed to nC$_{60}$-28L ranged between -27.4% and +3.6% with the largest population increase at the 1.0 mg/L concentration and the largest decline at the 100 mg/L concentration, suggesting a relationship between population and nC$_{60}$ concentration (Figure 4-2). No statistically significant difference was identified between control and any sample or within groups, suggesting no relationship between *S. aureus* population and nC$_{60}$-28L concentration.
Mean population changes for *S. aureus* exposed to fullerenol-PAI showed a population decline at all concentrations ranging from -0.5% at 1.0 mg/L to -39.2% at 1,000 mg/L (Figure 4-3). This fullerenol caused a statistically significant reduction of the population as compared to control at 1000 mg/L only, yet the differences in mean values for treated samples were not statistically significant from one another, indicating that a dose-response relationship was indefensible. Mean population changes for *S. aureus* exposed to fullerenol-CA ranged between –82.9% and +47.0% with the largest growth observed at the 1.0 mg/L concentration and the largest decline seen at the 1,000 mg/L concentration (Figure 4-3). This fullerenol caused a statistically significant reduction of the population as compared to control at 100 and 1000 mg/L only, which were also statistically different from samples at lower concentrations (≤ 10 mg/L) indicating a dose-response relationship. Regression analysis indicated a general trend of declining population with increasing concentration and an LC$_{50}$ estimate of 92.8 mg/L.

Mean population changes for *S. aureus* exposed to TBAH ranged between -60.0% and +97.1% with the population generally declining with concentration (Figure 4-4). The treated samples were statistically different from control at all concentrations except 1.0 mg/L. Furthermore, the 0.1 samples were significantly different from the 1.0, 10, 100, and 1,000 mg/L samples, indicating that a dose-response relationship was identified. The LC$_{50}$ for TBAH determined through regression analysis was estimated to be 8.09 mg/L. Mean population changes for *S. aureus* exposed to NaOH ranged between -79.3% and -15.1% with the population generally declining with concentration (Figure 4-4). Treated samples were statistically different from control at all concentrations except 1.0 mg/L. Furthermore, the 0.1 and 1.0 mg/L samples were statistically different from the 10, 100, and 1,000 mg/L samples, indicating that not only that NaOH had an adverse effect on *S. aureus* but a dose-response relationship was identified.
The LC$_{50}$ for NaOH determined through regression analysis was estimated to be 28.8 mg/L. Mean population changes for *S. aureus* exposed to MeOH ranged between +10.1% and +68.6% with the population increasing with concentration from the lowest concentration (0.1 mg/L) until 10 mg/L, then declining (Figure 4-4). The control was significantly different from the 0.1, 1.0, and 100 mg/L samples; no other significant difference was found. It was concluded that NaOH stimulated *S. aureus* at concentrations between 1.0 mg/L and 100 mg/L with an optimum concentration for growth found at 10 mg/L.

Mean population changes for *S. aureus* exposed to by-product #2 (BP #2) ranged between -24.5% and +27.3% with growth at concentrations $\geq$ 100 mg/L and population decline at concentrations $\leq$ 10 mg/L (Figure 4-5). No statistically significant difference was found between any data at any concentration or with controls. Mean population changes for *S. aureus* exposed to by-product #3 (BP #3) ranged between +40.0% at 1,000 mg/L concentration and +77.7% at 0.1 mg/L concentration (Figure 4-5). Statistical difference was found between control and concentration $\leq$ 100 mg/L concentrations, indicating BP #3 stimulated *S. aureus* populations, yet no significant differences were found between concentrations.

**Bacterial Cell Membrane Integrity Assays**

Fluorescence results from the flow cytometric analysis of the LIVE/DEAD BacLight Bacterial Viability Assay were quantified with the intensity recorded through the red filter (ordinate) as a function of the intensity recorded through the green filter (abscissa) for both “live” (suspended in NaCl solution) and “dead” (suspended in isopropyl alcohol) cells. Dead cell samples experienced $> 98.5\%$ mortality for both *P. aeruginosa* and *S. aureus* (data not shown), indicating successful demonstration of cell membrane damage through the stain of DNA with the membrane-impenetrable propidium iodide.
Figure 4-6 depicts distinct groupings of *P. aeruginosa* and *S. aureus* cells exposed to dyes in the absence of light above and below unity that were gated and quantified. The groupings to the lower right, labeled “APOPTOTIC”, indicated high SYTO 9 dye green-channel fluorescence with compromises yet functional cell membranes, and the grouping to the upper left, labeled “NECROTIC”, indicated high propidium iodide staining, an indication of traumatic cell activity. Calculation of *P. aeruginosa* cells located within each gated region indicated that 72.5% of the cells were without traumatic membrane damage, while an additional 9.2% demonstrated necrotic membrane failure. The remaining 18.3% of the cells fluoresced at both red and green wavelength intensities, suggesting that the cell functions were unaffected. Calculation of the *S. aureus* cells indicated that 54.9% of the cells were without membrane damage, while an additional 42.2% demonstrated necrotic membrane damage. The remaining 2.9% of the cells fluoresced at equal red and green wavelength intensities, suggesting that the cells were healthy.

Figure 4-7 depicts *P. aeruginosa* and *S. aureus* cells exposed to dyes in the presence of light. Calculations of *P. aeruginosa* cells indicated 59.2% experienced necrotic membrane damage and apoptotic effects were exerted on 6.7% of the population; the remaining 34.08 experienced no significant cell damage. In contrast, 96.5% of *S. aureus* experienced traumatic cell membrane damage with 1.1% demonstrating apoptotic effects and 2.4% exhibiting no adverse effects.

**Algal Chronic Toxicity Assays**

Mean population changes for *P. subcapitata* exposed to nC$_{60}$-14L ranged between -14.9% and +4.3% with the largest growth seen at the 100 mg/L concentration and the largest decrease seen at the 1.0 mg/L concentration (Figure 4-8). No statistically significant difference was found between any data at any concentration or with controls. Mean population changes for *P. subcapitata* exposed to nC$_{60}$-28L ranged between −16.7% and +8.5% with the largest growth seen at the 0.1 mg/L concentration and the largest decrease seen at the 10 mg/L concentration.
Statistically significant population reduction was noted between concentrations ≥ 10 mg/L and controls, though no differences were found within treatments.

Mean population changes for *P. subcapitata* exposed to fullerenol-PAI ranged between -83.3% and +5.2% and caused a statistically significant reduction of the population as compared to control at 100 and 1000 mg/L only (Figure 4-8). The 1,000 mg/L sample was statistically different from all other samples including control, suggesting that this fullerenol exhibited an effect on *P. subcapitata* with a demonstrated dose-response relationship. Regression analysis estimated an LC$_{50}$ of 299.2 mg/L. Mean population changes for *P. subcapitata* exposed to fullerenol-CA ranged between -55.7% and +37.8% with the largest growth observed at the 0.1 mg/L concentration and the largest decline seen at the 1,000 mg/L concentration (Figure 4-8). Statistically significant difference was found between control and all concentrations except 10 mg/L with samples with concentrations ≤ 10 mg/L significantly different that samples with ≥ 100 mg/L concentration, indicating that a dose-response relationship was established. Regression analysis indicated a general trend of declining population with increasing concentration and an estimate of LC$_{50}$ = 1,462 mg/L, which was outside the range of tested concentrations.

Mean population changes for *P. subcapitata* exposed to TBAH ranged between -78.6% and -1.6% with the largest decline at 1,000 mg/L and the smallest decline at 0.1 mg/L. All samples ≥ 1.0 mg/L were statistically different from control and significantly different from one another, indicating a dose-response relationship between TBAH and *P. subcapitata*. Regression analysis estimated an LC$_{50}$ of 39.4 mg/L. Mean population changes for *P. subcapitata* exposed to NaOH ranged between +43.3% and +76.9% with all samples statistically different from control but not different from one another (Figure 4-9), indicating that the presence of NaOH fostered growth regardless of concentration (up to 1,000 mg/L). Mean population changes for *P.*
*P. subcapitata* exposed to MeOH ranged between -93.9% and -28.2% with the largest decline at 1,000 mg/L and the smallest decline at 1.0 mg/L (Figure 4-9). All samples were statistically different from control and the higher concentrations (≥ 10 mg/L) were significantly different from lower concentrations (≤ 1.0 mg/L), indicating a dose-response relationship between MeOH and *P. subcapitata* and an estimated LC$_{50}$ of 8.43 mg/L.

Mean population changes for *P. subcapitata* exposed to by-product #2 (BP #2) ranged between +33.5% at the lowest concentration (0.1 mg/L) and +24.6% at the highest concentration (1,000 mg/L), as shown in Figure 4-9. No statistical difference occurred between control and any samples but BP #2 was observed to stimulate algal populations at all concentrations. Mean population changes for *P. subcapitata* exposed to by-product #3 (BP #3) ranged between -32.9% at 1,000 mg/L concentration and -8.7% at 10 mg/L concentration (Figure 4-9). No statistically significant difference was found between any data at any concentration or with controls. Regression analysis indicated that the population generally decreased with increasing concentration but no reliable threshold concentrations were able to be determined.

**Invertebrate Acute Toxicity Assays**

Mean mortality rates of *C. dubia* exposed to nC$_{60}$-14L ranged between 2.2% and 18.6% (Figure 4-10). No statistically significant difference was found between any data at any concentration or with controls. Mean mortality rates of *C. dubia* exposed to nC$_{60}$-28L ranged between 7.1% at 0.01 mg/L and 33.9% at 100 mg/L (Figure 4-10). Statistical significance was found between control and the 100 mg/L sample; additionally, 100 mg/L samples were statistically different than 0.01, 0.1, and 10 mg/L samples, indicating that nC$_{60}$-28L affected *C. dubia* with a dose-response relationship. It should be noted that significant impact only occurred at the highest concentration, which was three to six orders of magnitude higher than background levels of fullerene in the aquatic environment, indicated by Perez et al. (239) as 0.13 µg/L.
Mean mortality rates of *C. dubia* exposed to fullerenol-PAI ranged from 11.3% at 0.1 mg/L and 93.3% at 1,000 mg/L (Figure 4-10). Statistical difference was noted with control ≥ 10 mg/L, while 100 and 1,000 mg/L samples were significantly different than lower concentrations (≤ 1.0 mg/L), suggesting a dose-response relationship between this fullerenol and *C. dubia*. Probit analysis was used to estimate an LC<sub>50</sub> of 31.05 ± 2.92 mg/L. Mean mortality rates of *C. dubia* exposed to fullerenol-CA ranged between 7.1% at 0.1 and 1.0 mg/L and 93.1% at 1,000 mg/L. Statistical difference was noted between control and samples with concentrations ≥ 100 mg/L. The 1,000 mg/L samples were significantly different than 0.1, 1.0, and 10 mg/L samples while 100 mg/L samples were significantly different that 0.1 and 1.0 mg/L samples, suggesting a dose-response relationship between this fullerenol and *C. dubia*. Probit analysis was used to estimate an LC<sub>50</sub> of 45.15 ± 3.83 mg/L.

Mean mortality rates of *C. dubia* exposed to TBAH ranged between 3.6% and 100%. All samples ≥ 10 mg/L were statistically different from control and significantly different than samples < 10 mg/L, establishing a dose-response relationship between TBAH and *C. dubia* (Figure 4-11). Probit analysis was used to estimate an LC<sub>50</sub> of 1.81 ± 0.17 mg/L. Mean mortality rates of *C. dubia* exposed to NaOH ranged between 47.2% at 1,000 mg/L and 4.4% at 0.1 mg/L (Figure 4-11). Statistical differences were noted between control and higher concentration samples (≥ 10 mg/L), which were significantly different than lower concentration samples (≤ 1.0 mg/L), suggesting a dose-response relationship between *C. dubia* and NaOH. Probit analysis was used to estimate an LC<sub>50</sub> of 1,956 ± 189.70 mg/L, which must be greeted with skepticism as the highest concentration tested was 1,000 mg/L. Mean mortality rates of *C. dubia* exposed to MeOH ranged between 4.4% and 2.8% (Figure 4-11). No samples were
significantly different than control, indicating that MeOH did not cause an adverse biological effect on C. dubia, even at 1,000 mg/L.

Mean mortality rates of C. dubia exposed to by-product #2 (BP #2) ranged between 2.2% at 0.1 mg/L and 72.2% at 1,000 mg/L (Figure 4-11). Statistical differences were noted between control and samples ≥ 10 mg/L, and concentrations ≥ 100 mg/L showed significant differences with samples ≤ 10 mg/L, suggesting a dose-response relationship between C. dubia and BP #2. Probit analysis was used to estimate an LC$_{50}$ of 44.45 ± 5.96 mg/L. Mean mortality rates of C. dubia exposed to by-product #3 (BP #3) ranged between 4.4% at 0.1 mg/L and 97.8% at 1,000 mg/L (Figure 4-11). Statistical difference was noted between control and samples ≥ 100 mg/L with concentrations ≥ 100 mg/L exhibiting differences with concentrations < 100 mg/L, suggesting a dose-response relationship between C. dubia and BP #3. Probit analysis was used to estimate an LC$_{50}$ of 22.70 ± 1.62 mg/L.

**Discussion**

It can be seen from Table 4-1 that none of the water-stirred fullerene affected either bacteria species at environmentally relevant concentrations (239). Samples of nC$_{60}$-28L had statistically greater adverse impact than nC$_{60}$-14L on P. aeruginosa at all concentrations and on S. aureus at concentrations ≥ 10 mg/L. The biological effect on bacteria caused by the two fullerenol species used in this study, laboratory-prepared and commercially-available, did not differ significantly from one another, although they both showed statistically significant reductions in population compared to control (fullerenol-PAI at 1,000 mg/L; fullerenol-CA ≥ 100 mg/L). Fullerenol species demonstrated significantly more adverse biological effect than nC$_{60}$-14L (≥ 1 mg/L) and, while impacts were statistically indifferent from those of nC$_{60}$-28L (0.1 to 100 mg/L), estimated toxicity threshold concentrations were generally lower for fullerenol species than for nC$_{60}$-28L. The nC$_{60}$ samples caused no substantial impact to either
algae or daphnids, although one-third of C. dubia experienced significantly higher mortality with \( nC_{60}-28L \) than with \( nC_{60}-14L \) at concentrations \( \geq 100 \text{ mg/L} \). The biological effect to algae and daphnids did not differ significantly between 14-day and 28-day water-stirred \( nC_{60} \). Fullerenol significantly impacted algal cultures at concentrations \( \geq 100 \text{ mg/L} \) (\( \geq 1,000 \text{ mg/L} \) for commercially-available material) and daphnids at concentrations \( \geq 10 \text{ mg/L} \).

Previous investigations into the toxicity of fullerene species have elucidated numerous deficiencies in the body of knowledge, producing conflicting results that necessitate more critical and comprehensive research into the factors and mechanisms of potentially adverse biological effects. Studies of the biological impact of \( nC_{60} \) on bacteria have focused mostly on dispersible fullerene created with the use of the solvent tetrahydrofuran (THF-\( nC_{60} \)), which has been shown to have toxicity threshold ranging from \(< 1 \text{ mg/L} \) (36, 53) to no inhibition at 2,000 mg/L (117) on a variety of bacteria including \( S. aureus \). One research team indicated an EC\(_{50}\) of 0.82 mg/L on \( Vibrio fischeri \) caused by sonicated-in-toluene fullerene (240). Reports that examined the biological effect of \( aqu-nC_{60} \) on bacteria indicated an MIC of 0.4 mg/L (36) and no population reduction at 26 mg/L (241) on \( Bacillus subtilis \) while another noted no significant metabolic reduction in an unspecified soil community at 50 ppm after 23 days as determined by carbon dioxide production (242). Gao et al. (106) investigated the biological effect of \( nC_{60} \) on \( P. subcapitata \), the only report identified on the subject, indicating an LC\(_{50}\) = 0.139 mg/L for THF-\( nC_{60} \). Reports involving \( nC_{60} \) and invertebrates indicated threshold toxicity levels on \( D. magna \) from an LC\(_{50}\) of 0.45 mg/L (243) to 0.8 mg/L (105) for THF-\( nC_{60} \) and, an LC\(_{50}\) of 10.5 mg/L (109) to an LC\(_{40}\) of 35 mg/L (27) for \( aqu-nC_{60} \). Tao (244) observed that \( D. magna \) continually consumed THF-\( nC_{60} \) agglomerates and were able to clear their digestive canal completely within 24 hours once removed from the fullerene suspension. Several researchers examined the impact
of fullerenol on bacteria with results of normal growth at 5 mg/L for *Escherichia coli* and *B. subtilis* (53), no significant mortality *E. coli* of at 20 mg/L (245), no inhibition of *E. coli* or *Bacillus* sp. at 2,000 mg/L, and MICs of 2,000 mg/L for *S. aureus* and *Staphylococcus epidermidis* and 1,000 mg/L for *Pseudomonas acnes* (117). No previous reports were identified investigating the biological effect of fullerenol on algae and only one examined the impact on *Daphnia pulex*, indicating statistically significant mortality at 100 mg/L (102).

Several findings from the biological assays deserve additional analysis including: 1) differential biological responses among fullerene species; 2) differential biological responses between dark and light assays; 3) differential biological responses between commercially-available and laboratory-prepared fullerenol; and, 4) the impacts caused by fullerenol synthesis by-products and reactants. Determination of the causes of biological effects requires consideration of the mode of toxicity, the factors affecting biological activity, points of impact, and the potential mechanisms resulting in adverse impact. Mode of toxicity refers to whether the effect is acute or chronic, lethal or sublethal, reversible or irreversible. This investigation examined aspects of toxicity using two assays isolating acute effects (bacteria: sublethal; invertebrates: lethal) and two assays assessing chronic, or apoptotic, effects (bacteria and algae: sublethal).

The primary mechanisms of toxicity identified in previous studies included oxidation of lipids (20, 23, 35, 105, 113, 128, 146, 156, 158, 193, 246-251), proteins (36, 70, 84, 114, 252-254), enzymes (193, 255), and amino acids (18, 20, 256), which compromised the immediate viability of the cell, resulting in a necrotic response, and binding to the membrane exterior (254, 257) and to nucleic acids (14, 38, 147, 245, 258), disrupting critical cell functions and leading to apoptosis. Most of these mechanisms required fullerene to be activated, either as a catalyst for
reactive oxygen species (ROS) generation or directly as a reagent itself, both of which have been attributed to fullerene photo-reactivity. This study explored the feasibility of applying these theories as well as other potential mechanisms such as fullerene complexation with nutrients and fullerene membrane shielding to prevent nutrient exchange to experimental observations. Since some analytes stimulated populations, mechanisms of beneficial biological effects such as ROS scavenging, fullerene as a nutrient, and eustressed responses also were contemplated.

**Differential Biological Responses among Fullerene Species**

**nC$_{60}$-14L**

It has been proposed that Gram-negative bacteria were more resistant to derivatized fullerene due to interception of fullerene species by flagella or lipopolysaccharides, which were not present in their Gram-positive counterparts (259, 260). While Gram-negative bacteria superiority has been demonstrated in terms of resistance to THF-nC$_{60}$ (36), no single report reviewed provided information on the impacts of aqu-nC$_{60}$ on both bacteria types. Since results on the biological effect of fullerene prepared with THF must be considered suspect, this investigation provides the first comparative analysis of the toxicological impacts on a variety of bacterial types without the influence of exigent compounds.

$nC_{60}$-14L caused a moderate increase to Gram-negative *P. aeruginosa* populations yet caused a statistically significant increase of Gram-positive *S. aureus* populations at higher concentrations (≥ 10 mg/L). The bacteria population assays were conducted in the absence of light, so photo-active mechanisms were not considered. Perhaps the simplest explanation for biological effect could be shielding of bacteria from their environment, especially nutrients, by deposited fullerene molecules, adhered to the surface as a result of intermolecular forces. Calculations based on known concentrations indicated that $10^6$ fullerene molecules existed per cell in the bacterial assays at 0.01 mg/L concentration based on a mean starting population of 7 x
10^4 cells per mL. At 100 mg/L, concentration increased to 10^{10} molecules per cell, potentially covering 20.1% of the entire surface of S. aureus cells (5.3% of P. aeruginosa), assuming all the material adhered to bacteria. No indication of fullerene shielding was found by other researchers as a proposed mechanism of toxicity.

Utilization of fullerene species as a nutrient by bacteria could be accomplished through either of two mechanisms. First, extracellular digestion of fullerene molecules would require secretion of capable enzymes and adsorption into the cell through selectively-permeable, protein-based pores in the cytoplasmic membrane. The other method of consumption would require the bacteria to perform endocytosis, which has been documented in Gram-negative bacteria (261), although not P. aeruginosa, and no reports were viewed that indicated its occurrence in Gram-positive bacteria. Bacteria were cultured and exposed to fullerene species in tryptic soy broth and plated on tryptic soy agar, providing an ideal nutrient environment, which suggested fullerene as a nutrient was not a necessary exposure pathway. Complexation of fullerene with nutrients presented a potential vehicle for ingestion as tryptic soy broth consists of amino acids, monosaccharides, lipids, and minerals. Additional research as to whether fullerene species were utilized by cells as a nutrient source or complexed with ubiquitous nutrients may seek to employ radioisotope-labeled fullerene (^{13}C_{60}) to assess assimilation into cell material as such an approach would facilitate conclusive results and hasten investigative efforts as to potential biochemical mechanisms. Substitution of ^{13}C_{60} in lieu of C_{60} would require the use of ^{13}C-NMR, which would enable differentiation of carbon from fullerene and the organic carbon of the test organism.

It was noted through FTIR analysis that epoxide moieties were present in nC_{60}-14L but absent in 28-day samples; furthermore, no fullerenol samples or by-products were shown to
contain epoxides, suggesting that epoxylated $nC_{60}$-14L was capable of unique biological interactions. Kahan et al. (262) indicated epoxylated compounds were uniquely capable of apoptotic impacts on $S. aureus$ by blocking the cytoplasmic enzyme pyruvyl transferase, which initiated the production of peptidoglycan. In the Gram-positive $S. aureus$, peptidoglycan functioned as a protective barrier that entrapped potential toxicants and initiated apoptosis through production of peptidoglycan hydrolase upon contact with xenotoxins such as polystyrene (263). Apoptotic effects associated with cell maintenance inhibition were not observed in epoxylated aqu-$nC_{60}$ assays. In other studies, molecules with epoxide moieties have been shown to react with and alter cysteine, an amino acid essential to the structural integrity of proteins, and in the production of glutathione, which has been shown to facilitate ROS quenching (264, 265). In a comparative study, epoxylated compounds were shown to prevent the production of glutathione more effectively than non-epoxylated counterparts (266). The presence of a epoxylated fullerene species ($nC_{60}$-14L) may have inhibited the production of glutathione and caused greater impact to both bacteria than materials without epoxide groups (i.e. $nC_{60}$-28L and fullereno1), resulting in increased ROS production and decreased populations compared to control. Conversely, data indicated that the populations of both species increased, suggesting that ROS quenching may have been a mechanism leading to the biological effect.

Prior to interaction with surface proteins or intracellular enzymes, a potential toxicant would need to interact with the outer surface of the cell structure, which consisted of lipopolysaccharides in $P. aeruginosa$ (Figure 4-12), and peptidoglycan (Figure 4-13) and teichoic acid structures (Figure 4-14) of $S. aureus$. The ability of epoxylated compounds to adhere to hydroxyl sites on lipopolysaccharides has been demonstrated as a species-specific method for identification of Gram-negative bacteria including $P. aeruginosa$ (257, 267, 268),
indicating a potential for epoxylated fullerene to bind to *P. aeruginosa* in a similar fashion.

Peptidoglycan and teichoic acid, components of the outer portion of Gram-positive membranes, were similar to lipopolysaccharide of the Gram-negative membrane in that the backbone of each was comprised of saccharide- or reduced-saccharide-containing chains with amino acid side groups. Since epoxylated compounds were shown to bond to saccharide groups in lipopolysaccharide, a potential existed for epoxylated fullerene to bind to reduced-saccharide groups in peptidoglycan and teichoic acid of the *S. aureus*. The precise mechanism of adhesion has not been identified; however, three possible pathways including hydrogen bonding, deprotonation, or dehydroxylation were explored (Figure 4-15).

The formation of hydrogen bonds between a saccharide hydroxyl unit and the epoxide moiety of fullerene (a) presented a feasible mechanism for epoxylated fullerene attachment, although no reports have presented such molecular interactivity. Xiao *et al.* (269) described hydrogen bonds between derivatized fullerene species as a mechanism for dimerization in an organic solvent, although hydrogen bonding would not ensure functional disruption of the membrane component (i.e. lipopolysaccharide, peptidoglycan, or teichoic acid) and its involvement in adhesion remains unresolved. Alternately, surface group transformation has shown to be a common occurrence with derivatized fullerene. Chemical transformations required to incorporate fullerene species into amino acids would necessitate deprotonation of amino acid hydroxyl groups (b), which is energetically disfavored to the removal of the entire hydroxyl group; hence, proton addition was determined not to be a probable mechanism of adhesion.

Adhesion through dehydroxylation of a saccharide would occur through the formation of an ether bridge and the release of a hydroxyl ion (c). The increase in hydroxyl ions in solution
caused by dehydroxylation would result in an increase in pH of the solution. At a concentration of 100 mg/L, conservatively assuming 1% derivatization occurred (110) and 1% of the material reacted, the pH of the system would have only increased from 7.30 to 7.33, based on ion concentration calculations. The pH of the bacterial population assays was analyzed (Figure 4-16), indicating a nominal decrease in hydroxyl concentration of the $P. \text{aeruginosa}$ cultures at 10 mg/L and 100 mg/L and suggesting hydroxylation was not the dominant mechanism for adhesion. However, pH of the controls also decreased, and significantly less than the pH of the treated samples at ≥ 10 mg/L. Furthermore, the pH of 100 mg/L cultures was significantly higher than for the 10 mg/L assays, indicating the presence of $nC_{60}$ in samples ameliorated the decrease in pH in a manner that reflected a relationship with concentration, providing support for the proposition that $nC_{60}$ adhesion caused by dehydroxylation of lipopolysaccharide was a feasible mechanism of action.

Binding of fullerene material to lipopolysaccharide, peptidoglycan, or teichoic acid would have two important consequences. First, $nC_{60}$ would lose its ability to migrate closer to the cell membrane, where it might react with the lipid bilayer or penetrate into the cell through a protein pore or through diffusion across the polar region of the lipid matrix. It should be noted that the ability of the protective mechanisms of bacteria to capture $nC_{60}$ molecules did not preclude the ability for some $nC_{60}$ to reach the lipid membrane or to achieve cellular penetration. However, effects of lipid membrane damage or intercellular $nC_{60}$ activity were not recorded. The second impact of $nC_{60}$ adhesion was that affected lipopolysaccharide molecules would lose structural integrity, causing biomolecular disassociation with the membrane. The loss of lipopolysaccharide molecules would instigate production of new cell material and reproduction through the release of enzymes, compensating for the adverse environmental conditions. As
seen in this investigation, both bacteria responded to adverse environmental modification (i.e. the presence of epoxylated fullerene species) with the eustressed response of increased cell material production, as evidenced by increased overall populations. The biological expressions were slightly different due to a slower growth rate in *P. aeruginosa*, which has a growth rate constant of 1.02 h\(^{-1}\) (270), compared to *S. aureus*, which has a growth rate constant of 0.86 h\(^{-1}\) (271). In an eustressed condition (272), with an increased, eustressed growth rate, *S. aureus* increased cell population over control while the impact experienced by *P. aeruginosa* was generally less pronounced, suggesting that the Gram-positive bacteria were more sensitive to epoxylated *nC\(_{60}\) than the Gram-negative bacteria. One possible conclusion was that peptidoglycan or teichoic acid was more susceptible to epoxylated fullerene adhesion than lipopolysaccharide; however, experiments would need to be established to determine the specific mechanisms of such interactions.

*nC\(_{60}\)-28L*

While *nC\(_{60}\)-14L* stimulated cellular activity, *nC\(_{60}\)-28L* affected a moderate population decline compared to control in both bacteria populations, with statistically significant decreases at concentrations ≥ 10 mg/L in *P. aeruginosa*. Again, mechanisms involving photo-induced reactivity were not considered due to the absence of light in the bacterial chronic toxicity assays. FTIR analysis of *aqu-nC\(_{60}\)* samples indicated that their chemical compositions were identical in terms of the number of di-oxygenated carbon (hemiketal) sites but 28-day stirred material had 15% more mono-oxygenated groups. The nominal difference in the number of surface groups was not expected to explain the limited cell disruption difference observed; however, the absence of epoxylated groups warranted independent analysis of previously examined pathways. Of identified dark-phase mechanisms, only *aqu-nC\(_{60}\)* binding to intracellular nucleic acids remained possible. However, prior to intracellular interactivity, the material must have penetrated the cell,
either by initiating damage to the cytoplasmic membrane, diffusion, or through active transport. Published reports on the activity of fullerene membrane damage, outside that which was caused by adhesion to extracellular material, focused primarily on lipid peroxidation and protein denaturation, both of which relied on the reactivity of fullerene, either in terms of photo-activated fullerene as a generator of ROS or through photo-induced reactivity of fullerene species. The proposed mechanisms of lipid peroxidation (Figure 4-17) and protein denaturation through the oxidation of cysteine (Figure 4-18) were comparable, whether caused by ROS or a reactive species of fullerene, yet both were omitted from consideration as a mechanism of $nC_{60}$-28L toxicity under the premise that they were photo-dependent and the results analyzed were obtained through dark-phase assays.

The ability of a molecule or cluster of molecules to diffuse across a membrane was a function of relative concentrations, chemical compatibility, and size. As it was assumed that bacteria do not possess background fullerene, any concentration gradient involving $nC_{60}$ would tend from the aqueous medium into the cell. Chemical compatibility includes issues related to the hydrophilic-lipophilic balance, electrical charge, and complexation potential. While underivatized fullerene molecules were observed to be decidedly hydrophobic, $nC_{60}$ possesses the capacity for hydrophilicity, demonstrated by remaining in aqueous solution during the organic solvent phase-transfer processes used to determine concentration, as shown previously. Based on examination of physical models of derivatized fullerenes and the limited number and type of functional groups on the surface of $nC_{60}$, regions of the carbon cage may retain their hydrophobic nature, displaying amphiphilic behavior, similar to a surfactant, which would enable persistence in both polar and non-polar media such as that found in and around cell membranes.
The mean hydrodynamic diameter of $nC_{60}$-14L agglomerates determined through electron microscopy was 118 nm while $nC_{60}$-28L was reported to be 108 nm with no significant difference between stirring duration; reported values for $aqu-nC_{60}$ were in the range of 35 nm to 235 nm (35-41, 47, 49). It is important to note that the polar head groups of the phospholipids contained within each bacteria species differed considerably. The polar heads of $P. aeruginosa$ Lipid-A (Figure 4-19) were estimated to measure 1.4 nm across and those of $S. aureus$ lysyl-phosphatidylglycerol (Figure 4-20) were far smaller, suggesting any possible penetration was expected to be more likely in Gram-positive bacteria due to the size of the polar head groups, but, more importantly, that the potential for a 100-nm agglomerate to penetrate through the tightly-packed lipid membrane through diffusion of either bacteria was highly suspect. While means are important parameters in the analysis of particle size, it should be noted that the distribution of $aqu-nC_{60}$ agglomerate sizes observed ranged from 2.2 nm to $> 1 \mu m$, indicating that even while most $aqu-nC_{60}$ agglomerates were much too large to penetrate a cell membrane, there existed a small percentage ($< 1\%$) that were adequately small ($< 5 \text{ nm}$) in diameter to be absorbed into the cell through the polar head region of the lipid bilayer membrane. A 5-nm agglomerate of fullerene is roughly the size of some enzymes (137, 273-275) and, if chemically compatibility does not inhibit interaction, would be able to permeate a cell membrane in a fashion similar to an enzyme. Molecular dynamic simulations have demonstrated underivatized fullerene diffusivity with penetration into the bilayer lipid membranes through the lipophilic heads of the membrane lipid molecules (273-275). Wong-Ekkabut (137) demonstrated the potential for a 10-molecule (1.7-nm diameter) cluster of fullerene to penetrate a lipid bilayer, disaggregating upon reaching the hydrophobic interior region of the membrane.
Benyamini et al. (254) explored the affinity of fullerene species with membrane proteins by simulating docking sites on numerous proteins, indicating complexation with several amino acids including one present in lipopolysaccharide (L-alanine) and several present in peptidoglycan and teichoic acid (L-glutamine, L-glycine, and L-lysine), concluding that both underivatized and derivatized fullerene formed complexes with amino acids and that derivatized fullerene generally displaying higher affinity than underivatized fullerene. Appendix E provides detailed schema of amino acids associated with microbiological species. It was reasonable to suspect that similar complexation occurred between nC<sub>60</sub>-28L and amino acid side groups of extracellular lipopolysaccharide, peptidoglycan, or teichoic acid as well as with surface membrane proteins. Whereas epoxylated nC<sub>60</sub> was shown capable of forming a chemical bond that disrupts the functional capacity of the extracellular material, the complex of non-epoxylated fullerene derivatives and extracellular material was described as non-chemically bonding (254), suggesting that no functional disruption occurred. However, extracellular sequestration of the fullerene species may have served to reduce the effective concentration of toxicants approaching the cytoplasmic membrane, ameliorating impact.

Eddaoudi et al. (276) studied active transport phenomena of a polymerized membrane in response to fullerene species, noting limited penetration due to the relatively long time required for passage but, more importantly that the presence of fullerene material blocked pores and slowed transport of other materials. Kraszewski et al. (277) used molecular dynamic simulation to investigate the potential for C<sub>60</sub> to enter cells using ionic channels, specifically three protein-based potassium (K<sup>+</sup>) channels, indicating a high affinity between the carbon cages and the proteins, and suggesting that fullerene species could inhibit nutrient exchange by impeded
passage of nutrients. Additional investigation of active transport mechanisms involving fullerene species is warranted to explore the potential for intracellular toxicity.

Endocytosis, complexation with surface membrane proteins, diffusion, and/or active transport may have permitted $nC_{60}$ penetration into the cytoplasm, where it interacted with internal structures. Fullerene has been shown to have adverse effects to nucleic acids either through photo-activated generation of singlet oxygen (278) or independent of ROS (279). Takenaka et al. (280, 281) noted DNA cleavage and apoptotic cell death while Isakovic et al. (70) claimed no DNA fragmentation by either underivatized or derivatized fullerene. Researchers have demonstrated the stability of a fullerene complex with the guanine base (Figure 4-21) both through experimental efforts that proposed a single electron transfer mechanism (279-281) and through molecular dynamic simulation (282). Zhao et al. (165) demonstrated that fullerene had a greater affinity for guanine than for itself, indicating adhesion occurred either at the hydrophobic end of the chain or within minor grooves of the helical structure. Yamakoshi et al. (174, 283) induced DNA cleavage in the presence of $C_{60}$ upon photo-irradiation but were unable to demonstrate fullerene biological activity when nicotinamide adenine dinucleotide (NAD), a reducing agent critical to metabolic activity, was eliminated from the irradiated system. This result suggested that fullerene species were not solely responsible for nucleic acid disruption but that NAD, which is a requisite cellular molecule and would be omnipresent in all biological systems, demonstrating that nucleic acid binding was a feasible mechanism. These findings indicated that nucleic acid transcription was susceptible to interference, impeding the production of cell material, especially new nucleic acids and proteins, resulting in an apoptotic response.
It was proposed that \( nC_{60}-28L \) caused population decrease through apoptotic action as a result of membrane penetration through protein complexation and subsequent disruption of cellular maintenance resulting from binding with the guanine base of nucleic acids. Additionally, complexation of \( nC_{60}-28L \) with nutrients and the blocking of pore-proteins by agglomerates of fullerene may have exacerbated the effect. The response increased with concentration, suggesting a dose-response relationship, which was proposed to be dampened due to non-traumatic complexation of \( nC_{60}-28L \) with amino acid side groups in the extracellular material of both species. \( P. \ aeruginosa \) demonstrated increased population reduction compared to \( S. \ aureus \), suggesting that \( nC_{60}-28L \) exhibited greater complexation with peptidoglycan or teichoic acid than with lipopolysaccharide, thus more fullerene was immobilized to penetrate the cell and impart damage to nucleic acids. Another potential explanation for the difference resulted from the structure of \( P. \ aeruginosa \) membrane proteins, which may have greater fullerene affinity than those of \( S. \ aureus \), permitting a larger number of molecules access to the cytoplasm and exert nucleic acid damage.

**Laboratory-prepared fullerenol**

The chemical compositions of \( nC_{60}-28L \) and fullerenol-PAI were surprisingly similar, as was previously demonstrated, with water-stirred fullerene having a molecular structure of \( C_{60}H_{24}O_x(OH)_y \) \((x + y = 33)\) with six hemiketal groups and fullerenol-PAI having \( 26 \pm 4 \) total surface groups, of which 10 to 17 were hemiketal. \( nC_{60}-14L \), with a determined composition of 29 surface groups (six hemiketal), did not differ considerably from either \( nC_{60}-28L \) or fullerenol-PAI with the exception of the epoxylated moieties. The similarities in identified surface group compositions led to the hypothesis that biological species would reflect a similar response to these fullerene derivatives. In fact, the bacterial response to fullerenol-PAI was statistically indifferentiable from that of \( nC_{60}-28L \).
Fullerenol was reported to be soluble in water (73, 176, 214), although it has been shown to agglomerate at higher concentrations (> 10 mg/L) in aqueous solution both experimentally in this investigation and elsewhere (29) and has been confirmed through computer simulation (277). Some researchers have suggested that fullerenol prevented oxidative stress caused by lipid peroxidation and protein denaturation by quenching radical species (285, 286). Schreiner et al. (177) studied the potential for fullerene species to serve as a nutrient source for fungi, which were able to mineralize the carbon cage to water and carbon dioxide using energy from degraded carbon bonds to fuel other metabolic processes. Data generated through this research indicated that, at concentrations ≥ 20 mg/L, fullerenol molecules form agglomerates. However, at more dilute concentrations (< 20 mg/L), fullerenol maintained a molecular dimension of roughly 1.34 nm (Figure 3-1), leading to a hypothesis that the biological response was different on either side of the agglomeration-divide. Again, the hypothesis was not supported by experimental evidence, as no intra-species statistical difference was seen between any of the fullerenol-PAI treatment concentrations or bacterial species.

Bacterial chronic toxicity assays were performed in the absence of light, indicating that photo-active reaction pathways were not available mechanisms for biological impacts. Fullerenol was shown to be non-epoxylated, indicating that chemical binding to lipopolysaccharide, peptidoglycan, or teichoic acid as proposed previously was not feasible. The non-reactive mechanisms of extracellular amino acid complexation indicated for nC_{60}-28L were potential protective processes for both bacteria against fullerenol, suggesting that some fullerene material may have been removed from circulation by interaction with extracellular material and unavailable to impart adverse to the cell membrane or internal structures. However, impacts
were seen at most concentrations, several of which were statistically significant, questioning whether sequestration of fullerenol actually influenced results.

The error sum of squares describing the differential biological response of bacteria between fullerenol and \( nC_{60} \)-28L was 0.6% at the four overlapping concentrations (0.1 to 100 mg/L), suggesting that the two materials acted very similarly toward Gram-negative bacteria at all concentrations. Mechanisms identified for bacteria exposure to \( nC_{60} \)-28L included complexation to extracellular material, membrane penetration through complexation with membrane proteins, and nucleic acid binding and functional disruption (apoptosis). The major difference in chemical structures of \( aqu-nC_{60} \) and fullerenol resided in the number of hemiketal groups, six for both \( nC_{60} \) species and 14 ± 4 for fullerenol. Given that a mono-oxygenated surface group may or may not be hydroxylated, the presence of di-oxygenated hemiketal demonstrates inclusion of a known quantity of functional hydroxyl groups, in this case between 10 and 18 definitive hydroxyl moieties with the potential for an addition 12 hydroxyls from mono-oxygenated carbons.

Since the source of oxygenated compounds in the fullerenol synthesis process was highly concentration (25M) NaOH, as compared to atmospheric oxygen and dissociated hydroxyl ions from slightly acidic water used in the synthesis of \( aqu-nC_{60} \), it was more probable from a source-concentration perspective that a larger number of mono-oxygenated groups in fullerenol were hydroxyl in comparison to its water-stirred counterpart. The larger number of expected hydroxyl groups in fullerenol may have been responsible for the differential impact upon \( S. \) aureus, causing the Gram-positive bacteria to increase production of cellular material (272). Such a response may be indicative of fullerenol adversely impacting the peptidoglycan-teichoic acid
matrix, signaling the cell to reproduce, without ever effectively permeating the lipid bilayer and causing necrotic impacts.

**Dark and Light Assays**

**Bacterial cell membrane integrity assays**

Subsequently analyses of the biological effects of fullerenol were conducted using dye assays to assess cell membrane penetration. Bacteria cultures treated with 100 mg/L fullerenol for 24 hours under dark-conditions were exposed to membrane-selective nucleic acid dyes in the dark to elucidate whether membranes were compromised causing necrosis and whether impacts to nucleic acids resulted in apoptotic cell damage. Through the dark-phase LIVE/DEAD *BacLight* Bacterial Viability Assay, it was seen that fullerenol-PAI impacted Gram-positive bacteria to a greater extent than Gram-negative bacteria in dark-phase reactions. The augmented necrotic effect on cells caused by exposure to light indicated the capacity of photo-active fullerenol to increase incapacitation in both species. Dark-phase fullerenol activity appeared to be predominantly apoptotic while light-phase fullerenol activity appeared to be predominantly necrotic, indicating potentially different mechanisms of toxicity. Photo-physical properties of both *nC*<sub>60</sub> and fullerenol have been documented ([13, 24, 25, 65, 67, 76, 113, 114, 120-122, 128, 129, 287]) and biological/biomedical application continues to be a major research focus to harness these characteristics ([14, 146, 250]). Examination of photo-active toxicity mechanisms involving fullerene species have focused on the ability of fullerene species to be elevated to an excited state, subsequently generating ROS, which have been proposed to be direct reagents in biological processes. Ground-state fullerene, *¹C*<sub>60</sub>, has been shown to become excited to *¹C*<sub>60</sub>* when irradiated; this excited molecule displayed an estimated lifetime of only 1.3 nm, but transforms automatically to *³C*<sub>60</sub>*; a species that persisted as long as 100 μs ([15, 38, 74]):

\[
¹C_{60} + h\nu \rightarrow ¹C_{60}^* \rightarrow ³C_{60}^*
\] (4-1)
Several studies have suggested that excited-state fullerene impacted cell membranes through ROS-induced lipid peroxidation (38, 110, 112, 115, 147, 155, 156, 288-290). Tang et al. (291) indicated destruction of the outer cell membrane was due to ROS, although these claims have been brought into question due to the potential for fullerene to interfere with the results of dye-based assays (149, 292). Vileno et al. (64) described mechanisms of fullerene-spurred ROS generation through two scenarios: in the first, atomic particle transfer yielded superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radicals (OH$^*$):

\[
3C_{60}^- + 3O_2 \rightarrow C_{60} + O_2^-
\]  \hspace{1cm} (4-2)

\[
C_{60} + O_2^- \rightarrow C_{60}^- + O_2^- + 2H^+ \rightarrow C_{60} + H_2O_2
\]  \hspace{1cm} (4-3)

\[
C_{60} + H_2O_2 \rightarrow C_{60} + OH^* + OH^-
\]  \hspace{1cm} (4-4)

and, in the second, resonant energy transfer generated singlet oxygen (O$_{1}^{2}$).

\[
3C_{60}^- + 3O_2 \rightarrow C_{60} + O_2
\]  \hspace{1cm} (4-5)

Peroxidation of lipids (Figure 4-16) by fullerene species has been suggested as the primary mechanism for toxicity for both water-stirred fullerene and fullerol (35, 105, 113, 128, 145, 146, 193). Radicalized species have been shown to disrupt normal function of phospholipids by cleaving protons on the non-polar alkyl chain, thus radicalizing the molecule (99, 112, 146, 156, 230, 246). Sequential addition of a di-oxygen molecule and electron transfer with an unaffected lipid yielded lipid peroxide with reduced hydrophobic functionality and a destabilized lipid radical, which was likely to be re-oxidized and transformed to another reduced-capacity lipid peroxide. Lipid peroxidation generated a positive-feedback system, where the by-products of peroxidation included additional reactive species, facilitating additional peroxidation. Lipid peroxidation caused cell trauma initially due to a loss of osmotic pressure, followed by inability
to contain intracellular material, at which point, the impact was expressed as necrotic death (246-250).

Amino acids form proteins by linking together in a chain, connecting from hydroxyl group to amine group, with amino acid side-chains, responsible for secondary protein folding, which leaves scant opportunity for fullerene species to impact polypeptide chains directly (293). Proteins utilize amino acids such as cysteine, a common, sulfur-based component of *P. aeruginosa* protein c551 and Staphylococcus protein A, to facilitate the formation of tertiary structures (294, 295). Fullerene-induced protein denaturation occurred through oxidation of cysteine (Figure 4-17), which was caused by the addition of superoxide at the sulfur end groups of the amino acid, forming sulfenic acid, with conversion to either cystine, a dimeric amino acid, or cysteic acid through a sulfinic acid intermediary (296). Protein denaturation diminished the capability of proteins to maintain their designated conformation, upon which the lock-and-key mechanisms of proteomics rely. Leon *et al.* (297) examined the potential for fullerene to interact with 20 primary amino acids, showing high affinity for half of them including asparagine, cysteine, leucine, and glutamine, all amino acids present in subject bacterial proteins. Several reports have cited fullerenol-induced protein denaturation through oxidation of cysteine, resulting in apoptotic cell damage (70, 297, 298). Denaturation of cell membrane proteins can result in immediate loss of cell function by abandoning position in the cell membrane, effectively creating holes in the membrane for seepage of intracellular material, and relinquishing control of active transport mechanisms responsible for nutrient uptake and integrity of the cell membrane. Perhaps the most invasive scenario occurred when denatured proteins instigated functions that were not warranted or bound to sites that inhibited cell functions, spurring apoptotic effects such as teratogenesis, mutagenesis, or carcinogenesis.
Impacts to bacteria populations observed upon exposure to fullerol in light-phase were overwhelmingly necrotic, suggesting that irradiation facilitated traumatic cell damage. Lipid peroxidation and protein denaturation were consistent with the results of light-phase dye assays, indicating that irradiation of fullerene promoted necrotic bacterial cell death in both Gram-negative *P. aeruginosa* and Gram-positive *S. aureus*. Numerous studies have examined the potential for fullerene species to be used in photo-dynamic therapy (132, 138, 299) in attempts to harness the photo-active capacity of fullerene species. Recently, fullerol molecules were shown to release combustible levels of energy when irradiated by a low-intensity ($10^2$ W cm$^{-2}$), monochromatic (785 nm) laser (215). Release of energy by an irradiated derivatized fullerene molecule within a cell, inside a cell membrane, attached to the exterior, or in close proximity to the cell membrane may prove to impact the functions of a cell detrimentally. Cells could be affected acutely through molecular cleavage or chronically through transcription interference and disruption of energy and nutrient supplies. Currently, the potential of fullerene photo-induced combustion is being researched, but it may be discovered that some of the toxicity ascribed to the production of ROS or direct reactivity of fullerol may be attributable to the ability of fullerol to release high levels of energy at a specified location upon exposure to light. Future research activities may seek to use concentrated infrared to assess the production or reduction of ROS and to attempt to induce biological responses. Confocal microscopy may prove to be a useful analytical method for obtaining instantaneous data to observe the changes in cells exposed to different wavelength radiation in the presence of fullerene species.

Impacts to bacteria populations during dark-phase exposure were shown through dye studies to encompass slower-rate apoptotic damage such as modification to nucleic acids as well as necrotic damage possibly including both lipid peroxidation and protein denaturation. The
difficulty of assessing the necrotic mechanisms observed stemmed from the limitation that lipid peroxidation and protein denaturation were indicated to occur only upon exposure to light, which excited the fullerene species and possibly generated ROS. According to the premise that these oxidation processes occurred only in the presence of radicals, either reactive oxygen or fullerene species, the necrosis observed in dark-phase assays, especially in the case of *S. aureus*, was not caused by photo-activated fullerene species but, rather, through an alternate mechanism. Da Ros and Prato (15) observed cellular damage but no intracellular generation of ROS by fullerenol and Sayes *et al.* (110) indicated the production of ROS in the presence of *n*C₆₀ was independent of irradiation. Complicating the ledger, Kamat (124) indicated that lipid peroxidation via *n*C₆₀ was caused by the generation of singlet oxygen while oxidation caused by fullerenol was a function of radical peroxy and hydroxyl generation. Alternately, Isakovic (70) suggested that oxidation caused by *n*C₆₀ was through the generation of ROS while membrane damage caused by fullerenol was not related to ROS.

To explain dark-phase necrotic damage, photo-independent mechanisms of fullerene reactivity were examined. Several reports have proposed that fullerene species became excited through Fenton’s reaction (193, 300, 301), utilizing hydroxyl and perhydroxyl radicals generated in the decomposition of hydrogen peroxide present in the aqueous medium:

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^{\cdot} + \text{OH}^{-} \quad (4-6)
\]

\[
^{1}\text{C}_{60} + \text{OH}^{\cdot} \rightarrow ^{3}\text{C}_{60} + \text{OH}^{\cdot} \rightarrow ^{3}\text{C}_{60}^{\cdot} + \text{OH}^{-} \quad (4-7)
\]

\[
\text{Fe}^{3+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{2+} + \text{OOH}^{\cdot} + \text{H}^{+} \quad (4-8)
\]

\[
^{1}\text{C}_{60} + \text{OOH}^{\cdot} \rightarrow ^{3}\text{C}_{60} + \text{OOH}^{\cdot} \rightarrow ^{3}\text{C}_{60}^{\cdot} + \text{OOH}^{-} \quad (4-9)
\]

or through a Fenton-type reaction, without the benefit of metallic ions (83, 302):

\[
\text{C}_{60} + \text{H}_2\text{O}_2 \rightarrow \text{C}_{60}^{\cdot} + \text{OH}^{\cdot} + \text{OH}^{-} \quad (4-10)
\]
Cooper and Zika (303) identified hydrogen peroxide as a naturally occurring constituent of surface and ground water, which may not be removed during deionization and likely was present in trace amounts in laboratory water. Additional hydrogen peroxide was generated through metabolic activity (e.g. xanthine oxidase, an enzyme that metabolizes guanine-based xanthine) in S. aureus (293) and P. aeruginosa (304). Further, iron ions were present in bacterial assays through the inclusion of tryptic soy media. The limited amount of hydrogen peroxide may have provided explanation of the limited biological effects.

Fenton’s reaction was applied to the derivatized fullerene species in this investigation:

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^+ + \text{OH}^- \quad (4-12)
\]

\[
[C_{60}\text{H}_x\text{O}_y\text{OH}]^+ + \text{OH}^- \rightarrow [C_{60}\text{H}_x\text{O}_y\text{OH}]^+ + \text{OH}^- \quad (4-13)
\]

\[
\text{Fe}^{3+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{2+} + \text{OOH}^+ + \text{H}^+ \quad (4-14)
\]

\[
[C_{60}\text{H}_x\text{O}_y\text{OH}]^+ + \text{OOH}^+ \rightarrow [C_{60}\text{H}_x\text{O}_y\text{OH}]^+ + \text{OOH}^- \quad (4-15)
\]

or, through a fullerenol-based Fenton-type reaction:

\[
C_{60}\text{H}_x\text{O}_y\text{OH} + \text{H}_2\text{O}_2 \rightarrow [C_{60}\text{H}_x\text{O}_y\text{OH}]^+ + \text{OH}^+ + \text{OH}^- \quad (4-16)
\]

\[
[C_{60}\text{H}_x\text{O}_y\text{OH}]^+ + \text{H}_2\text{O}_2 \rightarrow C_{60}\text{H}_x\text{O}_y\text{OH} + \text{OOH}^+ + \text{H}^+ \quad (4-17)
\]

An alternate mechanism for radicalization of fullerene species was proposed, suggesting that inherent reactivity of fullerene species can be found resulting from the collisions of these highly electrophilic molecules (37). An underivatized C\text{60} molecule had 30 potential electron-pair bonding sites, located at the carbon double bonds between hexagonal faces (77); derivatized molecules had fewer potential electron bonding sites as some site were occupied, but potential for electron addition remained. Fullerene molecules exchanged charge continuously from the aqueous medium, accumulating and releasing electrons upon collision with other fullerene
molecules. The icosahedral symmetry molecular group theory permits the highest energy level (corresponding to \( L = 5 \) for atomic orbital theory) to be split to \( H_u \), \( T_{1u} \), and \( T_{2u} \) energy levels, which, at ground-state (\(^1\)C\(_{60}\)), indicates ten paired electrons in the five-available \( H_u \) orbitals (305). The excited, triplet-state fullerene (\(^3\)C\(_{60}\)) was created by promotion of a \( H_u \) electron to the \( T_{1u} \) orbital (HOMO-LUMO energy gap = 1.5 eV). The charge necessary to promote the electron was proposed to hail from the accumulation of electrons from the aqueous solution by the highly electrophilic fullerene.

It was deemed feasible that colliding fullerene molecules became excited to the triplet state through charge exchange and generation of an exciton (electron-hole pair):

\[
^1\text{C}_{60} + ^1\text{C}_{60} \rightarrow ^3\text{C}_{60} + ^3\text{C}_{60} \rightarrow ^3\text{C}_{60}^* + ^3\text{C}_{60}^* \quad (4-18)
\]

Upon subsequent collision with an oppositely-charged fullerene molecule, recombination occurred, returning the electron back to the paired-electron configuration and, ultimately, to the ground state:

\[
^3\text{C}_{60}^* + ^3\text{C}_{60}^- \rightarrow ^2\text{C}_{60} + ^3\text{C}_{60} \rightarrow ^1\text{C}_{60} + ^1\text{C}_{60} \quad (4-19)
\]

or, alternately, to an unpaired-electron configuration, yielding a radicalized fullerene and a triplet state molecule, which would return, ultimately, to the ground state:

\[
^3\text{C}_{60}^* + ^3\text{C}_{60}^- \rightarrow ^3\text{C}_{60}^* + ^3\text{C}_{60} \rightarrow ^3\text{C}_{60}^* + ^1\text{C}_{60} \quad (4-20)
\]

As another potential pathway, the collision could occur with an unadulterated molecule in a likewise fashion, yielding a radical fullerene and a hole:

\[
^3\text{C}_{60}^* + ^1\text{C}_{60} \rightarrow ^3\text{C}_{60}^* + ^3\text{C}_{60} \rightarrow ^3\text{C}_{60}^* + ^3\text{C}_{60}^* \quad (4-21)
\]

Similarly, collision of fullerenol molecules could create a radicalized fullerenol molecule:

\[
\begin{align*}
\text{C}_{60}\text{H}_{2}\text{O}_x(\text{OH})_y + \text{C}_{60}\text{H}_{2}\text{O}_x(\text{OH})_y & \rightarrow [\text{C}_{60}\text{H}_{2}\text{O}_x(\text{OH})_y]^\cdot + [\text{C}_{60}\text{H}_{2}\text{O}_x(\text{OH})_y]^\text{-} \quad (4-22) \\
[\text{C}_{60}\text{H}_{2}\text{O}_x(\text{OH})_y]^\cdot + [\text{C}_{60}\text{H}_{2}\text{O}_x(\text{OH})_y]^\text{-} & \rightarrow [\text{C}_{60}\text{H}_{2}\text{O}_x(\text{OH})_y]^\text{2+} + \text{C}_{60}\text{H}_{2}\text{O}_x(\text{OH})_y \quad (4-23)
\end{align*}
\]
or a radical plus a fullerene molecule with an electron hole:

\[ [C_{60}H_{2}O_x(OH)]^+ + C_{60}H_{2}O_x(OH) \rightarrow [C_{60}H_{2}O_x(OH)]^+ + [C_{60}H_{2}O_x(OH)]^* \]  \hspace{1cm} (4-24)

The creation of an additional fullerene molecule with an electron hole indicated the potential for subsequent radicalization reactions within the mixture, permitting an environment with spontaneous formation of reactive fullerene species.

The presence of radical fullerene species has been shown to initiate radical reactions directly upon cellular material (e.g. lipid peroxidation) or to generate radical oxygen species that executed biochemical processes, causing damage to lipids, proteins, enzymes, and amino acids. Additional investigation is warranted to assess the feasibility of these alternate mechanisms of fullerene species reactivity including electron-spin resonance (ESR) to assess the presence of radical fullerene species, although experiments will need to determine how to radicalize fullerene in an oxic environment without generating radical oxygen species as presence of the latter would confound results. One method could be to use a known scavenger of ROS that is indifferent to excited-state fullerene species so that ESR will yield only results reflective of fullerene radicals. An additional option is to study the potential for tracking transformation pathways of fullerene species using radio-labeled $^{13}$C$_{60}$ as a raw material, eliminating confounding elements related to the presence of biological carbon.

**Algal chronic toxicity assays**

Neither aqu-\(n\)C$_{60}$ material assayed caused a statistically significant effect on *P. subcapitata* at any concentration between 0.01 and 100 mg/L. Fullerenol-PAI caused no statistical difference at lower concentrations (\(\leq 10\) mg/L); however, significant population reductions were noted at 100 mg/L (-27.0%) and 1,000 mg/L (-83.3%). At lower concentrations (\(\leq 10\) mg/L), it was concluded that fullerenol was not present in adequate mass to impact algal cells. At higher concentrations (\(\geq 100\) mg/L), an adequate quantity of material was available to become
sufficiently detrimental to the algae, imparting an observable, measureable, and adverse impact. As neither $nC_{60}$ samples caused an adverse biological effect to *P. subcapitata* while the effect of fullerenol was statistically significant at the higher concentrations, it was apparent the impacts of the two materials were distinct despite chemical similarity. Investigation of potential mechanisms of biological impact included issues related to nutrients, nutrient complexation, shielding, and fulleren species photo-activity.

Algae used carbon dioxide as a carbon source, not organic carbon, thus, consumption of fulleren species for sustenance was not anticipated. Algae, however, do absorb minerals and complexation of fulleren with the magnesium, calcium, sodium, potassium, zinc, iron, cobalt, and copper as well as the counter ions borate, chloride, nitrate, sulfate, phosphate, molybdate, selenate, and carbonate, which are all present in the algal PAAP culture medium, has been documented (94, 306, 307), presenting nutrient complexation as a plausible pathway for biological impact and warranting subsequent investigation. Absent from the literature reviewed were accounts of fulleren species reacting with carbon dioxide. Regarding shielding of nutrients, generalized calculations indicated a maximum of 1.2% of the surface of the algae would be covered by fullerenol at a concentration 1,000 mg/L with an established initial population of 10,000 cells per mL and, assuming 100% adhesion, an area comparable to less than the soles of the feet on a human body would be impacted; hence, nutrient shielding was dismissed as a potential mechanism of toxicity.

While the impact of inhibiting nutrients does not seem a plausible cause of growth inhibition, the potential for inhibiting radiation available to *P. subcapitata* as a result of culture media being infused with fulleren species implored examination. *P. subcapitata* contain the photo-sensors chlorophyll A and B, which have been shown (308) to absorb wavelengths in the
blue (peaks at $\lambda = 430$ and $\lambda = 480$ nm) and yellow (peaks at $\lambda = 662$ and $\lambda = 630$ nm) spectral regions while photo-inactive in the green range (500 nm to 600 nm). *P. subcapitata* also contain $\beta$-carotene, another photo-sensor for biosynthesis, which displayed absorbance peaks at 280, 420, and 490 nm and was photo-inactive at $\lambda > 520$ nm (308). *aqu-nC60* was shown to have ultraviolet/visible light spectroscopy peaks near $\lambda = 360$ nm and $\lambda = 525$ nm, neither of which corresponded to the activity of either chlorophyll or $\beta$-carotene, suggesting no conflict of wavelengths. Neither fullerenol-PAI nor fullerenol-CA displayed peaks in the obtainable range of available equipment. Potential impacts of fullerenol-absorbed radiation in this range of wavelengths included reduction of total radiation absorbed by algae or a photo-active, biological effect from fullerenol (308). A fullerenol-caused reduction in the total radiation received by algae may have prevented adequate photosynthetic growth, as demonstrated in the reduced populations. Some interference with algal photo-receptors at the higher energy wavelengths ($\lambda < 500$ nm) was possible; however, fullerenol absorbance at $\lambda < 400$ nm was much greater than in the range of algal photo-sensors, suggesting that the maximum capacity for interference was not attained, especially at lower concentrations ($\leq 10$ mg/L). At higher concentrations ($\geq 100$ mg/L), fullerenol altered the color of the PAAP media, imparting a distinct amber tone, suggesting little to no absorbance in the 560 nm to 635 nm wavelength range. The lower light collection range of the photo-sensors ($\lambda = 420$ nm to 490 nm) may have been impacted marginally, although the effect was not confirmed.

An alternate explanation to biological response may be found in the photo-activity of fullerenol and its ability to inflict lipid (peroxidation) and protein (denaturation) damage to cells exposed to radiation by direct oxidation or through ROS generation. Research has demonstrated photo-induced production of ROS in fullerenol solutions, indicating the generation of singlet
oxygen ($^1\text{O}_2$), superoxide ($\text{O}_2^-$), and hydroxyl radicals ($\text{OH}^-$) using a lamp that emitted wavelengths between 310 nm and 400 nm (238) and superoxide ($\text{O}_2^-$) using wavelengths between 350 nm and 400 nm (128). Keren (309) indicated photo-induced oxidative inactivation of algal was limited by the transient interaction of singlet oxygen with chloroplasts, specifically the D1 protein involved in photosynthesis. Metzler (310) noted that fullerol-induced lipid peroxidation increased the rate of electron transfer across the cell membrane and disrupted potential difference, causing algae to lose control of membrane diffusion and active transport. Vannini (311) indicated oxidative stress to chlorophyll $a/b$ binding protein caused by photo-activated xenotoxins that were able to penetrate the cell, indicating that penetration was achieved without inactivating the cell, which permitted longer-term damage such as chloroplast protein denaturation. As a defense mechanism to ward of oxidative stress, algae generated enzymatic antioxidants that included superoxide dismutase, catalase, glutathione peroxidase, ascorbate peroxidase, thioredoxin, peroxiredoxin, and glutathione reductase (312). More detailed study of the interaction between fullerene species and these enzymes is warranted to determine the potential for impact on primary ecological producers.

**Invertebrate acute toxicity assays**

The most apparent impetus for the response of *C. dubia* to fullerene species was that assays contained no source food (algae, wheat germ, or yeast-cereal-tetramin mixture), only the nutrient salts contained within the culture media (sodium bicarbonate, calcium sulfate, magnesium sulfate, and potassium chloride). While issues related to fullerene-nutrient complexation and shielding may offer some indication as to why increased fullerene species caused increased mortality, a more direct justification could be found in daphnid ingestion carbonaceous material directly into the alimentary canal. Several groups have identified ingestion of fullerene species by daphnids (27, 104, 240, 313-315), however, complete clearing
after removal from the fullerene suspension was noted (240), suggesting no nutritional assimilation occurred. While no daphnid-specific food was present in any of the assays including control, the presence of consumable fullerene species may not lead to adverse biological effects. Oberdorster et al. (27) noted that daphnids have been observed ingesting silt and clay sediments as part of normal nutritional behavior, which impacted longer-term biological indicator functions such as feeding rate, body growth, and brood size (315). Lovern et al. (314) noted that daphnids selected food based on size, shape, and texture, indicating fullerene species used in invertebrate assays resembled desired food sources. Rosencranz et al. (315) observed daphnids ingested particles as large as 70 μm and have been known to consume bacteria with diameters greater than 200 nm; based on these observations, all fullerenol and a vast majority of the nC₆₀ was determined to be ingestible physically by C. dubia.

This investigation demonstrated daphnids consumed derivatized fullerene species (Figure 4-21), though no information was sought as to whether biological assimilation of the material occurred. Additional analysis on the use of fullerene species as a nutrient source for daphnids may require use of radio-labeled ¹³C₆₀ as stock material to determine uptake and metabolism of fullerene species. However, the mere presence of fullerene species within the alimentary canal of the daphnids in the presence of radiation suggested that biological damage could be facilitated by photo-activity. It was deemed plausible that the reactivity of fullerene species lent to the decline in viability even at lower concentrations. Since aqu-nC₆₀ caused minimal mortality over control, it was suggested this material was not photo-active. It was postulated that daphnids expended energy in an attempt to digest fullerene without a return on their energy investment; essentially, the invertebrates died from eating material without nutritional value. While nutrient complexation and shielding may have lent to the increase mortality witnessed with fullerenol-
PAI at ≥ 100 mg/L, it was proposed further that ingestion of fullerol by *C. dubia* and exposure to light enabled photo-active processes to occur, causing immobilization or mortality at high concentrations.

**Commercially-available and Laboratory-prepared Fullerol**

It was observed from impacts to unicellular organisms that laboratory-prepared fullerol (fullerenol-PAI) and commercially-available fullerol (fullerenol-CA) caused different biological effects. The populations of both bacteria were adversely affected to a greater degree with fullerenol-CA than with fullerenol-PAI at ≥ 100 mg/L. In algal assays, fullerenol-CA caused a statistically significant increase in population compared to control at lower concentrations (≤ 1 mg/L) with as much as an 33.7 ± 4.5% increase over control at 0.1 mg/L. Such an increase presented low-concentration commercially-available fullerol as a potential stimulant for algal growth; given the current advent in the use of algal products as viability industrial fuel sources, such development warrants further investigation. More detailed investigation of fullerenol-CA component data indicated that, of the two commercial samples analyzed, material from BuckyUSA behaved analogously to fullerol synthesized from raw materials (fullerenol-PAI), leaving commercially-available Nano-C fullerol as the anomalous component. Fullerenol-Nano-C caused increased algae populations over control of 83.4 ± 12.8% at 0.01 mg/L and 60.0% ± 13.6% (*n* = 3).

It was hypothesized differential toxicity between fullerenol-Nano-C and the other fullerol products was caused, in part, by the presence of residual reactants in fullerenol-Nano-C, as was shown previously. Although not analyzed as part of this study, toluene was found to have an LC$_{50}$ of 213 mg/L for *P. aeruginosa* (316) while *S. aureus* experienced 50% mortality in five hours due to the proliferation of branched-chain lipids when exposed to 5 mL toluene on a culture plate (317). Toluene was reported to have an EC$_{50}$ for *P. subcapitata* ranging from 12.5
mg/L (318) after a 72-hour exposure to 9.4 mg/L (319) after a 96-hour exposure and an LC₅₀ ranging from 310 mg/L (320) to 4,005 mg/L (321) when D. magna was exposed for 48 hours. Toluene was used as a solvent to disperse underivatized fullerene but was phase separated after addition of TBAH and NaOH. Due to phase-transfer of fullerene material, it was determined that no toluene remained in the synthesis products. This finding was verified by FTIR analysis of BP #1 (Figure 3-13), which showed only peaks consistent with toluene, as well as the solids remaining after phase separation (data not shown), labeled as Mix-2 in Figure 3-1, and final fullerenol products, all of which showed no presence of toluene.

NaOH stimulation to algae was not unexpected since algal prefer media with pH in the range of 7 to 9; NaOH has been as an alkalinity enhancer in biological assays and NaOH for the investigation generated pH ranging from 7.5 at 0.01 mg/L to 12.3 at 1,000 mg/L. Adverse effects at higher NaOH concentrations seen in both bacteria were attributed to hyper-alkalinity, causing an imbalance of osmotic pressure and resulting in cell lysis (310). A similar response was observed in C. dubia, although not to the same degree of severity. Warne and Schifko (322) reported an EC₅₀ of 40.38 mg/L for NaOH acting on C. dubia and Anderson (323) reported a toxicity threshold for NaOH on D. magna of 156 mg/L based on population immobilization, figures that are not inconsistent with the findings of this study.

Several reports have indicated threshold toxicity of MeOH on aquatic species including an LC₅₀ of 66.0 mg/L with P. aeruginosa (316), an EC₅₀ > 60.4 mg/L with P. subcapitata (324), an EC₅₀ ranging from 20,793 to 24,500 mg/L with D. magna (325-327), and an EC₅₀ of 21,402 mg/L with D. pulex (326, 327). MeOH has been cited as causing reduced chlorophyll content in P. subcapitata (308), lending credence to the finding of an LC₅₀ of 8.43 mg/L. It was postulated that MeOH acted as a carbon source for bacteria, a practice utilized in wastewater treatment
systems, albeit primarily for anaerobic microbes (328), as well as for the daphnids, who may have utilized MeOH as a carbon source in lieu of alternate carbon sources in the culture medium.

Very little information was available in literature or in available toxicity databases (ECOTOX, TOXNET) regarding the biological effects of TBAH. The only article reviewed regarding TBAH toxicity and bacteria, algae, or invertebrates indicated that *P. aeruginosa* experienced 1.7 to 3.0 times the toxic effect as control and *S. aureus* experienced 1.8 to 3.5 times the toxic effect as control when exposed for 24 hours to 3.0 mg on a culture plate. The United States Occupational Health and Safety Administration (OSHA) mandated Materials Safety Data Sheet indicated only an LC$_{50}$ for rats at > 90 mg/kg (30 mg ingested for an average adult rodent or 6.1 g per average adult human); otherwise there were no outstanding health or safety concerns pertaining to or regulations controlling TBAH. TBAH proved to be the most detrimental substance assayed during these experiments (Table 4-1) and warranted additional scrutiny. FTIR analysis displayed a distinct peak located at 1379 cm$^{-1}$ in only TBAH and fullerenol-Nano-C. Subsequent investigation showed the same 1379 cm$^{-1}$ peak in a mixture of only C$_{60}$ and TBAH but not in underivatized C$_{60}$, providing indication that TBAH was present in fullerenol-Nano-C. TBAH residuals in fullerenol-Nano-C were proposed as the reason for differential toxicity with both fullerenol-PAI and fullerenol-BuckyUSA, causing a eustressed increase in *P. subcapitata* population at lower concentrations but a decrease in destructive fullerenol photo-activity in both algae and daphnids at higher concentrations either by binding to the fullerenol at activity sites or by inhibiting the interaction of the fullerenol with activity sites on or within organisms. This analysis, in conjunction with the earlier discovery that commercially-available fullerenol was not solely C$_{60}$(OH)$_{24-26}$ or C$_{60}$(OH)$_{18-22}$ per label literature but more along the lines of C$_{60}$H$_{x}$O$_{y}$(OH)$_{z}$
with a maximum $x$ of 20 for the former and 12 for the latter, suggested that chemical composition may have impacted biological response.

**Fullerenol Synthesis By-products and Reactants**

Fullerenol synthesis generated three by-products, which were defined as the material remaining after the end-products were generated, as depicted in Figure 4-3. It was hypothesized upon visual inspection of evaporated filter residue that BP #2 would contain underivatized C$_{60}$, nestled amidst underivatized aggregates and unable to achieve adequate contact with the reactants, while BP #3 would contain only trace amounts of TBAH and fullerenol complexed with MeOH residues. Upon examination of the FTIR spectra (Figure 3-13 and Figure 3-14), TBAH was only present in BP #2 and NaOH was present in both BP #2 and BP #3. It was hypothesized further that BP #2 would impart biological effect in a manner consistent with both TBAH and NaOH while BP #3 would act similar to NaOH. BP #2 did not have marked impact on either bacteria population while BP #3 stimulated *S. aureus* at all concentrations and caused significant decline in *P. aeruginosa* at 1,000 mg/L. TBAH stimulated *S. aureus* at trace concentrations but caused dramatic decline at higher concentrations whereas MeOH stimulated Gram-positive bacteria, indicating BP #3 displayed biological effects similar to both NaOH and MeOH. Algal populations were stimulated by BP #2, mimicking the impacts of the NaOH assays, while BP #3 caused population declines reminiscent of TBAH and MeOH, although not as a severe at higher concentrations ($\geq$ 100 mg/L). The invertebrates were adversely impacted by both by-products at $\geq$ 100 mg/L ($\geq$ 10 mg/L for BP #2) and, based on its lethality to *C. dubia*, TBAH present in the by-products was assigned as the cause of toxicity to daphnids. Examination of FTIR spectra (Figure 3-12 and Figure 3-14) indicated that peaks found in fullerenol were also present in the spectra of by-products, suggesting some impacts inflicted on *C. dubia* may have been attributable to fullerenol photo-activity.
Summary

Neither aqu-nC₆₀ sample was shown to have significant adverse effect on any biological species at concentrations as high as 100 mg/L while growth stimulation was observed in S. aureus and, to a lesser extent, in P. aeruginosa after exposure to aqu-nC₆₀-14L. These impacts were prominent at higher concentrations, possibly as a eustressed response to epoxylated moieties binding to extracellular material resulting in lipid dissociation and apoptosis. Fullerenol demonstrated statistically significant reductions of bacteria populations, with commercially-available fullerenol demonstrating a greater adverse effect than material produced in the laboratory. Fullerenol reduced algae and daphnid populations significantly at concentrations greater than 100 mg/L and 10 mg/L, respectively, with greater adverse effect on bacteria than either of the water-stirred samples. Fullerenol adversely impacted algae and daphnids whereas aqu-nC₆₀ did not have an adverse impact. Adverse impacts of fullerenol to bacteria were predominantly apoptotic in the absence of light but overwhelmingly necrotic in the presence of light, indicating fullerenol acted through multiple toxicological mechanisms. Since adverse impacts were documented in the absence of light, it was proposed that penetration of material led to the dark-phase nucleic acid binding and, subsequently, apoptosis. Furthermore, it was proposed that fullerene derivatives possessed inherent reactivity mechanisms, which may include Fenton’s reactions utilizing iron within the culture media, a Fenton-type reaction, where trace hydrogen peroxide formed through metabolic activity spur activity, or fullerene species excitation was induced through charges released upon agglomerate collision. One commercially-available fullerenol sample was found to have different biological impacts than another commercially-available material and laboratory-prepared fullerenol, which was attributed to impurities in commercially-available material, specifically assigned to the presence of TBAH. Also, fullerenol synthesis by-products were shown to impart a biological response caused by
reactants (TBAH or MeOH) as well as by the photo-activity of derivatized fullerenol species transferred to the by-products during purification processes.

While several potential mechanisms were assessed for biological feasibility given the parameters of the assays conducted, additional investigation into other mechanisms such as nutrient shielding, nutrient complexation, and the use of fullerene as a nutrient may enhance future understanding of organism response. Nutrient shielding investigations may utilize scanning electron microscopy (SEM) to visually observe the surface of cell, although care must be taken to utilize a conducting coating to ensure proper imagine; \(^{13}\text{C}_60\) utilized within SEM may prove to generate dynamic images. Examination of fullerene complexation with nutrient would entail a matrix design whereby the fullerene species in question would be exposed a suite of nutrients found in assay protocols or within natural systems and assessed for complexation through mass spectrometry. It was shown that daphnids ingested fullerene species, though their fate after consumption remained unconfirmed and analysis may be facilitated through the use radio-labeled \(^{13}\text{C}_60\) to assess biological assimilation. Future investigations may be undertaken to qualify and quantify the interaction of epoxylated fullerene with cellular material, specifically membrane structures, to verify if these fullerene species have definable adsorptive capacity. Interaction between fullerene species and biological molecules such as bacterial and algal enzymes may elucidate biomedical applications previously undiscovered that could inhibit adverse health effects, as has been shown in viral and neurological diseases. In a manner similar to nutrient complexation, fullerene species could be exposed to isolated membrane biochemicals and reactions could be observed using mass spectroscopy. The presence of radical fullerene species has been shown to initiate radical reactions directly upon cell material (e.g. through lipid
peroxidation) or to generate radical oxygen species both in the absence and presence of light, resulting in cell damage.
Table 4-1. Summary of estimated toxicity thresholds for select organisms exposed to fullerene species, synthesis by-products, and synthesis reactants.

<table>
<thead>
<tr>
<th>Biological Species</th>
<th>nC$_{60}^a$</th>
<th>Fullerenol$_d^a$</th>
<th>By-Products$_d^a$</th>
<th>Synthesis Constituents$_d^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14L</td>
<td>28L</td>
<td>PAI</td>
<td>CA</td>
</tr>
<tr>
<td>$P$. aeuriginosa</td>
<td>ND$_b$</td>
<td>1,336</td>
<td>2,409.0</td>
<td>785.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ND$_c$</td>
<td>ND$_c$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>36.8</td>
<td>51.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>45.50</td>
</tr>
<tr>
<td>$S$. aureus</td>
<td>ND$_b$</td>
<td>ND$_c$</td>
<td>92.8</td>
<td>ND$_b$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ND$_b$</td>
<td>ND$_b$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>28.8</td>
<td>ND$_b$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8.09</td>
</tr>
<tr>
<td>$P$. subcapitata</td>
<td>ND$_b$</td>
<td>ND$_b$</td>
<td>299.0</td>
<td>1462.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ND$_b$</td>
<td>ND$_b$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ND$_b$</td>
<td>ND$_b$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8.43</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>39.40</td>
</tr>
<tr>
<td>$C$. dubia</td>
<td>ND$_b$</td>
<td>ND$_b$</td>
<td>31.1</td>
<td>45.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>44.5</td>
<td>22.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1,956.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ND$_b$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.81</td>
</tr>
</tbody>
</table>

$^a$ All figures represent LC$_{50}$ in mg/L.

$^b$ ND1 – LC$_{50}$ not determined due to mathematical limitations.

$^c$ ND2 – LC$_{50}$ not determined due to infeasible concentration (> 10,000 mg/L).
Figure 4-1. Response of *Pseudomonas aeruginosa* (ATCC No. 15442) and *Staphylococcus aureus* (ATCC No. 6538) to *aqu-nC$_{60}$* at $t = 14$ and 28 days (14L and 28L). Error bars indicate standard deviation. Asterisk indicates statistically significant difference from control ($\alpha = 0.05$).

Figure 4-2. Response of *Pseudomonas aeruginosa* (ATCC No. 15442) and *Staphylococcus aureus* (ATCC No. 6538) to fullerenol synthesized by “modified” Kitazawa method (214) (PAI), and commercially-available fullerenol (CA). Error bars indicate standard deviation. Asterisk indicates statistically significant difference from control ($\alpha = 0.05$).
Figure 4-3. Response of *Pseudomonas aeruginosa* (ATCC No. 15442) and *Staphylococcus aureus* (ATCC No. 6538) to sodium hydroxide (NaOH), methanol (MeOH), and tetrabutylammonium hydroxide (TBAH). Error bars indicate standard deviation. Asterisk indicates statistically significant difference from control ($\alpha = 0.05$). Bracketed sets indicate statistically significant difference between bacteria species.
Figure 4-4. Response of *Pseudomonas aeruginosa* (ATCC No. 15442) and *Staphylococcus aureus* (ATCC No. 6538) to by-products of fullerenol. Synthesized by “modified” Kitazawa method (214) (BP #2 and BP #3). Error bars indicate standard deviation. Asterisk indicates statistically significant difference from control (α = 0.05). Bracketed sets indicate statistically significant difference between bacteria species.
Figure 4-5. Response of bacteria to fullerol synthesized by “modified” Kitazawa method (214) (fullerol–PAI #1) at 100 mg/L quantified from LIVE/DEAD flow cytometry assay in dark exposure conditions. A) *Pseudomonas aeruginosa* (ATCC No. 15442). B) *Staphylococcus aureus* (ATCC No. 6538). Each point represents a single bacteria cell.
Figure 4-6. Response of bacteria to fullerol synthesized by “modified” Kitazawa method (214) (fullerenol-PAI #1) at 100 mg/L quantified from LIVE/DEAD flow cytometry assay in light exposure conditions. A) *Pseudomonas aeruginosa* (ATCC No. 15442). B) *Staphylococcus aureus* (ATCC No. 6538). Each point represents a single bacteria cell.
Figure 4-7. Response of *Pseudokirchneriella subcapitata* to aqu-nC₆₀, at *t* = 14 and 28 days (14L and 28L), fullerenol synthesized by “modified” Kitazawa method (214) (fullerenol-PAI), and commercially-available fullerenol (fullerenol-CA). Error bars indicate standard deviation.

Figure 4-8. Response of *Pseudokirchneriella subcapitata* to by-products of fullerenol. Synthesized by “modified” Kitazawa method (214) (BP #2, BP #3), sodium hydroxide (NaOH), methanol (MeOH), and tetrabutylammonium hydroxide (TBAH). Error bars indicate standard deviation.
Figure 4-9. Response of *Ceriodaphnia dubia* to *aqu-nC*$_{60}$, at $t = 14$ and 28 days (14L and 28L), fullerenol synthesized by “modified” Kitazawa method (214)(fullerenol-PAI), and commercially-available fullerenol (fullerenol-CA). Error bars indicate standard deviation.

Figure 4-10. Response of *Ceriodaphnia dubia* to by-products of fullerenol. Synthesized by “modified” Kitazawa method (214)(BP #2, BP #3), sodium hydroxide (NaOH), methanol (MeOH), and tetrabutylammonium hydroxide (TBAH). Error bars indicate standard deviation.
Figure 4-11. Schematic of lipopolysaccharide of Pseudomonas aeruginosa. Saccharides: D-man = D-mannose; D-fuc = D-fucose; D-glc = D-glucose; D-rha = D-rhamnose; D-gal = D-galactose; D-hep = D-heptose; KDO = 3-deoxy-D-manno-octulosonic acid; NAG = N-acetyl-D-glucosamine. L-ala = L-alanine. P = phosphatidyl group.
Figure 4-12. Schematic of teichoic acid of *Staphylococcus aureus*. Chains of teichoic acid are formed through phosphate groups. Multiple hydroxyl binding sites are located on reduced saccharide group.

Figure 4-13. Schematic of peptidoglycan of *Staphylococcus aureus*. NAM = N-acetylmuramic acid; NAG = N-acetyl-D-glucosamine. Amino acids: L-ala = L-alanine; D-glu = D-glutamine; L-lys = L-lysine; D-ala = D-alanine; L-gly = L-glycine. Chains of peptidoglycan are formed through proteins, interconnected with amino acid cross links. Multiple hydroxyl binding sites are located on reduced saccharide group.
Figure 4-14. Proposed adhesion reactions of epoxylated $a\nu-nC_{60}$-14L. Shown with representative saccharide unit contained in lipopolysaccharide of Pseudomonas aeruginosa and peptidoglycan or teichoic acid of S. aureus. Mechanism depicted as hydrogen bond (a), addition (b), and dehydroxylation (c).
Figure 4-15. pH of *Pseudomonas aeruginosa* cultures. Measurements shown were prior to treatment (t = 0) and after 24-hour assay without treatment (Control), at 10 mg/L, and 100 mg/L. Error bars indicate standard deviation.
Figure 4-16. Schematic of oxidation process with lipid molecule. Shown as initiated by triplet-state fullerene (1), and initiated by a hydroxyl radical (2). Product a indicates a destabilized lipid radical and is likely to be re-oxidized. Product b indicates a lipid peroxide molecule with reduced functional capacity.

Figure 4-17. Schematic of oxidation of cysteine. End products shown are cystine (a) and cysteic acid (c) through a sulfinic acid intermediary (b).
Figure 4-18. Schematic of *Pseudomonas aeruginosa* cell membrane phospholipid unit (Lipid-A).
Figure 4-19. Schematic of a typical cell membrane phospholipid unit (lysyl-phosphatidylglycerol) for Gram-positive *Staphylococcus aureus*.
Figure 4-20. Schematic of guanine. Base-pair hydrogen bond locations, and potential adhesion sites for fullerene species, denoted by dashed lines.

Figure 4-21. Micrograph of *Ceriodaphnia dubia*. White arrows indicate beginning and end of gastro-intestinal tract full of fullerenol.
CHAPTER 5
CONCLUSIONS AND FUTURE RESEARCH AVENUES

Conclusions

In terms of the time-evolution analysis of $nC_{60}$, which offered the benefit of simulating natural conversion of $C_{60}$ to a dispersible form in a natural body of water or within a treatment facility, $aqu-nC_{60}$ concentration showed marked increase over time, indicating that with turbulence and time, underivatized fullerene may convert to a water-stable form, facilitating transport into living cells within natural and man-made systems. The agglomerate size of a cluster of fullerene molecules appeared dependent on mechanical agitation energy, as was apparent in the consistent size of the agglomerates throughout the stirring period. The implication of this determination was that more turbulent flows, whether within a stream or in a treatment facility, will determine the size of the agglomerate, with higher mechanical energy inputs as seen in water and wastewater treatment facilities generating smaller, more difficult to remove, and potentially more biologically-active agglomerates. Although pH of the starting suspensions were influenced by purification of the type of water used for suspension preparation (i.e. de-ionized water), suspensions slowly reached a near-neutral pH over time while zeta potential was negatively charged and stable. The chemical composition of $aqu-nC_{60}$ was assessed and found to alter over time, with the disappearance of epoxide moieties, a reduction of di-oxygenated hemiketal structures, and an increase in the total number of surface groups over time, and with mono-oxygenated groups, either hydroxyl and ether, dominating the chemical composition profile. The presence of light during the synthesis process spurred surface groups derivatization at all times throughout the stirring.

Ultimately, the number and type of the mono-oxygenated carbon site may be the most important factor in the interaction between fullerene species and biological organisms. While it
was demonstrated through FTIR that mono-oxygenated epoxide groups disappeared from aqu-

$nC_{60}$ samples some time after 14 days of stirring, technological limitations prohibited
differentiation of mono-oxygenated surface groups between hydroxyl and ether moieties, a
distinction that may hold the answer for the mechanisms of biological interaction. Additional
methodological advancements in analytical chemistry are required to determine with confidence
the specific surface groups affiliated with derivatized fullerene, without which advancement of
fullerene derivative physicochemical and biological research likely will remain stagnated.

Bacteria populations were shown to have differential biological response to the presence of
$nC_{60}$-14L and $nC_{60}$-28L. It was proposed that both *P. aeruginosa* and *S. aureus* experienced
adverse effects, which were manifest in the eustressed response of stimulated cell reproduction in
the case of epoxylated $nC_{60}$-14L, due to chemical binding of fullerene species to saccharide
groups in the extracellular material of the cell membrane. Furthermore, the decline in population
in the case of non-epoxylated $nC_{60}$-28L, due to membrane penetration through protein
complexation, potentially resulted in nucleic acid binding, with apoptosis observed at the highest
concentrations (> 10 mg/L). Laboratory-synthesized fullerenol caused a moderate decline of *P.
aeruginosa* populations at all concentrations while *S. aureus* populations increased significantly
at all but the highest concentration (1,000 mg/L) during dark-phase exposure. The proposed
biological response of *P. aeruginosa* implicated a membrane penetration-protein complexation-
nucleic acid binding pathway in a similar manner of $nC_{60}$-28L, which was not unexpected given
the uniformity of chemical composition.

Upon exposure to light and through dye assays, it was determined that photo-activity
spurred necrotic cell death such as that caused by lipid peroxidation and protein denaturation.
Further investigation discovered that toxicological impacts previously attributed to photo-
activation occurred in the absence of light, suggesting activation by Fenton-type reactions or from the release of internal energy from diffusive collisions, yielding radicalized fullerene species that can impart effect directly or through generation of ROS. Light-phase algal and daphnid assays showed no significant adverse effect to aqu-$n$C$_{60}$ exposure but some photo-active response from both laboratory-prepared and commercially-available fullerenol, with an LC$_{50}$ of 31.1 mg/L attributed to fullerenol-PAI acting on C. dubia. Commercially-available fullerenol, specifically the material obtained from Nano-C, demonstrated increased eustressed conditions and inhibition of photo-active properties, which was attributed to the presence of impurities. Finally, the by-products from fullerenol synthesis process contained not only TBAH and NaOH, which were shown to have adverse biological effects themselves, but also fullerenol, which may have added to the toxicity response at higher concentrations as a result of photo-activity.

Fullerene species may impact biota directly through a number of mechanisms such as binding to the lipophilic outer surface of the bilayer membrane or through non-chemical means such as penetrating the cell membrane to oxidize lipids and proteins, interfering with nucleic acid functions, or through photo-induced fullerenol ignition. Additionally, fullerene species may affect organisms indirectly by interfering with nutrient supplies through mineral or carbohydrate complexation, through inhibition of ingestion pathways, or by disrupting necessities such as sunlight. Fullerene reactivity, especially activities that promote necrosis (i.e. lipid peroxidation and protein denaturation), was presented in previous reports as primarily a photo-induced phenomenon; however, this study has shown that processes detrimental to the immediate viability of organisms (i.e. necrotic cell death) occurred in both dark and light, although necrotic impacts were more pronounced upon exposure to radiation.
Neither bacteria nor algae expressed high sensitivity to *aqu-nC*$_{60}$ or fullerenol with the exception of one commercially-available product, which was attributed to impurities. For laboratory-prepared fullerenol, the lowest estimated threshold toxicity level was in excess of 1,000 mg/L and 299 mg/L for bacteria (excluding impure samples) and *P. subcapitata*, respectively. No toxicity thresholds were found for *C. dubia* in terms of water-stirred fullerene, but an LC$_{50}$ of 31.1 mg/L was determined for fullerenol, primarily due to the invertebrates choking on the material but also attributable, in part, to photo-activity. Based on elevated toxicity threshold values for *aqu-nC*$_{60}$ species and the associated lack of pronounced dark-phase impact, it was determined that fullerene posed no immediate adverse biological effect on the species examined at environmentally relevant concentrations. However, light-phase assays suggested fullerenol caused necrotic and apoptotic cell death in bacteria, algae, and invertebrates at concentrations that should allow those in positions of regulatory authority to take heed to potential ecological impacts.

None of the biological species examined displayed alarming toxicity to the derivatized fullerene. Previous reports indicated that *nC*$_{60}$ was orders of magnitude more toxic than the polyhydroxylated fullerenol counterpart, a result that was not supported by the results presented from this study. Efficient fullerene derivatization required non-polar solvents for synthesis and results suggested that residual non-polar solvent artifacts may be responsible for the adverse biological effects observed. Recent reports regarding *nC*$_{60}$ toxicity and the potential toxicological responsibility being attributed to THF degradation products were supported by the results of the assays; that is, *aqu-nC*$_{60}$ did not cause adverse biological impacts at concentrations deemed relevant to normal ecological systems. However, at higher concentrations, which might be expected in industrial processes or through accidental release, an adverse effect was
demonstrated. The information presented within this report might be deemed important by those working with the material at high concentrations, both in terms of ecological safety and operational efficiency.

**Future Research Avenues**

One of the difficulties identified through this research was the inability to determine with certainty the exact quantity of individual surface groups that comprised the material being examined. It was uncertain whether or not there was modified risk potential with the addition or subtraction of surface groups, whether they were hydroxylated, epoxidized, etherized, or existed hemiketal moieties. Presently, efforts to develop a method to conclusively identify the chemical composition continue and potential areas of scientific study for derivatized fullerene species are expanding as each new report is published.

Future research activities regarding fullerene, its derivatives and synthesis by-product may seek to refine the relationship between stirring velocity and particle size and shape by altering mechanical energy infusion and recording changes in particle size. Development of a standard for morphological configurations of fullerene agglomerates may adopt existing methodologies for other nanoparticles such as titanium dioxide, gold, or quantum dots. Early formation mechanisms, which is the period before the first examination period (t < 2 days), may seek to use flow-through sampling to facilitate recordation of particle size information. Special attention should be paid to the first few hours of stirring, as this timeframe indicates initial conditions from which subsequent comparison can be established. While there are no “real-time” methods of morphology examination, samples can be desiccated upon removal from the bulk to obtain as realistic a representation of shape transformation over time.

Information pertaining to the hydrogenation of the carbon cage may provide insight to the pre-condition of surface derivatization and may be achieved through the use of H-NMR,
although assessing conformation on the surface may still remain for technological advanced yet realized. The use of electron-spin resonance (ESR) may provide data on the radicalization of fullerene species, permitting research to enhance the understanding of dark-phase oxidation of lipids and proteins. Additional study of toxicological effects of fullerene species may seek to replicate ecological conditions perhaps with inclusion and control of electrolytic concentration, and examining the physical stability, chemical reactivity, and capacity for photo-activation.

Further, conducting synthesis with controlled and altered the atmospheric temperature or with additional purification steps such as additional methanol washes or use of size-exclusion separator, will provide data on the potential for environmental formation of derivatized fullerene.

The Kitazawa method utilized for alkaline-generation of fullerenol would benefit from refinement. Experiments designed to improve efficiency of the method may include more dilute reaction vessels facilitated by a larger volume of toluene, use of more absorbent desiccants and higher temperatures to ensure more effective drying, use a larger volume of MeOH to promote more efficient contact with fullerenol and higher dilution of the suspension prior to filtration. Sensitivity analyses of varied parameters should record effects observed in each permutation. Electron-spin resonance (ESR) may be employed to assess the presence of radical fullerene species, which will need to control the oxic environment without generating radical oxygen species, perhaps through the use of a known scavenger of ROS that is indifferent to excited-state fullerene species.

Substituting $^{13}$C$_{60}$ as a raw material instead of unmodified fullerene may prove to eliminate the inability to differentiate between carbon from organic sources and that assimilated through exposure to fullerene species, ultimately answering the question as to whether or not fullerene is utilized as a nutrient source. Examination of fullerene complexation with nutrients
and other biochemicals such as amino acids, enzymes, proteins, and nucleic acids would entail multiple preparations and determination of chemical binding through mass spectrometry. Nutrient shielding investigations may utilize scanning electron microscopy (SEM) to assess the impact of high concentrations and fostering understanding of adsorption mechanisms.

This report has determined that adverse biological effects of hydroxylated fullerene species are not significantly different than other naturally occurring carbon such as graphite. However, complex matrices can be found in natural and industrial biological systems. Researchers are continuing to develop programs to investigation the interaction of fullerene with naturally occurring waters. Some reports have noted that fullerene can interfere with natural processes; such examination will continue to expand on an as-needed basis with most of the new work being spurred by new applications and interaction potentials. Overall, this study contributes new knowledge to the emerging field of environmental nanotechnology and reported results are expected to be beneficial to the efforts of other researchers, the environment, and the fullerene industry.
Underivatized fullerene was examined upon receipt for purity, which was claimed by the manufacturer (Nano-C, Westwood, Massachusetts) to be 99.5%, of which the remainder was stated to be other fullerene molecules (i.e. \( C_{70} \) and \( C_{60}O_x \)). Purity analyses were conducted using matrix-assisted laser desorption/ionization (MALDI) mass spectrometry, high pressure liquid chromatography (HPLC), Raman spectroscopy, Fourier-transform infrared (FTIR) spectroscopy, and quantum mechanical calculations to determine the expected vibrational modes for underivatized \( C_{60} \). Results from samples were compared to existing literature to determine confirmation of purity.

**Manufacturer Data**

The mass spectrum and HPLC chromatograph for the underivatized \( C_{60} \) were obtained from Nano-C, Inc. (Westwood, Massachusetts) and are provided in Figures A-1 and A-2. The mass spectrum indicated a primary peak at \( m/z = 721.4171 \), which was within an acceptable shift (< 0.02%) from the anticipated 720.654. Also visible in the mass spectrum was a small, unlabeled peak around 735 and a small, labeled peak at 1418 \( m/z \), which correspond to \( C_{60}O \), and the \( C_{60}-C_{60} \) dimer. In addition, peaks at 500 and 971 \( m/z \) were unidentified. The 971 \( m/z \) peak corresponded to \( C_{60}O_{16} \), but determination of the presence of such a molecule without the noted presence of other intermediary oxygenated species required additional contemplation. Absent from the spectrum was a peak at 840 \( m/z \), which would correspond to \( C_{70} \), often found in small yet detectable quantities from the arc-vaporization synthesis process (1). The HPLC chromatograph depicted a single peak at approximately 8 minutes, suggesting singularity in chemical composition. Nano-C, Inc. indicated a chemical composition of 0.23% for \( C_{60}O \), 0.39% for \( C_{60}-C_{60} \) dimer, and 0.04% \( C_{70} \).
Raman and Fourier-transform Infrared Spectroscopy

Infrared spectroscopy was used to assess $C_{60}$ vibrational energies, or modes, which provided insight into the compositional structure of the material analyzed. Applied radiation scattered upon impact with $C_{60}$ aggregates either elastically or inelastically, termed Rayleigh and Raman scattering, respectively. The intensity of scattered energy, either elastic or inelastic, reflected activity at distinct energy levels, with each bond within a molecule emitting a specific energy depending on the atoms examined, facilitating chemical composition determination. The data was collected into a spectrum over the entire scanned energies and characteristics, or “fingerprints”, were used to identify compounds by comparing individual bonds within the molecule. Rayleigh scattering was measured using Fourier transform infrared (FTIR) spectroscopy and Raman scattering was measured using Raman spectroscopy with Rayleigh filters.

The FTIR spectrum for the underivatized $C_{60}$ sample depicted two primary peaks at 1183 and 1429 cm$^{-1}$ and two less prominent peaks at 1539 cm$^{-1}$ and 2328 cm$^{-1}$ (Figure A-3). The minimum wavenumber scanned with the Thermo Electron Magna 760 was 650 cm$^{-1}$, occluding potential peaks at lower wavenumbers. The Raman spectrum (excitation wavelength $\lambda = 785$ nm) of underivatized $C_{60}$ on a 100-silica substrate showed the presence of ten peaks at the following wavenumbers: 278, 431, 498, 712, 778, 1103, 1250, 1426, 1469, and 1580 cm$^{-1}$ (Figure 3-4).

Pertaining to vibrational modes, Fowler and Ceulemans (91) indicated that fullerene follows the general localized $\pi$-orbital configuration and can be expressed through reducible representations:

$$\Gamma(C_{60}) = \Gamma_{\pi} + \Gamma_{R} + \Gamma_{T}$$  \hspace{1cm} (A-1)
where \( \Gamma_\sigma = \) vibrational modes, \( \Gamma_R = \) rotational modes, and \( \Gamma_T = \) translational modes. As the investigation focused on FTIR and Raman spectroscopy to assess chemical composition through vibrational modes, rotational and translational reducible representations were ignored, resulting in:

\[
\Gamma(C_{60}) = \Gamma_\sigma(C_{60}) \tag{A-2}
\]

The general configuration for the vibrational modes was expanded to include specific irreducible representations through the use of icosahedral point group symmetry (\( I_h \)), yielding the following bonding orbitals:

\[
\Gamma_\sigma(C_{60}) = A_g + T_{1u} + T_{2u} + G_g + G_u + H_g + H_u \tag{A-3}
\]

where each term represents an irreducible representation of the molecular orbital energy state (A for \( l = 1 \), T for \( l = 2 \), G for \( l = 3 \), and H for \( l = 4 \)) and the subscripts, \( g \) and \( u \), correspond to “gerade” (paired) and “ungerade” (unpaired) symmetry, respectively. Researchers have found that not all vibrational modes were Raman or Rayleigh (FTIR) active (91, 330, 331). The active vibrational modes for \( C_{60} \) were represented by the following expression:

\[
\Gamma_\sigma(C_{60}) = A_g + H_g + T_{1u} \tag{A-4}
\]

where the FTIR-active vibrational modes were limited to the \( T_{1u} \) irreducible representation while Raman-active vibrational modes corresponded to \( A_g \) and \( H_g \) irreducible representations.

Based on previous investigations of \( C_{60} \) using FTIR spectroscopy, \( T_{1u} \) vibrational modes were active at 523-526, 573-576, 1180-1183, 1429-1430, 1539, and 2328 cm\(^{-1}\) (45, 87, 332, 333). Peak locations at 526, 576, 1183, and 1429 cm\(^{-1}\) were reflective of carbon-carbon bonding. Peaks at 1539 and 2328 cm\(^{-1}\) can be attributed to gases clathrated within the fullerene crystalline structure, identified as \( O_2 \) and \( CO_2 \), respectively (68). Bethune et al. (163) reported that other fullerene species such as \( C_{70} \) were FTIR-active at 1134, 1414, 1430 and 1460 cm\(^{-1}\) but not 1183
or 1429 cm\(^{-1}\), which provided support for the claim of no contamination. Although the lower wavenumber peaks were occluded by equipment cut-off, the consistency of the location of the visible measurements with published results and the lack of other notable peaks indicated that only C\(_{60}\) was present in the sample.

Previous reports indicated that \(A_g\) vibrational modes were found to be Raman-active at two wavenumbers, 498 and 1470 cm\(^{-1}\), and \(H_g\) vibrational modes were found to be Raman-active at eight wavenumbers corresponding to 273, 432, 711, 775, 1101, 1251, 1426, and 1577 cm\(^{-1}\) (69, 163, 330, 333). The Raman-active peaks from the experimental data were consistent with previous reports without additional notable peaks, providing indication that the material examined consisted only of C\(_{60}\).

**Quantum Mechanical Simulation**

A quantum mechanical software package, Gaussian03, was used to analyze virtual molecules. The C\(_{60}\) molecule was first analyzed to assess the lowest energy configuration, after which vibrational energies, examining both FTIR- and Raman-active modes. While the experimental data ranged from 650 to 4000 cm\(^{-1}\), the Gaussian03 data ranged from 500 to 1800 cm\(^{-1}\), which included higher wavelengths (i.e. lower wavenumbers) and two prominent peaks omitted in the experimental investigation at 532 and 570 cm\(^{-1}\). The FTIR spectrum from the computer simulation using Gaussian03 (Figure A-5) showed a considerable amount of noise compared to the experimental data as well as that of the literature. Additional peaks were shown at 1149 and 1453, and cm\(^{-1}\) with other unlabeled peaks that do not correspond to FTIR-active vibrational modes (i.e. 501, 570, 661, 694, 716, 736, 1018, 1208, 1301, 1479, and 1610 cm\(^{-1}\)).

The Raman spectrum from the computer simulation using Gaussian03 showed two peaks at 460 and 1475 cm\(^{-1}\) that correspond to \(A_g\) vibrational modes, based on prominence, and eight peaks at 228, 368, 709, 805, 1120, 1208, 1403, and 1573 cm\(^{-1}\) that correspond to \(H_g\) vibrational
modes (Figure A-6). A shift in the peaks from the reported values of 40-50 cm\(^{-1}\) for many of the peaks was noted in results reported in the literature from both experimental and modeling activities. Additionally, the Raman activity returned from Gaussian03 did not correspond to that of the literature in terms of magnitude; for example, the three vibrational mode peaks with the greatest magnitude from the experimental data were at 273, 432, and 1470 cm\(^{-1}\) while Gaussian03 returned only two peaks, 460 and 1525 cm\(^{-1}\), more magnanimous than the rest.

The differences in the spectra may be attributable to the equipment used in spectroscopy; however, for all experimental data to differ consistently from the modeled data suggests that the algorithm used by Gaussian03 to generate the vibrational mode wavenumbers and magnitude differs from the algorithm used within Raman spectrometers or, alternately, that the FTIR equipment displayed a consistent shift. Perhaps the most visible discrepancy between the Gaussian03 data and the other data sources was seen at the intensities between 661 and 736 cm\(^{-1}\), which graphically dominate the spectrum but provide only a non-active mode at 668 cm\(^{-1}\) and a Raman-active mode at 709 cm\(^{-1}\) (334-336). Investigation on the algorithms utilized in the various software programs available was outside the scope of this project. Upon examination of the visual transformation of the Gaussian03 output data using ChemCraft version 1.6 (Grigory A. Zhurko, Moscow, Russia), it was found that the wavenumbers with the peaks of greatest magnitude did not correspond directly to the wavenumber that produced the same vibrational response as indicated in the literature or in the experimental results (Table A-1).

Further analysis of the purity of the underivatized fullerene was conducted through inductively couple plasma (ICP) to assess the presence of metals, possible artifacts of the arc-vaporization synthesis process (J). No metals were detected, which was consistent with the claims in the manufacturer’s labeling material.
The mass spectrum provided by Nano-C suggests the presence of more constituents than stated and in quantities questioning the claim of 99.5% purity. To facilitate further chemical determination, Nano-C additionally provided a high pressure liquid chromatograph for the sample material obtained, which suggested a singular constituent with no detectable contamination. Independent experimental investigation through FTIR and Raman showed agreement with literature for peak location. Quantum mechanical calculations for both FTIR and Raman also provided additional substantiation of chemical composition, although notable differences were observed with Raman-active vibrational modes occurring experimentally at 278, 431, and 498 cm\(^{-1}\). Based on consistencies in peak location with the literature, the absence of additional peaks found with other fullerene species, and the lack of other compounds present as determined through ICP and HPLC analysis, the claim of 99.5% purity of the sample was not refuted.
Table A-1. Comparison of Raman ($A_g$ and $H_g$ modes) and FTIR ($T_{1u}$ modes) vibrational mode peaks from experimental and computer modeling.

<table>
<thead>
<tr>
<th>Activity</th>
<th>Current Research</th>
<th>Experimental</th>
<th>Modeled</th>
</tr>
</thead>
<tbody>
<tr>
<td>$H_g$</td>
<td>278</td>
<td>272</td>
<td>Peaks$^a$</td>
</tr>
<tr>
<td>$H_g$</td>
<td>431</td>
<td>432</td>
<td>Modes$^b$</td>
</tr>
<tr>
<td>$A_g$</td>
<td>498</td>
<td>498</td>
<td></td>
</tr>
<tr>
<td>$T_{1u}$</td>
<td>525</td>
<td>523</td>
<td></td>
</tr>
<tr>
<td>$T_{1u}$</td>
<td>576</td>
<td>572</td>
<td></td>
</tr>
<tr>
<td>$H_g$</td>
<td>712</td>
<td>711</td>
<td></td>
</tr>
<tr>
<td>$H_g$</td>
<td>778</td>
<td>775</td>
<td></td>
</tr>
<tr>
<td>$H_g$</td>
<td>1103</td>
<td>1101</td>
<td></td>
</tr>
<tr>
<td>$T_{1u}$</td>
<td>1183</td>
<td>1180</td>
<td></td>
</tr>
<tr>
<td>$H_g$</td>
<td>1250</td>
<td>1251</td>
<td></td>
</tr>
<tr>
<td>$H_g$</td>
<td>1426</td>
<td>1426</td>
<td></td>
</tr>
<tr>
<td>$T_{1u}$</td>
<td>1429</td>
<td>1430</td>
<td></td>
</tr>
<tr>
<td>$A_g$</td>
<td>1469</td>
<td>1470</td>
<td></td>
</tr>
<tr>
<td>$H_g$</td>
<td>1580</td>
<td>1577</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Calculated vibrational modes (Gaussian – peaks) at 278, 431, and 498 cm$^{-1}$ provide poor agreement with literature and experimental values outside of a 5% tolerance.

$^b$ Calculated vibrational mode (Gaussian – modes) at 498 cm$^{-1}$ provides poor agreement with literature and experimental values outside of a 5% tolerance.
Figure A-1. Fourier-transformed infrared (FTIR) spectrum of C$_{60}$. Samples obtained from Nano-C, Inc. (Westwood, Massachusetts).

Figure A-2. BioRaman IR spectrum of C$_{60}$. Samples obtained from Nano-C, Inc. (Westwood, Massachusetts).
Figure A-3. Gaussian03-calculated FTIR spectrum for $\text{C}_{60}$.

Figure A-4. Gaussian03-calculated Raman spectrum for $\text{C}_{60}$. 
APPENDIX B
DESCRIPTION OF SURFACE GROUPS

A considerable amount of research has examined derivatized fullerene with an understanding that the most common, stable, or perhaps most applicable oxygenated state was hydroxylated (25, 30, 47, 82, 83, 176, 218, 231). Hydroxylation of fullerene consisted of transformation of the \( \pi \)-bond between to two adjacent carbon atoms, located at the intersection of two hexagonal faces, to a \( \sigma \)-bond, with the bonding of a tertiary hydroxyl group on each carbon site.

Despite many manufacturers presenting their products as comprised of only fullerene and hydroxyl groups, researchers have shown a more complex cage surface, focusing attention on the processes involved in the transformation of the fullerene surface. These species may provide insight not only into additional physicochemical properties but also to the potential biological and ecological effects. An alternate mono-oxygenated surface group investigated was epoxide, which formed \( \sigma \)-bonds with two carbons. The carbon-oxygen-carbon angle within an epoxide typically was represented as 59.2 degrees (210), indicating that the other bond angles are 60.4 degrees each, forming a dense moiety. The bond length between \( \pi \)-bonded carbons in fullerene has been reported to be 135 pm but the epoxide requires 147 pm, introducing not only 25 kcal/mol of additional localized strain due to bond-angle constraints (210) but also regional strain due to the stretching of the carbon-carbon bond. A third mono-oxygenated group considered was ether, which could have evolved from epoxide, with one major difference being the carbon-carbon \( \sigma \)-bond. Ether group formation removed angle strain but introduced stretching since the preferential carbon-oxygen-carbon bond of ether was reported to be 110 degrees (210), necessitating 160 pm between formerly-bound carbon sites, 25 pm greater than for underivatized C\(_{60}\). While the addition of an epoxide would create structural strain within the
carbon cage due to an uneven distribution of bond dissociation energies, formation of a cage-compromising ether moiety might be thought as more destabilizing, given that the carbon-carbon bond was replaced by an oxygen bridge. However, due to the acute angle formed between the carbon-carbon-oxygen, structural strains were expected to be more pronounced with epoxide. Hence, ether formation was anticipated to be more prevalent than epoxide, although epoxide formation was determined to be a prerequisite condition.

Di-oxygenated hemiketal surface groups consisted of a single oxygen atom bonded to two adjacent carbon atoms at the intersection of two hexagons, similar to an ether group, with the addition of a secondary hydroxyl group bonded to one of the etherized carbons (30, 31, 75). The carbon atom associated with only the oxygen formed a π-bond with one adjacent carbon atom and a σ-bond with the other carbon atom. The carbon atom associated with the hydroxyl group maintained its σ-bonds with the adjacent carbon atoms. An alternate hemiketal formation was proposed to consist of a central oxygen molecule flanked by a hydroxyl group on each of the carbon atoms at the hexagonal intersection, establishing σ-bonds with the adjacent carbons in the form of a polyol structure. The single-hydroxyl hemiketal configuration appeared to be more likely to form due to potential steric interference in the polyol hemiketal. No indication of polyol hemiketal structures, or a preferred configuration on such a structure, was found in the literature.

Electronic rearrangement within hemiketal structure resulted in a combination of a di-oxygenated carbonyl and a hydroxyl group (multiple bond configurations are shown in Figure 2-27). A carbonyl group, comprised of a π-bonded oxygen, required carbon to break a σ-bond with an adjacent carbon, which produced a slightly modified, and distinguishable, bond energy. The attachment of a carbonyl surface group to a C_{60} molecule resulted in a fracture of the carbon
cage with unsupported, double-bonded appendages, which may have promoted fullerene destabilization. Due to cage fracturing and a lack of literature that indicated such degradation of the carbon cage, formation of carbonyl surface groups was considered unlikely. Furthermore, the formation of carboxylic acid surface groups was considered unlikely due to steric interference, fracturing of the carbon cage, and the creation of a double \( \pi \)-bonded condition on the facing carbon.
APPENDIX C
INTERACTIONS CONTROLLING FULLERENE AGGREGATION

Attractive intermolecular forces have been related to Newton’s gravitational force ($F_g$):

$$F_g = \frac{GM_1M_2}{r^2} \quad \text{(C-1)}$$

where $G$ = gravitation constant, $M_1$ and $M_2$ = masses of the bodies, and $r$ = distance between the bodies, as the interaction potential ($F_r$):

$$F_r = \frac{nCm_1m_2}{(r^{n+1})} \quad \text{(C-2)}$$

where $n$ = number of interactions, $C$ = intermolecular force constant, and $m_1$ and $m_2$ = mass of the interacting molecules. However, it was shown that, with intermolecular distances and with an array of interacting molecules ($n > 3$), alternate expressions are more suitable.

By considering the finite volume of molecules and the attractive forces between them, van der Waals proposed that the non-ideal behavior of molecules in gas can be characterized through use of a modified Universal Gas Law:

$$(P + \frac{a}{n^2V^2})(V - nb) = nk_B T \quad \text{(C-3)}$$

where $P$ = pressure, $V$ = volume, $k_B$ = Boltzmann’s constant, $T$ = temperature, $n$ = number of moles, and $a$ and $b$ = constants that compensated for interactive forces, $a$, and finite volume, $b$ (176). Recognizing that ($a / n^2V^2$) represented the inverse of the interactive force causing disruption, subsequent analysis of the mostly commonly confronted short-range attractive forces, that was those related to dipole-dipole, dipole-induced dipole, and induced dipole-induced dipole interaction, were found to be analogous to another Newtonian principle, that is, Coulomb’s relationship of electrostatic particles:

$$F = k_e \frac{q_1q_2}{r^2} \quad \text{(C-4)}$$
where $k_e = \text{Coulomb's proportionality constant, and } q_1 \text{ and } q_2 = \text{charges of the particles.}
Coulomb's Law can also be expressed in regard to an electrical field, such as the ubiquitous planetary electric field:

$$F = \frac{q}{4\pi\varepsilon_0 r^2} \quad (C-5)$$

where $q = \text{magnitude of the product of the charges and } \varepsilon_0 = \text{permittivity of free space.}$ In the case of intermolecular forces ($w_r$), charge ($q$) was equated to the square of the product of the dipole or induced dipole moments, relative permittivity ($\varepsilon_r$) was introduced to compensate for the impacts of the medium, both terms were squared to include translational impacts, the Boltzmann's constant, temperature, and an amalgamated constant were introduced to account for environmental conditions, and the short-range of the force was considered through an augmentation of the translational parameter. The resulting expressions for the three potential attractive van der Waals forces were for dipole-dipole interactions, termed Keesom forces, which are most applicable in terms of hydrogen bonding:

$$w_r = -\frac{1}{3k_B T(4\pi\varepsilon_0 \varepsilon_r)^2} \left[ \frac{1}{r^6} \right]$$

(C-6)
dipole-induced dipole interaction, termed Debye forces, which are significant in terms of polarizability, as with an electrical field:

$$w_r = -\left[ \frac{1}{(4\pi\varepsilon_0 \varepsilon_r)^2} \right] \left[ \frac{1}{r^6} \right]$$

(C-7)
and, induced dipole-induced dipole interaction, termed London forces:

$$w_r = -\left[ \frac{1.5(\alpha_{01}\alpha_{02})}{(4\pi\varepsilon_0 \varepsilon_r)^2} \right] \left[ \frac{1}{r^6} \right]$$

(C-8)
where $u_1$ and $u_2 = \text{dipole moments, } \alpha_0, \alpha_{01}, \alpha_{02} = \text{polarizabilities of the induced dipoles, } I_1 \text{ and } I_2 = \text{ionization potentials, and the other terms were as previously defined. Given that both Keesom and Debye forces demanded that at least one of the interacting molecules maintained a permanent dipole, and London forces described induced dipoles, which all molecules were
capable of achieving given adequate conditions. London forces provided the greatest contribution to the total attractive van der Waals forces as well as provided the strongest interaction. It was anticipated that all three forces were involved with C_{60} aggregation and agglomeration since C_{60} molecules have been shown to have localized electron-rich and electron-poor regions (91, 93, 114), suggesting polarity as well as polarizability. Additional development of the models (337) led to a single representation for all three van der Waals forces, which simplified calculations to an acceptable tolerance of error. Thus, van der Waals force (F_H) was defined as:

\[ F_H = - \frac{AR}{12\pi r^2} \]  

which expressed the force as a function of the radius of the particle (R), the distance separating the centers of mass (r), and a substance-specific parameter, Hamaker’s constant (A):

\[ A = \pi^2 C \rho_1 \rho_2 \]  

where C = intermolecular force constant, and \( \rho_1 \) and \( \rho_2 \) = number of atoms within the interacting molecules (338). The Hamaker’s constant was reported to be 6.7 x 10^{-21} J for C_{60} (178) and 4.62 x 10^{-20} J for water (172). It should be noted that the relationship for van der Waals forces was similar to the work of cohesion, which provided the foundation for particle interaction and was defined as:

\[ W_c = A_{11} / (12\pi H_0^2) \]  

where \( H_0 \) = distance of separation between the two particles and \( A_{11} \) = Hamaker’s constant for a singular material. The work of cohesion is twice the surface energy, or surface tension (\( \gamma \)):

\[ W_c = 2\gamma \]  

Closely related to the work of cohesion is the work of attraction that describes the energy required to bring two dissimilar materials together:
\[ W_a = A_{12} / (12\pi H_0^2) \]  
(C-13)

where \( A_{12} \) = Hamaker’s constant for the two-material system.

In addition to the attractive forces were electrostatic forces that function based on the electrical charge of a particle of molecule. Electrostatic forces repelled in cases where electrical charges were similar and comprised of energy measured as a constant charge:

\[ W_{\sigma r} = [2Z^2\nu_i k_B T (e\Psi_s / k_B T)^2] \left[ 1/\kappa \right] \left[ 1 - \coth((\kappa - 2)/2) \right] \]  
(C-14)

or as a constant potential:

\[ W_{\psi r} = [2Z^2\nu_i k_B T (e\Psi_s / k_B T)^2] \left[ 1/\kappa \right] \left[ 1 - \tanh(\kappa r/2) \right] \]  
(C-15)

where \( Z = \) valency, \( \nu_i = \) ion concentration, \( e = \) electron charge, \( \Psi_s = \) Stern potential, and the Debye length \( (\kappa) \) is defined as:

\[ \kappa = (\sum \nu_i k_B T e / 4\pi e_0 k_B T)^{1/2} \]  
(C-16)

At larger distances, much greater than the Debye length, the charge and potential were equal and the relationship was simplified to:

\[ W_{\psi r} = W_{\sigma r} = (2\sigma_s^2 / 4\pi e_0 \kappa) e^{-kr} \]  
(C-17)

where the Grahame charge \( (\sigma_s) \) is defined as:

\[ \sigma_s = 4(2\nu_i k_B T e / 4\pi e_0 \kappa)^{1/2} \]  
(C-18)

and the reduced surface potential \( (\gamma) \) is defined as:

\[ \gamma = \tanh[Z e\Psi_s / 4k_B T] \]  
(C-19)

making the final expression for the electrostatic charge between two particles:

\[ W_{\psi r} = W_{\sigma r} = (64k_B T \nu_i \gamma^2 / \kappa^2) e^{-kr} \]  
(C-20)

It should be noted that the mathematical representations of intermolecular force assumed a spherical structure, which was not the case, as observed through TEM imaging.
DLVO (Derjaguin, Landau, Verwey, and Overbeek) theory indicated that the total interaction energy \( (U_r) \) of the system can be represented as a function of separation distance by a single relationship:

\[
U_r = -\left[ \frac{AR}{12\pi r^2} \right] + \left[ \frac{64\pi Rk_B T \gamma^2}{\kappa^2} e^{-r} \right] (C-21)
\]

where the first term describes the attractive van der Waals forces and the second term, modified to account for the per-unit work of the system \( (\pi R) \), describes the repulsive electrostatic forces. DLVO theory has been used to describe the interactions within aggregated \( C_{60} \) molecules as well as between \( nC_{60} \) agglomerates in liquid (28, 34, 46).

The intermolecular forces holding aggregates together were dependent on the size of the particle as well as the distance between the two molecules being held together. It followed that molecules at the center of a cluster were the most tightly bound, with intermolecular forces decreasing the greater the distance \( (r) \) from the center of mass a molecule was located:

\[
U_r \propto \frac{1}{r} \quad (C-22)
\]

or, in terms of the differential:

\[
dU_r / dr < 0 \quad (C-23)
\]

The energy added to the system via mechanical stirring was sufficient to increase collisions between clusters, which resulted in a shear force \( (F_s) \), with an associated shear work:

\[
W_s = \int F_s \, dx \quad (C-24)
\]

that served to break apart the clusters when:

\[
W_s > W_r \quad (C-25)
\]

The amount of shear at work in the stirred system was proportional to the amount of mechanical energy introduced, indicating that higher stirring velocities yielded a greater shear force. The molecules, or more likely a group of molecules forming a primary particle group, furthest from
the center of the cluster were dislodged when the shear force was greater than the van der Waals attractive forces ($F_r$):

$$F_s > F_r \quad \text{(C-26)}$$

Subsequent dislodgement occurred until the attractive forces were able to withstand the shear caused by stirring, forming a cluster within a size range that was related to the stirring velocity.
APPENDIX D
BIOLOGICAL SPECIES

There are two commonly identified bacteria types: Gram-negative and Gram-positive, corresponding to the response of the bacteria to hexamethyl pararosaniline chloride, a violet dye that adheres to peptidoglycan, an amino-acid/sugar polymer component of the cell wall of some bacteria. Gram-positive bacteria cell walls are primarily comprised of peptidoglycan, and consist of teichoic acid, a cationic binding site that fosters structural rigidity, while Gram-negative cell walls are comprised of only 10% to 20% peptidoglycan and is void of teichoic acid. Lipids, proteins, lipoprotein, and lipopolysaccharides comprise 80% of the cell walls of Gram-negative bacteria, while Gram-positive walls may contain up to 3% lipids and lipoproteins and are void of surface proteins and lipopolysaccharides. The thickness of the cell wall of Gram-positive bacteria is two to eight times that of Gram-negative bacteria, but the former is mono-layered while the latter has two distinct layers. The outer surface of *P. aeruginosa* is comprised of a double lipid bilayer membrane with a very thin (< 10 nm) layer of extracellular material that consists of lipopolysaccharide and selectively-permeable protein-based pores. Between the two lipid membranes resides the periplasmic space, in which are found lipoproteins, nutrient-binding proteins, and peptidoglycan, an amino acid- and saccharide-based polymer that provides rigidity to the membrane. Additional proteins are located within the inner membrane, which are designed to transfer nutrients into and waste material out of the cell. The primary binding protein in the *P. aeruginosa* membrane, cytochrome 551 (c551), consists of a polypeptide chain with amino acid (L-alanine, L-cysteine, L-histidine, and L-valine) side chains (339). The cell membrane of *S. aureus* is much thicker than that of its Gram-negative counterpart, in the range of 80 to 100 nm, despite having only a single lipid bilayer (340).
The difference in membrane thickness can be found in the lipoteichoic acid cell wall, of which 70% is peptidoglycan and the remainder is teichoic acid, a polysaccharide with ribitol (reduced ribose) side groups (341). The cytoplasmic membrane holds numerous structural and enzymatic proteins, the most common being Staphylococcus protein A, which possesses numerous side chains comprised of aspartic acid, leucine, lysine, and phenylalanine, which is attached to the phospholipid structure by sortase B, which is an enzyme comprised of cysteine (294). Despite the advantage in cell membrane thickness, Gram-positive bacteria typically are less resistant to antimicrobials (i.e. penicillin) and enzyme activity (i.e. lysozyme) than their fat-laden cousins (341).

Multiple researchers proposed that fullerene species caused lipid peroxidation (70, 112), suggesting that Gram-negative bacteria with the much higher percentage of lipid content would be more susceptible to fullerene activity. Three previous reports compared the biological effect of THF-nC₆₀ to each type of bacterial membrane. Gram-positive bacterium, Bacillus subtilis was shown to be more sensitive to THF-nC₆₀ than Gram-negative Escherichia coli, with the minimum inhibitory concentration (MIC) five times higher for the latter (36), while Fang et al. (99) demonstrated that the MIC of THF-nC₆₀ on B. subtilis was 50% higher than for Gram-negative Pseudomonas putida. Lyon and Alvarez (292) indicated that THF-nC₆₀ disrupted the membrane of B. subtilis while that of E. coli was not impacted significantly. No identified research has examined the impacts of aqu-nC₆₀, fullerol, or fullerol synthesis by-products on both Gram-negative and Gram-positive bacteria. Hence, both Gram-negative and Gram-positive bacteria are being investigated due to the differences between their cell membranes and to provide insight into the proposition that lipid peroxidation within the cell membrane is the mechanism of bacterial inhibition.
*Pseudomonas aeruginosa* is a rod-shaped, Gram-negative aerobic bacterium, approximately 0.3- to 0.8-μm in width by 1.0 to 1.2 μm in length, opportunistic to humans and plants, and found ubiquitously in soil and water (341). The bacteria have been found to be responsible for multiple mammalian ailments including pulmonary and urinary tract infections and osteomyelitis as well as one of the most common causes of nosocomial disease, especially in post-operative infections in patients who have undergone radial keratotomy surgery. *P. aeruginosa* has been shown to be resistant to numerous antibiotics and, thus, serves as an ideal indicator species for toxicological assays. The species, specifically strain ATCC (American Type Culture Collection) 15442, has been incorporated into numerous testing protocols including seven for the American Society for Testing and Materials, as well as more than a dozen independent protocols for the British Standards Institute, the Association of Analytical Communities (AOAC) International Testing, and the United States General Services Administration. Culture purity was ensured through the use of pseudosel agar, which selectively isolated *P. aeruginosa*. Due to facile adaptability to antibiotics, a new pellet of *P. aeruginosa* was prepared for each round of testing to ensure purity. Other researchers have documented the biological effects of fullerene species to other Gram-negative bacteria including *Escherichia coli* (36, 53, 99, 245, 292), *Pseudomonas putida* (240), and *Vibrio fischeri* (240), however, no toxicological data was reviewed pertaining to *P. aeruginosa*. Due to its ubiquity and relevance to human health, *P. aeruginosa* was selected for this investigation to supplement existing data on fullerene and its effects on ecological indicator species.

*Staphylococcus aureus* is a spherical, Gram-positive facultative aerobic bacterium, approximately 1-μm in diameter, which has significant impact to human health (341). *S. aureus* is found commonly in the nasal and oral cavities and throughout the gastrointestinal tract and can
cause a number of ailments including meningitis, mastitis, pneumonia, phlebitis, lesions, urinary tract infections, and nosocomial, post-procedural infection. *S. aureus* has been shown to be resistant to many antibiotics due to an enhanced polysaccharide microcapsule and surface proteins that mask its presence and enable it to secrete a number of different toxins. The strain ATCC 6538, a non-MRSA (methicillin-resistant *S. aureus*), has been incorporated into numerous testing protocols including twelve for the American Society for Testing and Materials, as well as more than two dozen independent protocols for the British Standards Institute, the AOAC International Testing, the United States Environmental Protection Agency and the United States General Services Administration. Other researchers have documented the biological effects of fullerene species to other Gram-positive bacteria including *Bacillus subtilis* (15, 36, 53, 99, 245, 292), *Streptococcus pyogenes* (342), and *Enterococcus Hirae* and *Enterococcus faecalis* (18); however, no toxicological data was reviewed pertaining to *S. aureus*. Due to its ubiquity, relevance to human health, and resistance to many known antibiotics, *S. aureus* was selected for this investigation to supplement existing data on fullerene and its effects on ecological indicator species.

*Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum*) is a rapidly growing, non-motile, photosynthetic algal species of the Cholorphyceae class, 40 to 60 μm in length, and commonly found in most freshwater sources as a primary producer (343). Algae are plants and have a cell wall, which is comprised primarily of glycoproteins including D-glucose, D-galactose, D-mannose, D-fucose, β-D-N-acetylglucosamine, β-D-N-acetylgalactosamine, β-D-Neu5Ac (sialic acid), and β-D-xylose. The cell wall also contains cellulose, mannan, and lipids, which provide structure for the glycoproteins (344, 345). Algae have a phospholipid bilayer with lipids, carbohydrates, and proteins embedded, that encompasses
a cytoplasm in which are found membrane-bound organelles including a nucleus, mitochondria, Golgi apparatus, ribosomes, storage vacuoles, and chloroplasts. The entire structure is encased in a semi-permeable calcium carbonate skeleton that is secreted around the cell wall.

Walsh and Merrill (346) indicated that *P. subcapitata* have a “medium sensitivity” to a wide range of toxicants and the species has proven easy to culture with a characteristic crescent-shape that makes it easily identified under a microscope (347). The algae has been shown to be easily quantified using most automated particle counters since it lacks complex surface structures that might enable chain formation (348). *P. subcapitata* was chosen as the primary producer species by the United States Environmental Protection Agency (US EPA) for the National Eutrophication Research Program to assess the biological effect of wastewater effluent (349) and has been continued to be used as the predominant algal indicator species, being included in the US EPA “Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Water to Freshwater Organisms”, the WET test, Method 1003.0 (350). Three reports and one dissertation were reviewed that utilized *P. subcapitata* as the indicator species for assessing the biological effect of fullerene species; however, only two (166, 194) examined *aqu-nC*$_{60}$ while another examined THF-*nC*$_{60}$ (103) and the last examined polyethylene glycol-coated *nC*$_{60}$ (351). Due to its ubiquity, sensitivity to adverse environmental conditions, its role as a foundation species, and inclusion in standardized protocols, *P. subcapitata* was selected for this investigation to supplement existing data on fullerene and its effects on ecological indicator species.

Bacterial and algal population assays provide information on the biological effects on single-celled species. The use of primary consumers as an indicator species offers information on how potential toxicants affect multi-cellular biota, providing insight as to how higher-order
species respond. Mount and Norberg (352) formalized the first invertebrate biological assessment assay using the cladoceran Ceriodaphnia reticulate in 1984. After additional investigation (238), the protocol was modified to utilize Ceriodaphnia dubia, a slightly smaller (≤ 1 mm length), but more widely-distributed, cladoceran than Ceriodaphnia reticulate. Others have incorporated the larger Daphnia magna into assays due to facile observation, as they measure roughly four to five times the length of Ceriodaphnia sp.; however, it has been determined that C. dubia are more environmentally-sensitive than their larger relative (353, 354), which provides a more discerning view of ecological impacts caused by potential toxicants. Measuring less than 1-μm in length, the adult female reproduces asexually roughly one week after hatching and lives approximately one month, making it ideal for short-term assessment of both acute and chronic biological impact. The US EPA (355) incorporated the methodology as Method 1002.0 of the “Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Water to Freshwater Organisms” and has been included in all revision (350). Other researchers have documented the biological effects of fullerene species to daphnid species, although many have been directed at the larger Daphnia magna and involving THF-nC₆₀ or son-nC₆₀ (104, 105, 108, 240, 313, 356). Others have looked at the impacts of aqu-nC₆₀ (27, 105, 108, 194) of D. magna. Klaper (102) used Daphnia pulex, another, smaller, and even more-ecologically sensitive than C. dubia, as indicator invertebrate, examining the biological effects of aqu-nC₆₀, THF-nC₆₀, and fullerenol. One report was found that utilized C. dubia (106); however, the analyte examined was THF-nC₆₀; no reports were identified that exposed C. dubia to aqu-nC₆₀ or fullerenol. Due to its use in standardized biological assays, C. dubia was selected for this investigation to supplement existing data on fullerene and its effects on ecological indicator species.
LIST OF REFERENCES


40. Bouchard, D.; Ma, X.; Isaacson, C. Colloidal Properties of Aqueous Fullerenes: Isoelectric Points and Aggregation Kinetics of C\textsubscript{60} and C\textsubscript{60} Derivatives. *Environmental Science and Technology* 2009, 43, 6597-6603.


48. Zhao, B.; He, Y.; Bilski, P.J.; Chignell, C.F. Pristine (C\textsubscript{60}) and hydroxylated [C\textsubscript{60}(OH)\textsubscript{24}] fullerene phototoxicity towards HaCaT keratinocytes: type I versus type II mechanisms. *Chemical Research in Toxicology*. 2008, 21, 1056-1063.


81. Wei, X; Wu, M.; Qi, L.; Xu, Z. Selective solution-phase generation and oxidation reaction of C\textsubscript{60}\textsuperscript{n-}\textsuperscript{2} (n = 1,2) and formation of an aqueous colloidal solution of C\textsubscript{60}. Journal of the Royal Chemistry Society, Perkin Transactions 2. 1997, 1389-1394.

82. Meier, M.S.; Kegel, J. Preparation and characterization of the fullerene diols 1,2-C\textsubscript{60}(OH)\textsubscript{2}, 1,2-C\textsubscript{70}(OH)\textsubscript{2}, and 5,6-C\textsubscript{70}(OH)\textsubscript{2}. Organic Letters. 2001, 3 (11), 1717-1719.


95. Giesy, J.P. Hormesis – does it have relevance at the population, community or ecosystem levels of organization? *Human and Experimental Toxicology*. 2001, 20, 517–520.


147. Oberdorster, E. Manufactured nanomaterials (C\textsubscript{60}) induce oxidative stress in the brain of juvenile largemouth bass. *Environmental Health Perspectives*. **2004.** 112 (10), 1058-1062.


166. Vadan, R. Photocatalytic Breakdown of Fullerene C_{60} Water Suspensions and Toxicity of C_{60} Breakdown Products to Green Algae (*Selenastrum capricornutum*). Dissertation submitted to Texas Tech University. **2010**.


183. Jones T.S.; Ashton M.R.; Richardson N.V.; Mack R.G.; Unertl W.N. The interaction of the polyimide precursors PMDA (1,2,4,5-benzenetetracarboxylic anhydride) and m-PDA (1,3-phenylenediamine) with Ni(110). Journal of Vacuum Science and Technology A. 1990, 8 (3), 2370-2375.


204. Cataldo, F. Raman spectra of C\textsubscript{60} fullerene photopolymers prepared in solution. *European Polymer Journal*. 2000, 36, 653-656.


305. Rioux, F. Quantum Mechanics, group Theory, and C_{60}. *Department of Chemistry, Saint John’s University, College of Saint Benedict*. **1994**. 1-8.


310. Metzler, D.M. Responses of *Pseudokirchneriella subcapitata* and Algal Assembly to Photocatalytic Titanium Dioxide Nanoparticles. Dissertation Submitted to the University of Delaware. **2010**.


314. Lovern, S.; Strickler, J.; Klaper, R. Behavioral and Physiological Changes in *Daphnia magna* when Exposed to Nanoparticle Suspensions (Titanium Dioxide, Nano-C_{60}, and C_{60}H_{x}C_{70}H_{x}). *Environmental Science and Technology*. **2007**. 41, 4465-4470.


273


Yang, X.; Edelmann, R.; Oris, J. Suspended C_{60} nanoparticles protect against short-term UV and fluoranthene photo-induced toxicity, but cause long-term cellular damage in *Daphnia magna.* *Aquatic Toxicity.* **2010.** **100,** 202-210.
BIOGRAPHICAL SKETCH

Paul Anthony Indeglia was born in Minneapolis, Minnesota to Robert Anthony and Katherine Lois Wanless Indeglia. He graduated with a degree in Architecture from the University of Notre Dame, after which he served in the United States Peace Corps, providing clean water and sanitation to rural populations. He obtained a Bachelor of Engineering in Environmental Engineering Sciences from the University of Florida and a Master of Science in Environmental System with a focus in International Development Technology from Humboldt State University. He worked for a number of engineering and environmental consultancy firms before forming The Peak Consulting Group, Inc. with divisions in civil and environmental engineering, community development, and renewable energy. He is a founding member of the Agency for Sustainable Systems in Science and Technology, a 501(c)3 organization based in Arcata, California, established to conducted applied research in appropriate technologies in the United States and around the world. He has been involved in infrastructure development projects in Africa, Asia, Central America, and the United States, and disaster relief programs in Africa, Asia, and Europe as well as for hurricane victims in Florida and Louisiana. He assisted in establishment of the Conserve Florida Water Clearinghouse, a legislatively-mandated resource for state-wide water conservation. In 2006, Paul was invited to obtain his Doctorate of Philosophy in Environmental Engineering Sciences from the College of Engineering at the University of Florida. He graduated in 2011.