Investigation of Enzymatically Synthesized Glycogen as a Novel Nanodendrimer for Therapeutic Delivery

By

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Abstract

The field of medicinal chemistry is ever expanding, designing and discovering new therapeutic strategies. Oftentimes, it is challenging for these therapeutics to undergo clinical translation due to ineffective administration or unwanted toxicity in vivo. As such, drug delivery vehicles are designed to overcome these hurdles, allowing for delivery to the site of action by improving biodistribution, protecting therapeutic cargo, and decreasing toxicity. The work presented here aims to investigate a naturally-derived carbohydrate nanodendrimer, enzymatically synthesized glycogen (ESG) for drug delivery. This nontoxic, highly-branched, glucose-based structure has interior void volumes to allow for cargo encapsulation as well as a large density of surface functionalities for chemical functionalization.

In order to non-covalently condense anionic cargo to ESG, a cationic modification was attempted using two different modification schemes. Incorporation of stable positively-charged quaternary ammonium groups was achieved using a one-step synthesis, resulting in cationic ESG (cESG) with a +20 mV zeta potential. To explore the potential of cESG for therapeutic gene delivery two different nucleic acid compounds, pEGFP-N1 plasmid and siRNA, were investigated for delivery to ovarian-derived cell lines in vitro. While effective condensation and low toxicity was seen with both formulations, cESG was not an efficient mediator for plasmid delivery. siRNA transfection was achievable using the complex, with varying efficacy in different cell types. In efforts to reveal why efficacy was cell-type dependent, the in vitro behavior of cESG was further characterized, revealing no difference in uptake between different cell types. Contrary to expected results, expression of glycogen breakdown enzymes also did not correlate to siRNA efficacy.

We then probed cESG for alternative delivery applications, attempting delivery of photosensitizers for photodynamic therapy (PDT). Encapsulation of a photosensitizer based on
hydrophobic interactions was inefficient, narrowly improving therapeutic response. But, charge-based encapsulation of a sulfonated porphyrin photosensitizer (TPPS) was more efficient and significantly improved therapeutic response compared to free compound. When this formulation was tested for therapeutic co-delivery with siRNA, the cESG-TPPS-siRNA complex successfully mediated protein expression and retained therapeutic efficacy for PDT. These studies establish ESG as a promising candidate for further investigation as a therapeutic delivery vehicle.
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# Table of Contents

Abstract ........................................................................................................................................ iii

Acknowledgments .......................................................................................................................... v

Table of Contents .......................................................................................................................... vi

List of Figures and Schemes ........................................................................................................... x

List of Tables ................................................................................................................................... xv

List of Abbreviations ...................................................................................................................... xvii

Chapter 1 - Enzymatically Synthesized Glycogen: A Potential Platform for Delivery of Therapeutics

1.1 Motivation ................................................................................................................................. 1

1.2 Design of Nanoscale Drug Delivery Vehicles ....................................................................... 2

1.3 Dendrimeric Drug Delivery ...................................................................................................... 6

1.3.1 Dendrimer Synthesis ......................................................................................................... 6

1.3.2 Considerations for in vivo Dendrimeric Delivery ................................................................. 9

1.3.3 Covalent and Non-Covalent Cargo Encapsulation ............................................................. 10

1.4 Glycogen .................................................................................................................................. 14

1.4.1 Glycogen in vivo ............................................................................................................... 14

1.4.2 Phytoglycogen ................................................................................................................... 15

1.4.3 Enzymatically Synthesized Glycogen (ESG) .................................................................. 18

1.5 Scope of the Thesis .................................................................................................................. 21

1.6 References ............................................................................................................................... 22

Chapter 2 - Cationic Modification of ESG for Use as a Delivery Vector

2.1 Introduction to Cationic Delivery Vectors for Gene Therapy ............................................... 35

2.2 Investigation of a Two-Step Cationic Modification of ESG .................................................. 39

2.2.1 Methods ............................................................................................................................ 40

2.2.2 Results and Discussion ...................................................................................................... 42

2.3 Investigation of GTMA Conjugation of ESG ...................................................................... 49

2.3.1 Methods ............................................................................................................................ 49

2.3.2 Results and discussion ........................................................................................................ 53
Chapter 3 - Investigation of cESG for Nucleic Acid Delivery

3.1 Introduction to Ovarian Cancer and Reactive Oxygen Species (ROS)..........................65
3.2 Role of Reactive Oxygen Species (ROS) and Mitochondrial Superoxide Dismutase (Sod2) in Cancer ............................................................................................................74
  3.2.1 Reactive Oxygen Species, Superoxide Dismutase, and Cancer .........................74
  3.2.2 Mitochondrial Superoxide Dismutase (Sod2) ......................................................77
3.3 Methods ......................................................................................................................80
  3.3.1 Materials ...........................................................................................................80
  3.3.2 cESG-Plasmid Condensation Reaction ............................................................81
  3.3.3 cESG-siRNA Condensation Reaction ...............................................................81
  3.3.4 Agarose Gel Retardation Assay .........................................................................81
  3.3.5 DLS and Zeta Potential Analysis .......................................................................82
  3.3.6 Cell Culture .......................................................................................................82
  3.3.7 Analysis of cESG-Plasmid Transfection Efficiency ...........................................82
  3.3.8 MTT Viability Assay ........................................................................................82
  3.3.9 Analysis of cESG-siRNA Uptake and Transfection Efficiency ...........................83
  3.3.10 Western Blotting Protocol .............................................................................84
  3.3.11 Statistical Analysis .........................................................................................84
3.4 Results and Discussion ..............................................................................................85
  3.4.1 cESG-Nucleic Acid Binding .............................................................................85
  3.4.2 cESG-pEGFP-N1 Transfection Efficiency and Toxicity ....................................88
  3.4.3 cESG-siRNA: Uptake, Transfection Efficiency, and Toxicity ...........................93
3.5 Conclusions ..............................................................................................................103
3.6 References ..............................................................................................................104

Chapter 4 - Characterization of cESG

4.1 Introduction ..............................................................................................................111
  4.1.1 Cellular Uptake Mechanisms ..........................................................................111
  4.1.2 Enzymes in Carbohydrate Breakdown ............................................................116
4.2 Methods .................................................................................................................119
Chapter 4 – Investigation of cESG for Imaging

4.2.1 Materials ......................................................................................................................119
4.2.2 cESG-Lucifer Yellow Reaction ..................................................................................120
4.2.3 Cell Culture ................................................................................................................120
4.2.4 cESG-LY Uptake Timecourse ...................................................................................120
4.2.5 cESG-LY Mechanistic Inhibitor Studies ...................................................................121
4.2.6 cESG-LY Uptake in Spheroids .................................................................................121
4.2.7 Uptake in Chorioallantoic Membrane (CAM) Tumor Model .................................122
4.2.8 Toxicity of ESG, cESG, and oxESG .........................................................................122
4.2.9 Enzymatic Breakdown Behavior of ESG and cESG ...............................................123
4.2.10 Real-time polymerase chain reaction (qPCR) .......................................................123
4.2.11 Statistical Analysis .................................................................................................124

4.3 Results and Discussion ...............................................................................................124
4.3.1 Time-dependent Cellular Uptake of cESG-LY ..........................................................124
4.3.2 Mechanisms of cESG-LY Cellular Uptake ................................................................129
4.3.3 Ability of cESG to Penetrate Three-dimensional Cell Culture Models ..................131
4.3.4 Toxicity Dose Curves of ESG, cESG, and oxESG ....................................................135
4.3.5 cESG Enzymatic Degradation ..................................................................................137

4.4 Conclusions ................................................................................................................140
4.5 References ..................................................................................................................141

Chapter 5 – Investigation of cESG for Photodynamic Therapy

5.1 Introduction to Photodynamic Therapy (PDT) ............................................................146
5.2 Methods .........................................................................................................................157
5.2.1 Materials ..................................................................................................................157
5.2.2 ESG- and cESG-THPP Loading ..............................................................................157
5.2.3 Cell Culture ..............................................................................................................157
5.2.4 Dark Toxicity of THPP Compounds .......................................................................158
5.2.5 Impact of Light Dosage and THPP treatment .........................................................158
5.2.6 TPPS Condensation of ESG- and cESG-siRNA .......................................................158
5.2.7 DLS and Zeta Potential Analysis .............................................................................159
5.2.8 Gel permeation chromatography (GPC) .................................................................159
5.2.9 Testing Efficacy of cESG-TPPS compounds for PDT ............................................160
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.2.10</td>
<td>Statistical Analysis</td>
<td>160</td>
</tr>
<tr>
<td>5.3</td>
<td>Results and Discussion</td>
<td>161</td>
</tr>
<tr>
<td>5.3.1</td>
<td>Exploration of ESG-THPP and cESG-THPP for PDT</td>
<td>161</td>
</tr>
<tr>
<td>5.3.2</td>
<td>ESG and cESG Encapsulation of TPPS</td>
<td>167</td>
</tr>
<tr>
<td>5.3.3</td>
<td>Exploration of cESG-TPPS for PDT</td>
<td>172</td>
</tr>
<tr>
<td>5.4</td>
<td>Conclusions</td>
<td>175</td>
</tr>
<tr>
<td>5.5</td>
<td>References</td>
<td>176</td>
</tr>
<tr>
<td>6.1</td>
<td>Combination of Traditional and Genetic Therapies for Cancer Treatment</td>
<td>183</td>
</tr>
<tr>
<td>6.2</td>
<td>Methods</td>
<td>187</td>
</tr>
<tr>
<td>6.2.1</td>
<td>Materials</td>
<td>187</td>
</tr>
<tr>
<td>6.2.2</td>
<td>cESG-TPPS-siRNA Condensation Reaction</td>
<td>188</td>
</tr>
<tr>
<td>6.2.3</td>
<td>DLS and Zeta Potential Analysis</td>
<td>188</td>
</tr>
<tr>
<td>6.2.4</td>
<td>Cell Culture</td>
<td>188</td>
</tr>
<tr>
<td>6.2.5</td>
<td>Ability of cESG-TPPS-siRNA to Knockdown Sod2</td>
<td>188</td>
</tr>
<tr>
<td>6.2.6</td>
<td>Efficacy of cESG-TPPS-siRNA for PDT</td>
<td>189</td>
</tr>
<tr>
<td>6.2.7</td>
<td>Investigation of Radical Generation Mechanism of TPPS in ES2 Cells</td>
<td>189</td>
</tr>
<tr>
<td>6.2.8</td>
<td>Statistical Analysis</td>
<td>190</td>
</tr>
<tr>
<td>6.3</td>
<td>Results and Discussion</td>
<td>190</td>
</tr>
<tr>
<td>6.3.1</td>
<td>cESG-TPPS siRNA Conjugation</td>
<td>190</td>
</tr>
<tr>
<td>6.3.2</td>
<td>Efficacy of cESG-TPPS-siRNA Co-delivery Treatment</td>
<td>192</td>
</tr>
<tr>
<td>6.4</td>
<td>Conclusions</td>
<td>198</td>
</tr>
<tr>
<td>6.5</td>
<td>References</td>
<td>198</td>
</tr>
<tr>
<td>7.1</td>
<td>Summary</td>
<td>202</td>
</tr>
<tr>
<td>7.1.1</td>
<td>Chemical modification of ESG</td>
<td>202</td>
</tr>
<tr>
<td>7.1.2</td>
<td>Application of cESG for Nucleic Acid Delivery</td>
<td>203</td>
</tr>
<tr>
<td>7.1.3</td>
<td>Application of cESG for Photosensitizer Delivery</td>
<td>204</td>
</tr>
<tr>
<td>7.2</td>
<td>Critical Assessment and Recommended Future Directions</td>
<td>204</td>
</tr>
<tr>
<td>7.3</td>
<td>References</td>
<td>209</td>
</tr>
</tbody>
</table>
# List of Figures and Schemes

<table>
<thead>
<tr>
<th>Chapter 1</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Figure 1.1</strong> Common nanoscale delivery vehicles ................................</td>
<td>5</td>
</tr>
<tr>
<td><strong>Figure 1.2</strong> Schematic of dendrimeric branching structure ..................</td>
<td>6</td>
</tr>
<tr>
<td><strong>Figure 1.3</strong> Two different synthesis schemes for dendrimers ................</td>
<td>7</td>
</tr>
<tr>
<td><strong>Figure 1.4</strong> Common commercially available G1 dendrimers explored for drug delivery applications</td>
<td>8</td>
</tr>
<tr>
<td><strong>Figure 1.5</strong> Glycogen formation from glucose in vivo .........................</td>
<td>16</td>
</tr>
<tr>
<td><strong>Figure 1.6</strong> Dendrimeric structure of phytoglycogen composed of glucose residues</td>
<td>17</td>
</tr>
<tr>
<td><strong>Figure 1.7</strong> Two ex vivo synthesis schemes for ESG ..........................</td>
<td>19</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Figure 2.1</strong> Steps necessary for vector mediated siRNA transfection .......</td>
<td>36</td>
</tr>
<tr>
<td><strong>Scheme 2.1</strong> A two-step cationic modification of ESG ..........................</td>
<td>39</td>
</tr>
<tr>
<td><strong>Figure 2.2</strong> FTIR spectra of initial periodate oxidation reaction of PG.</td>
<td>43</td>
</tr>
<tr>
<td><strong>Figure 2.3</strong> FTIR spectra of low temperature periodate oxidation reaction of PG.</td>
<td>44</td>
</tr>
<tr>
<td><strong>Scheme 2.2</strong> Hydroxylamine hydrochloride reaction with aldehydes resulting in acidification of the reaction solution</td>
<td>45</td>
</tr>
<tr>
<td><strong>Scheme 2.3</strong> Modification of glucose with GTMA modification of glycogen via an epoxide ring opening reaction</td>
<td>49</td>
</tr>
<tr>
<td><strong>Figure 2.4</strong> XPS spectra of ESG and cESG ......................................</td>
<td>54</td>
</tr>
<tr>
<td><strong>Figure 2.5</strong> FTIR spectra of cESG Compounds ..................................</td>
<td>56</td>
</tr>
<tr>
<td><strong>Figure 2.6</strong> FTIR of cESG with peak assignments ................................</td>
<td>57</td>
</tr>
<tr>
<td><strong>Figure 2.7</strong> Proton NMR of cESG indicates incorporation of ammonium groups</td>
<td>59</td>
</tr>
<tr>
<td><strong>Figure 2.8</strong> Scanning electron micrographs of unmodified ESG and cESG synthesized at 1:10 ratio ESG:GTMA</td>
<td>60</td>
</tr>
</tbody>
</table>
Figure 2.9 Atomic force micrographs of unmodified ESG and cESG........................................60

Figure 2.10 Transmission electron micrographs of unmodified ESG and cESG stained with methylamine tungstate.................................................................61

Chapter 3

Figure 3.1 Mechanisms of transcoelomic metastasis..........................................................70

Figure 3.2 Reactive oxygen species intermediates in the conversion of oxygen gas to water. .75

Fig 3.3 Structure of SOD isoforms with metal cofactors ..................................................76

Figure 3.4 CAM studies indicate Sod2 knockdown decreases tumor size and metastasis after a 7 day incubation.................................................................................79

Figure 3.5 Proposed dual role of Sod2 in OCCC tumorigenesis and metastasis. ..............80

Figure 3.6 Gel shift assay of cESG-plasmid complexes confirms condensation of plasmid to cESG. .............................................................................................................86

Figure 3.7 Gel shift assay of cESG-plasmid complexes at a 15:1 molar ratio cESG:plasmid confirms condensation of plasmid to cESG in as little as 10 min. .........................86

Figure 3.8 Gel shift assay of cESG-siRNA complexes confirms condensation of siRNA to cESG. .................................................................................................................87

Figure 3.9 Expression of GFP due to plasmid pEGFP-B1 transfection................................89

Figure 3.10 Theoretical diagram of cESG decoration of the pEGFP-N1 surface due to large size disparity between the particles.................................................................90

Figure 3.11 Theoretical incorporation of siRNA into cESG ..................................................91

Figure 3.12 Lipofectamine2000 condensation with plasmid increases toxicity. ...............92

Figure 3.13 cESG modification and plasmid condensation does not significantly impact toxicity. .............................................................................................................92

Figure 3.14 Uptake of fluorescently labeled cESG-siRNA by ES-2 ovarian clear cell carcinoma cells increases as siRNA per cESG particle decreases .........................................94

Figure 3.15 Uptake of cESG-siRNA correlates with Sod2 protein expression knockdown......95

Figure 3.16 cESG-siRNA facilitates sustained protein expression knockdown.....................97
Figure 3.17 cESG-siRNA displays significantly less cytotoxicity than Lipofectamine RNAiMAX ................................................................. 98

Figure 3.18 cESG-siRNA is equally effective if incubated for 1 day or 3 days ..................... 100

Figure 3.19 cESG-siRNA is less effective after storage ..................................................... 101

Figure 3.20 cESG-siRNA efficacy varies by cell type ....................................................... 102

Chapter 4

Figure 4.1 Endocytic and non-endocytic cellular uptake mechanisms ......................... 112

Figure 4.2 Endocytotic uptake mechanisms of mammalian cells with intracellular trafficking to the lysosome ................................................................. 113

Figure 4.3 Enzymatic degradation of glycogen .............................................................. 117

Figure 4.4 Alpha amylase degrades phytoglycogen and ESG into different components .... 119

Figure 4.5 cESGLY uptake over time is similar in Nose007, ES-2, Ovca420 ...................... 126

Figure 4.6 Percentage of cell population with cESGLY uptake increases over time in Nose007, ES-2, and Ovca420 cells ................................................................. 127

Figure 4.7 Quantification of percent cell population fluorescing as a function of incubation duration is not statistically different (p=0.05) in Nose007, ES-2 or Ovca420 cells ............ 128

Figure 4.8 Uptake of cESG-siRNA-Sod2-FAM (green) after 6 hours is not localized in the nucleus ........................................................................................................ 128

Figure 4.9 cESG-LY uptake is an energy dependent process, but not clathrin or caveolin mediated endocytosed in Nose007, ES-2, and Ovca420 cells ......................................................... 130

Figure 4.10 Uptake in ES-2 OCCC spheroids of treatment for 72 hours ...................... 132

Figure 4.11 Confocal z-stack of uptake of cESG-siRNA occurs throughout the spheroid, not observed in cESG treatment alone ......................................................... 132

Figure 4.12 cESG-siRNA-Sod2-FAM is taken up in individual tumor cells within an RFP expressing CAM tumor ........................................................................... 134

Figure 4.13 Treatment of CAM tumor with cESG-siRNA (scramble or targeted against Sod2) does not induce a statistically significant change in volume or mass ............. 135
Figure 4.14 Dose curves of 24 hour treatment with ESG, cESG, or oxESG in growth media and serum free media in Nose007, ES-2, or Ovca420 cells. .................................................................136

Figure 4.15 Representative degradation of ESG and cESG as a function of time after the addition of enzymes .................................................................137

Figure 4.16 Percent of particle remaining after enzymatic degradation with pullulanase and α-amylase. .................................................................138

Figure 4.17 AGL and PYGL display higher expression in Ovca420 high grade serous adenocarcinoma cells compared to ES-2 OCCC cells. .................................................................139

Chapter 5

Figure 5.1 Generation of free radicals from photosensitizers upon illumination by incident light by a type I (general ROS generating) or type II (singlet oxygen generating) mechanism .......147

Figure 5.2 Structure of p-THPP (5,10,15,20-Tetrakis(4-hydroxyphenyl)-21H,23H-porphine).153

Figure 5.3 Structure of H₂TPPS₄ Meso-tetra(4-sulfonatophenyl)porphyrin..........................155

Figure 5.4 Encapsulation of THPP (µM) in ESG (mM) plateaus at 50 µM THPP .................161

Figure 5.5 Encapsulation of THPP (µM) in cESG (mM) plateaus at 80 µM. .........................162

Figure 5.6. Dark toxicity of ESG, THPP and ESG-THPP treatment of Nose007 normal ovarian surface epithelial cells and ES2 OCCC .................................................................163

Figure 5.7. ESG encapsulation does not improve PDT response of THPP in Nose007 normal ovarian surface epithelial cells or ES-2 OCCC .................................................................165

Figure 5.8 cESG encapsulation does not improve PDT response of THPP in Nose007 normal ovarian surface epithelial cells or ES-2 OCCC .................................................................166

Figure 5.9 Representative GPC elution profile of ESG-TPPS and cESG-TPPS .................167

Figure 5.10 Coloration of ESG-TPPS stored at room temperature for several weeks differs from ESG-TPPS filtered and stored at 4 °C and cESG-TPPS stored at room temperature at increasing TPPS concentrations. .................................................................168

Figure 5.11 Concentration of cESG encapsulated TPPS determined using UV-Vis after GPC purification .................................................................169

Figure 5.12 Absorbance and fluorescence emission spectra at 420nm excitation of TPPS and cESG-TPPS formulations (3 µM TPPS) .................................................................171
Figure 5.13 MTT viability of cESG-TPPS treated or TPPS control treated ES-2 OCCC at a constant 0.7uM TPPS.................................................................173

Figure 5.14 MTT viability of cESG-TPPS treated ES-2 OCCC at constant concentration TPPS (0.6 µM). .........................................................................................174

Figure 5.15 MTT viability of cESG-TPPS treated or TPPS control treated ES-2 OCCC at constant concentration cESG (17 nM). .........................................................................................175

Chapter 6

Figure 6.1 Mechanism of tumor recurrence after PDT .................................................184

Figure 6.2 Treatment scheme of cESG-mediated siRNA and TPPS combination therapy for increased photodynamic efficiency ........................................................................187

Figure 6.3 Conjugation scheme of cESG encapsulation of TPPS followed by siRNA condensation ..........................................................................................................................190

Figure 6.4 Gel shift assay of cESG-TPPS-siRNA complexes at various molar ratios cESG-TPPS-siRNA confirms condensation with two different formulations of cESG-TPPS ..........191

Figure 6.5 cESG-TPPS and cESG-TPPS-siRNA are internalized by nearly 100% of the ES-2 cell population after a 72 h incubation with complexes ........................................................................193

Figure 6.6 cESG-TPPS-siRNA facilitates knockdown of Sod2 protein expression similarly to cESG-siRNA in ES-2 OCCC .............................................................................................................194

Figure 6.7 cESG-TPPS-siRNA shows no statistically significant effect in an in vitro PDT model. ..................................................................................................................................................196

Figure 6.8 Quenching of singlet oxygen radicals reduce the toxicity from PDT using cESG-TPPS in ES-2 OCCC. ......................................................................................................................198
List of Tables

Chapter 1

Table 1.1 Desired Characteristics for Drug Delivery .................................................................3

Table 1.2 Cleavable covalent linkages utilized for dendrimeric-cargo conjugates ..................12

Chapter 2

Table 2.1 Phytoglycogen oxidation at 30˚C. DLS and Zeta Potential ......................................43

Table 2.2 PG oxidation at 4 °C. DLS, Zeta potential, and Percent Modification .................46

Table 2.3 ESG oxidation at 4 °C. DLS, Zeta potential, and Percent Modification ...............46

Table 2.4 Investigation of Reaction Ratio on hydrazine linkage oxESG and Girard’s Reagent T .................................................................................................................................47

Table 2.5 Influence of reaction pH and purification method on hydrazine linkage of oxESG...48

Table 2.6 Impact of pH on cESG synthesis - Size, Zeta potential, and Carbon/Nitrogen Ratio.53

Table 2.7 Impact of Temperature on Synthesis of cESG: Size, Zeta Potential and Carbon/Nitrogen Ratio ..............................................................................................................................54

Table 2.8 Impact of Reactant Ratio on Synthesis of cESG: Size, Zeta Potential and Carbon/Nitrogen Ratio ..............................................................................................................................55

Table 2.9 ESG and cESG particle diameter calculated using various metrology techniques ....61

Chapter 3

Table 3.1 Prognosis of Ovarian Cancer by Stage ....................................................................66

Table 3.2 Genetic mutations associated with histological subtypes of epithelial ovarian cancer ........................................................................................................................................67

Table 3.3 Zeta potential of cESG-siRNA complexes formed at various ratios cESG to siRNA ........................................................................................................................................87
Chapter 4

Table 4.1 Uptake inhibitors with mechanism of action ..............................................................129

Chapter 5

Table 5.1 Clinical indications of photosensitizers used in United States clinical trials ..........149

Table 5.2 Size and Zeta potential of cESG-TPPS .......................................................................170

Chapter 6

Table 6.1 Size and zeta potential of cESG-TPPS-siRNA complexes with varying TPPS concentration ..................................................................................................................................................192

Chapter 7

Table 7.1 Efficacy of various TPPS-nanoformulations for photodynamic therapy in vitro ........208
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amylomaltase</td>
<td>AM</td>
</tr>
<tr>
<td>Anhydrous glucose units</td>
<td>AGU</td>
</tr>
<tr>
<td>Benzoporphyrin derivative</td>
<td>BPD</td>
</tr>
<tr>
<td>Branching enzyme</td>
<td>BE</td>
</tr>
<tr>
<td>Cancer antigen 125</td>
<td>CA 125</td>
</tr>
<tr>
<td>Cationic enzymatically synthesized glycogen</td>
<td>cESG</td>
</tr>
<tr>
<td>Chorioallantoic membrane</td>
<td>CAM</td>
</tr>
<tr>
<td>Dynamic light scattering</td>
<td>DLS</td>
</tr>
<tr>
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<td>EA-IRMS</td>
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<tr>
<td>Enhanced permeability and retention</td>
<td>EPR</td>
</tr>
<tr>
<td>Enzymatically synthesized glycogen</td>
<td>ESG</td>
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<tr>
<td>Fluorescein</td>
<td>FAM</td>
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<td>Folate receptor alpha</td>
<td>FRα</td>
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<td>Generation</td>
<td>G</td>
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<td>Glycidyltrimethylammonium chloride</td>
<td>GTMA</td>
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<td>Glycogen debranching enzyme</td>
<td>AGL</td>
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<td>Glycogen phosphorylase</td>
<td>GP</td>
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<tr>
<td>Glycogen phosphorylase liver isoform</td>
<td>PYGL</td>
</tr>
<tr>
<td>Human epidermal growth factor receptor 2</td>
<td>HER2</td>
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<tr>
<td>Human epididymal secretory protein</td>
<td>HE4</td>
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<tr>
<td>(hydroxymethyl) propanoic acid</td>
<td>HMPA</td>
</tr>
<tr>
<td>Term</td>
<td>Abbreviation</td>
</tr>
<tr>
<td>-------------------------------------------</td>
<td>--------------</td>
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<tr>
<td>Hypoxia-inducible factor</td>
<td>HIF</td>
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<tr>
<td>Intraperitoneal</td>
<td>IP</td>
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<tr>
<td>Intravenous</td>
<td>IV</td>
</tr>
<tr>
<td>Isoamylase</td>
<td>IAM</td>
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<tr>
<td>Lucifer yellow</td>
<td>LY</td>
</tr>
<tr>
<td>Matrix Metalloproteinase</td>
<td>MMP</td>
</tr>
<tr>
<td>Meso-tetra(4-sulfonatophenyl) porphyrin</td>
<td>TPPS</td>
</tr>
<tr>
<td>Molecular weight cut off</td>
<td>MWCO</td>
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<tr>
<td>Ovarian clear cell carcinoma</td>
<td>OCCC</td>
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<td>Oxidized enzymatically synthesized glycogen</td>
<td>oxESG</td>
</tr>
<tr>
<td>Oxidized phytoglycogen</td>
<td>oxPG</td>
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<td>Photodynamic therapy</td>
<td>PDT</td>
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<td>Photosensitizer</td>
<td>PS</td>
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<td>Phthalocyanine</td>
<td>Pc</td>
</tr>
<tr>
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<td>PLL</td>
</tr>
<tr>
<td>Poly(lactic-co-glycolic acid)</td>
<td>PLGA</td>
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<tr>
<td>Poly(methyl methacrylate)</td>
<td>PMMA</td>
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<tr>
<td>Polyamidoamine</td>
<td>PAMAM</td>
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<td>Polyethersulfone</td>
<td>PES</td>
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<td>PEI</td>
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<td>PLA</td>
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<tr>
<td>Polypropyleneimine</td>
<td>PPI</td>
</tr>
<tr>
<td>Term</td>
<td>Abbreviation</td>
</tr>
<tr>
<td>-----------------------------------------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Quaternary ammonium groups</td>
<td>Quats</td>
</tr>
<tr>
<td>Reactive oxygen species</td>
<td>ROS</td>
</tr>
<tr>
<td>Risk of ovarian malignancy algorithm</td>
<td>ROMA</td>
</tr>
<tr>
<td>Semi-quantitative real time reverse transcriptase polymerase chain reaction</td>
<td>sqRT-PCR</td>
</tr>
<tr>
<td>Short interfering ribonucleic acid</td>
<td>siRNA</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>SOD</td>
</tr>
<tr>
<td>Tetra(hydroxyphenyl)porphyrin</td>
<td>THPP</td>
</tr>
<tr>
<td>Tris-acetate-EDTA</td>
<td>TAE</td>
</tr>
<tr>
<td>Ultra low attachment</td>
<td>ULA</td>
</tr>
<tr>
<td>Vascular endothelial growth factor</td>
<td>VEGF</td>
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<tr>
<td>X-Ray photoelectron spectroscopy</td>
<td>XPS</td>
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CHAPTER 1.

ENZYMATICALLY SYNTHESIZED GLYCOGEN: A POTENTIAL PLATFORM FOR DELIVERY OF THERAPEUTICS

1.1 Motivation

As new medicinal compounds are designed and discovered, a constant challenge to clinical use is efficient and safe administration of the compounds in vivo. The overarching goal of drug delivery is to achieve specific delivery of therapeutics to their site of action in order to improve efficacy or decrease side effects. Administration of therapeutics extends to a range of cargo including not only traditional drugs, but also genetic therapies and imaging agents. Delivery vehicles may be tuned to specific cargo in order to improve a range of features from increasing solubility and biodistribution to decreasing systemic side effects of cytotoxic cargo. A variety of nanoscale delivery systems including liposomes, micelles, naturally-derived polymers, and inorganic nanoparticles have arisen to meet these challenges, in hopes of improving patient outcomes.

Among current drug delivery vehicles, highly branched dendrimeric polymers have attractive chemical characteristics. Notably, interior void volumes allow for encapsulation and a high degree of surface functional groups may be harnessed for chemical functionalization and targeting. Both hydrophobic and hydrophilic cargo have been delivered using covalent and non-covalent (affinity based) encapsulation. However, these synthetic systems suffer from biocompatibility issues, inducing toxicity due to a lack of degradability. Several strategies have been implemented to overcome this hurdle with varying success. Tuning size and surface charge may decrease toxicity, with smaller particles having lower charge densities exhibiting the lowest
toxicity.\textsuperscript{17,18} Introduction of degradable linkages may also decrease toxicity by improving clearance.\textsuperscript{19} We propose investigation of a carbohydrate nanodendrimer, enzymatically synthesized glycogen (ESG) as an alternate nontoxic material for delivery applications. This sub-50 nm glucose-based particle has a variety of applications, is FDA approved as a food and cosmetic ingredient, and is commercially available at low cost. To the best of our knowledge, ESG has not yet been investigated as a therapeutic delivery platform. \textbf{The central hypothesis of this dissertation is that the naturally derived, dendrimeric carbohydrate nanoparticle ESG can be utilized as a biocompatible therapeutic delivery vehicle}. The investigations within this study focus on non-covalent encapsulation of cargo into a cationically-modified ESG and determine the feasibility of the complex to mediate drug and gene delivery in an \textit{in vitro} cell culture model.

The following sections provide a brief introduction into; design of nanoscale drug delivery vehicles, dendrimeric drug delivery systems, as well as natural and enzymatically synthesized glycogens.

\subsection*{1.2 Design of Nanoscale Drug Delivery Vehicles}

Several properties need to be considered when designing drug delivery systems. The specific criteria vary based upon application, however some generally desirable features are outlined in Table 1.1. A major motivation for using drug delivery vehicles is to improve the bioavailability of their cargo. After intravenous (IV) administration many small molecules (less than 5 nm) are rapidly metabolized in the liver and cleared from the body by the kidneys, decreasing their therapeutic efficacy.\textsuperscript{20} Encapsulation of these agents in larger vehicles may increase circulation time, thus improving the chance of delivery to the target site. However, care must be taken that
this complex does not activate a phagocytic response by macrophages, which would result in rapid clearance.\textsuperscript{21} In conjunction with increasing circulation time, an ideal vehicle will be targeted to the site of action. This may be active targeting, such as including targeting ligands to overexpressed surface receptors at the disease site, or size-based passive targeting.\textsuperscript{22}

Table 1.1. Desired Characteristics for Drug Delivery. Adapted from Adair \textit{et al.}\textsuperscript{23}

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Desired Characteristic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size</td>
<td>10-200 nm</td>
</tr>
<tr>
<td>Material</td>
<td>Nontoxic, biodegradable</td>
</tr>
<tr>
<td>Stability</td>
<td>Limited agglomeration under physiological conditions – protein interactions, temperature, pH, ionic</td>
</tr>
<tr>
<td>Clearance</td>
<td>Long circulation half life Inherent clearance mechanism to prevent accumulation</td>
</tr>
<tr>
<td>Cargo Localization</td>
<td>Encapsulation more stable than surface decoration</td>
</tr>
<tr>
<td>Targeting</td>
<td>Active or passive targeting to site of action</td>
</tr>
<tr>
<td>Cargo Release</td>
<td>Triggered or controlled release at target tissue</td>
</tr>
</tbody>
</table>

In order to prevent toxic accumulation, vehicles eventually must be efficiently cleared from the body. It is important to note that clearance and toxicity are governed by a variety of factors such as particle composition, charge, and surface functionality.\textsuperscript{24} Advancement in nanotechnologies in the past 25 years has provided new avenues to tune these properties and design materials for delivery. Nanoparticles are attractive for \textit{in vivo} delivery as particles in the 10-200 nm diameter range have efficient clearance from the body. This small size also results in a relatively high specific surface area (surface area per unit volume), enhancing adsorption capabilities and subsequently increasing reactivity with the biological environment. The high density of surface groups allows for functionalization with passivating or targeting moieties in order to tune circulation behavior and encourage accumulation at the site of interest.\textsuperscript{14} Chemical modifications of nanomaterials may also be employed to decrease agglomeration or control release of cargo at the target tissue.
The properties of the vehicles must be tailored not only for \textit{in vivo} interactions, but also tuned to the chemical composition of the cargo. Several strategies for vehicle and material design have arisen in order to meet these needs (Figure 1.1). An in-depth review of these vehicles is outside the scope of this dissertation, but a numbers of reviews on specific delivery systems are available in the literature.\textsuperscript{1,4,25-29} The most common delivery systems for hydrophobic cargo are micelles and liposomes. Polymeric micelles are formed by amphiphilic surfactants that self-aggregate to form a hydrophobic shell surrounded by a hydrophilic corona, where hydrophobic drugs can solubilize in their cores.\textsuperscript{7} In liposomes, amphiphilic phospholipid-bilayer spheres, hydrophobic cargo may be solubilized in the membrane and hydrophilic cargo contained within the core.\textsuperscript{4} Liposomes can likely be considered the most successful drug delivery system thus far, with a dozen liposomal drug formulations currently FDA approved for therapy, and many more in clinical trials.\textsuperscript{4,30}

Hydrophobic compounds may also be encapsulated by the formation of inclusion complexes with linear or branched polymers.\textsuperscript{1} Traditional branched dendrimers, which will be discussed in more depth in the following section, can encapsulate either hydrophobic and hydrophilic cargo \textit{via} covalent or non-covalent design strategies and have attractive properties that can be taken advantage of for use as delivery vehicles.\textsuperscript{31} The high density of surface functional groups for dendrimers may not only allow for conjugation of therapeutics, but also of targeting moieties. The capacity to load multiple cargo of interest provides a potential platform for co-delivery of therapeutics and diagnostics.\textsuperscript{19}
A potential challenge to implementing dendrimers and other drug delivery vehicles is in vivo toxicity. For example, solid inorganic nanoparticles including metal nanoparticles and quantum dots are attractive imaging agents for “theranostic” delivery vehicles, in which therapeutics and imaging agents are delivered simultaneously. However many of these complexes suffer from toxicity issues, and thus have not been implemented in the clinic. In order to decrease toxicity, the surface properties of complexes may be tuned, using biocompatible polymers like polyethylene glycol (PEG). This strategy was successful in the first FDA approved nanovehicle, liposomal doxorubicin (Doxil®), resulting in decreased toxicity and increased circulation.
1.3 Dendrimeric Drug Delivery

1.3.1 Dendrimer Synthesis

The first iteration of dendrimers, called “cascade molecules” were synthesized by Vogtle et al. in 1978, comprised of PPI (poly(propylenimine)).\textsuperscript{35} This synthesis scheme was used as the template for the creation of more complex dendrimers. This new class of polymers were defined by having a core (initiator) which is built upon radially by layers (generations) of monomers, terminated by an exterior layer (Figure 1.2).\textsuperscript{36}

![Figure 1.2. Schematic of dendrimeric branching structure. Initiator core with monomeric layers (G1, G2, G3) with exterior layer of terminal groups](image)

The first synthetic dendrimers were built from the initiator core outward in concentric shells (divergent synthesis), a method which is still most popular in industrial synthesis. Convergent synthesis, developed in 1990,\textsuperscript{37} proceeds in the opposite direction, forming from the outermost surface inward toward a reactive focal point (Figure 1.3).\textsuperscript{38} The first dendrimers investigated, which are now all commercially available, were poly(propyleneimine) (PPI), polylysine (patented in 1981\textsuperscript{39}) and polyamidoamine (PAMAM) and polyesters (patented as “star
polymers” by the Dow Chemical Company in the early and mid-1980s\textsuperscript{40-43}; Figure 1.4). These materials have been most widely investigated for drug delivery applications. To date, dendrimers have been explored for a range of delivery routes including traditional intravenous (IV) therapy, as well as oral, transdermal, intraperitoneal, and ocular delivery.\textsuperscript{44,45}

Figure 1.3. Two different synthesis schemes for dendrimers. Divergent synthesis occurs outward from an initiator core and convergent dendrimer synthesis occurs radially from the surface towards a common focal point. Reproduced from Esfand \textit{et al.}\textsuperscript{38} Used with permission from Elsevier.
While not yet FDA approved, several dendrimeric delivery systems are in various stages of clinical trials. A phase I clinical trial is underway in Australia\textsuperscript{46} utilizing DEP\textsuperscript{™} docetaxel (Starpharma), a dendrimeric chemotherapeutic formulation which has shown to improve breast cancer response in preclinical studies.\textsuperscript{47} This same company also developed an antimicrobial poly(L-lysine) dendrimer decorated with naphthalene disulfonate, VivaGel\textsuperscript{®}, which is in phase III.
clinical trials for the prevention of sexually transmitted infections and bacterial vaginosis.\textsuperscript{47-49} Gadomer-17 (SH L 643A, Gd-DTPA-17) is a dendrimeric gadolinium-based contrast agent, which when used for human MR angiography, improved coronary artery imaging and improved the contrast-to-noise ratio.\textsuperscript{50,51} This formulation, commercially available through a Bayer subsidiary, has been used for research in the past decade, but has yet to undergo clinical trials for FDA approval.\textsuperscript{52-54}

1.3.2 Considerations for in vivo Dendrimeric Delivery

The \textit{in vivo} pharmacokinetics of dendrimers are affected by a variety of different properties. Dendrimers utilized for IV delivery interact with a variety of plasma proteins within the blood. These interactions may form complexes that impact not only protein structure but also alter the biodistribution of this new dendrimeric complex. For example, neutral PAMAM dendrimers interacts with serum albumin without affecting overall secondary protein structure,\textsuperscript{55} but corresponding cationic PAMAM dendrimers induce conformational changes in fibrinogen and clotting factors within the blood.\textsuperscript{56} Highly cationic dendrimers can also exhibit undesired toxicity through disruption of cellular membranes.\textsuperscript{57,58} This is a concern during circulation, as the rupture of red blood cells (ie. hemolysis) may occur. The hemolytic activity of dendrimers is not yet well characterized, but has shown dependence upon size, concentration, charge, and surface functionalization.\textsuperscript{59-61} These factors also highly impact dendrimer circulation time and clearance. In a rat study of the biodistribution of PAMAM G3 and G4 dendrimers, cationic dendrimers were rapidly cleared from circulation whereas their anionic counterparts had increased circulation. All particle formulations began accumulation within the liver, but the clearance of anionic particles was dependent upon size, a distinction not seen in cationic versions.\textsuperscript{17} This slight change in particle
formulation significantly changed particle behavior, highlighting the necessity for pharmacokinetic studies with cargo-containing formulations, which will affect in vivo behavior. For therapeutics, release of the drugs from the dendrimeric complex must also be investigated, a process highly dependent on the method of cargo encapsulation.\textsuperscript{18}

1.3.3 \textit{Covalent and Non-Covalent Cargo Encapsulation}

Dendrimeric cargo encapsulation can be accomplished through covalent or non-covalent means. For PAMAM and PPI dendrimer, the driving forces behind non-covalent encapsulation, in which a molecule is incorporated within the branching structure of the dendrimer, are hydrophobicity, charge, and hydrogen bonding.\textsuperscript{62} Electrostatic condensation with charged amines, within the complex or at the molecular surface, allows for the binding of anionic therapeutics or nucleic acids.\textsuperscript{63} Hydroxy-group terminated dendrimers may also undergo weak hydrogen bonding to further solubilize cargo.\textsuperscript{64} These non-covalent cargo solubilization strategies are highly dependent upon particle geometry, necessitating large enough void volumes to accommodate cargo. Smaller, lower generation, dendrimers often have larger void volumes, but can suffer from excess cargo leakage from the structure.\textsuperscript{65}

Non-covalent formulations must avoid immediate release and require long-term stability under physiological conditions in order to improve delivery in comparison to the “free” cargo molecule. The release of non-covalently bonded cargo is impacted by a variety of factors in vivo. The pH of dispersing media may affect the ionization of both dendrimer and cargo, inducing release of charge based encapsulations.\textsuperscript{66-68} Ionic concentration has a similar effect, in which dendrimer-drug complexes may be stable in aqueous solution but rapidly dissociate in ionic saline
During *in vivo* delivery, charged serum proteins such as albumin may act as counterions, displacing cargo from the dendrimer.\(^{70,71}\)

Covalent condensation between dendrimer and cargo may be employed as a strategy to avoid rapid cargo release in biological systems. In order to allow for eventual cargo release cleavable chemical linkages (Table 1.2) may bind cargo to the dendrimeric structure. These labile bonds may be cleaved by physiological triggers. For example, acid-labile hydrazine or ester functionalities may be used for drug release at lower pH in order to target solid tumors, as the extracellular pH of the tumor microenvironment is more acidic (pH 5.7-7.8) than normal tissues.\(^{72}\) A similar strategy may be employed for selective intracellular cargo release at endosomal and lysosomal pH 6 and 4, respectively.\(^{72}\) A caveat of this triggered release from covalent linkages is often the rate of release, culminating in insufficient concentrations of drug being delivered *in vivo*.\(^{73-77}\)

Non-degradable dendrimers may be insufficiently cleared by the body, leading to unwanted accumulation in the liver.\(^{15}\) *In vitro* studies indicate that the toxicity from cationic dendrimers is caused by disruption of biological membranes inducing necrosis via plasma membrane interactions\(^{15,16}\) or apoptosis via mitochondrial membrane disruption, which impairs mitochondrial function.\(^{78-80}\)

Biodegradable dendrimers may decrease this toxicity by breaking the complex into smaller fragments, which can be eliminated through existing metabolic pathways. The best polymer candidates for quick degradation are small hydrophilic dendrimers with interior cleavable linkages, the majority of which are ester-based.\(^{19}\) Bis-HMPA (2,2-bis(hydroxymethyl)propanoic acid) based dendrimers have been investigated for biocompatible dendrimer-based delivery of both watersoluble drugs\(^{81,82}\) and porphyrins for photodynamic therapy.\(^{83,84}\) Traditional ester-bonded bis-HMPA dendrimers are stable at acidic pH, similar to that of the endosome.\(^{85}\) Higher generation bis-HMPA dendrimers degrade at pH 9.\(^{86}\) but smaller G1 dendrimers can degrade at physiological
conditions (37 °C, pH 7.4) in under ten hours.\textsuperscript{87} Other monomers of interest for biodegradable dendrimer delivery include natural biological metabolites, such as succinic and adipic acid,\textsuperscript{88,89} cationic bis(2-hydroxy-ethyl)-amino]acetic acid tert-butyl ester,\textsuperscript{90} or carbohydrates.

Table 1.2. Cleavable covalent linkages utilized for dendrimeric-cargo conjugates

<table>
<thead>
<tr>
<th>Bond Type</th>
<th>Degradation Trigger</th>
<th>Citation</th>
</tr>
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<tbody>
<tr>
<td>Cis-aconityl</td>
<td>Acid Labile</td>
<td>91,92</td>
</tr>
<tr>
<td>Hydrazine</td>
<td>Acid Labile</td>
<td>93-95</td>
</tr>
<tr>
<td>Carbamate</td>
<td>Acid Labile</td>
<td>93,96,97</td>
</tr>
<tr>
<td>Esters</td>
<td>Esterase-catalyzed hydrolysis</td>
<td>97-101</td>
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<tr>
<td>Disulfide Bond</td>
<td>Glutathione reduction</td>
<td>101,102</td>
</tr>
<tr>
<td>Peptides</td>
<td>Enzymatic</td>
<td>92,95,103-109</td>
</tr>
</tbody>
</table>
Szoka et al. have done extensive research on the use of biodegradable dendrimer complexes for delivery to cancer.\textsuperscript{81,93,110-113} An ester-amide degradable dendrimer was synthesized and linked to doxorubicin via a pH sensitive hydrazine linkage for treatment of solid tumors. In vivo assessment of toxicity using these dendrimers indicated high tolerability in mice, with accumulation in the tumor rather than liver or kidneys. When the efficacy of the complex for the treatment of colon carcinoma was compared against Doxil, a liposomal doxorubicin nanoformulation FDA approved for human use, they were statistically equivalent, with 90% of mice tumor free 60 days after treatment.\textsuperscript{87} This pH responsive hydrazine linkage was also utilized in conjunction with a polyester-poly(ethylene oxide) dendrimer for delivery of doxorubicin. Mice treated with this formulation also had tumor regression, with 100% survival for mice over the course of the 60 day experiment.\textsuperscript{96} This indicated that the biodegradable dendrimer delivery system is a feasible alternative carrier in a clinical setting, improving drug response over that seen in free formulation.

Hyperbranched polymers have also been successful for improving drug delivery. Classified as dendrons or dendrites, these polymers have a branching, tree-like structure, but lack the radial symmetry of true dendrimers. One of the most commonly researched dendrons for delivery applications is polyglycerol (reviewed here\textsuperscript{114}). These 5-10 nm globular macromolecules have demonstrated improved delivery in vivo of the chemotherapeutic doxorubicin\textsuperscript{115} and multimodal imaging agents\textsuperscript{116} compared to unencapsulated compound. Other hyperbranched dendrons have been synthesized for delivery applications from many of the same polymers as “true” dendrimers including PMMA,\textsuperscript{117,118} polyesters,\textsuperscript{119} polyethylene oxide (PEO),\textsuperscript{120} and polyphosphate.\textsuperscript{121} Dendrites have less steric hindrance, which is advantageous for drug conjugation and encapsulation.\textsuperscript{122} To date, the only discovered nanoscale dendrimeric architectures in nature are
composed of polysaccharides. These highly branched complexes are also classified as dendrites, as they lack the symmetry of synthetic dendrimers.

1.4 Glycogen

Naturally-derived dendrimeric glycogen is attractive for delivery applications due to inherent biodegradability and low cytotoxicity. These dendritic carbohydrates are degraded by enzymes for glycogen metabolism, releasing non-toxic glucose or oligonucleotides. Carbohydrate chemistry is well established, and in conjunction with the high specific surface area of the nanoscale architecture, allows for a range of potential modification schemes to introduce either covalent or non-covalent cargo encapsulation. Compared to synthetic dendrimers, these natural dendrimers are also more economical. These properties provided the major motivations for the study of this material as a delivery vehicle.

1.4.1 Glycogen in vivo

One of the major biological functions of carbohydrates is energy storage. Starch is the primary storage carbohydrate for plants and is composed of linear α(1→4) amylose chains and branched amylopectin. In starch, amylopectin has α(1→6) branches every 24-30 glucose residues. Glycogen, the storage polysaccharide for animals, has an analogous structure, with a higher amylopectin branching density every 8-12 glucose residues. Starch, in general, has been utilized for pharmaceutical applications since the 1930s, primarily as a binding agent but with a recent emphasis placed on therapeutic delivery.

Glycogen, found primarily in muscle and liver cells, is stored as β-particles (21 nm), which form larger granules of 20-40 particles. A variety of enzymes are necessary for glycogen synthesis and degradation. The synthesis of glycogen may occur in a variety of mammalian tissues, but is most common in the liver as a mechanism for glucose storage. Glycogen synthesis in vivo
is highly regulated by enzymes including hexokinase, phosphoglucomutase, and UDP-glucose pyrophosphorylase (Figure 1.5). During formation, the nonreducing end of a glycogen chain is elongated with glucose, via an α(1→4) linkage. Branching α(1→6) linkages are made by glycogen branching enzyme which catalyzes the transfer of the final 6 to 7 glucose residues from the nonreducing end of a chain to a C-6 hydroxyl group of an interior glucose residue within a nearby chain. This branching increases solubility and the number of nonreducing ends.126

When the glucose stored in glycogen is needed to generate energy for the cell, the molecule must undergo degradation. Glycolysis, a major mechanism to harness energy (ATP) from glucose, requires free glucose. The enzyme glycogen phosphorylase hydrolyzes α(1→4) bonds, resulting in the release of a glucose-1-phosphate from the non-reducing end. Therefore highly branched glycogens, with more non-reducing ends, undergo quicker degradation. This process can occur until an α(1→6) bond is reached, at which point a secondary debranching enzyme is necessary to hydrolyze the linkage.126 We anticipated that these enzymes would provide a potential degradation mechanism for carbohydrate-based delivery vehicles.

1.4.2 Phytoglycogen

Phytoglycogen is a glucose storage conformation discovered in plants which mimics the structure of glycogen from animal organs.127 First described in 1939 by Morris and Morris,128 phytoglycogen is a highly branched α(1→4)(1→6) glucan, forming 30-100 nm diameter dendrimeric nanoparticles (Figure 1.6). Phytoglycogen is abundant and can be readily extracted from sweet corn kernals,128 but may also be harvested from a variety of sources including barley,129 rice,130 and even algae.131 The average molecular weight and branching characteristics of natural glycogens are dependent on their source. For example, sweet corn phytoglycogen is three times the molecular weight of oyster phytoglycogen (19800 kDa and 6010 kDa respectively). Average
chain lengths are normally in the 9-13 residue range with a dense core and a less dense exterior region. Although phytoglycogen has been used for 75 years, new and improved methods for extraction and purification continue to be explored and patented.

Figure 1.5. Glycogen formation from glucose in vivo.
As a carbohydrate particle, phytoglycogen has been investigated for use in food science. Applications include use as an additive in food products to maintain crispiness, such as ready-to-eat cereals,\textsuperscript{134,135} and use as an emulsifier.\textsuperscript{136} Yao \textit{et al.} were able to improve the emulsification properties of phytoglycogen by octenyl succinate modification.\textsuperscript{137} This platform was applicable as not only an emulsifier but also for delivery. For example, when tested as a vehicle for the antimicrobial peptide nisin, encapsulation prolonged the efficacy of nisin over free peptide.\textsuperscript{138} A cationic phytoglycogen was created by modifying the octenyl succinate modified particle with positively charged quaternary ammonium groups. When used as a delivery vehicle for a vaccine antigen (anthrax recombinant protective antigen) in a murine model, the particle significantly enhanced the activation of dendritic cells by the vaccine with no observed systemic effects in mice.\textsuperscript{139} This highlights the applicability of phytoglycogen as a non-toxic delivery vector, in this case \textit{via} intramuscular injection for delivery to dendritic cells.
1.4.3 **Enzymatically Synthesized Glycogen (ESG)**

*In vitro* enzymatically synthesized phytoglycogen mimetics were developed in order to achieve large-scale, reproducible production of uniform particle distributions. Like *in vivo* synthesis, this process takes advantage of enzymatic activity to generate the glycogen structure. In 1943, Cori and Cori discovered that an α(1-4) linear polysaccharide similar to amylose could be created using extracted muscle phosphorylase and the substrate glucose-1-phosphate. Upon addition of “a supplementary enzyme which is present in liver and heart extracts” a branched glycogen was formed.\(^{140}\) Currently, Cori’s method (GP-BE method) is performed using α-glucan phosphorylase (GP, EC 2.4.1.1) and branching enzyme (BE, EC 2.4.1.18; Figure 1.7). This method closely mimics *in vivo* glycogen synthesis by glycogen synthase and branching enzyme (Figure 5).\(^{132}\)

In order to improve yield and tune the molecular weight of synthetic glycogens, Kajiura *et al.* investigated the use of various BE to synthesize glycogen from native starch. For this method, starch or dextrin was broken into short chain amylose by isoamylase (IAM, EC 3.2.1.68) and then re-branched using branching enzyme and amylomaltase (AM, EC 2.4.1.25; Figure 7).\(^{132}\) Previously, glycogen could not be synthesized from short chain amylose using branching enzyme. This novel process was created by screening branching enzymes extracted from a variety of sources, with differing activities and substrate specificities based upon origin. There are over 280 known protein sequences for branching enzymes, with wide diversity in protein structures, which account for this differing activity.\(^{141}\) The choice of branching enzyme may be used to manipulate molecular weight, structure, and enzymatic degradation behavior of ESG.\(^{142}\) Amylomaltase was used to catalyze intra- and inter-molecular glucan transfer, increasing efficiency and producing
higher molecular weight glycogens than those attained using BE alone. The IAM-BE-AM method is used to synthesize commercially available ESG (Bioglycogen™, Glico Nutrition Co.).

**Figure 1.7.** Two *ex vivo* synthesis schemes for ESG. Traditional GP-BE synthesis occurs from the substrate glucose-1-phosphate whereas the IAM-BE-AM method utilizes a starch starting material.
The branching density of glucose chains within glycogen is of importance for the physiological behavior of the compound. The activity of amylase and transferase enzymes, integral to carbohydrate metabolism, are dependent upon these chain lengths. In conjunction with the overall molecular weight of the complex, the structure of ESG can also affect antibody\textsuperscript{143} and immune response to the particle.\textsuperscript{144} Structural parameters used to compare phytoglycogen to ESG are the overall average $\alpha(1\text{-}4)$ chain length, as well as interior and exterior chain lengths. These are similar for both natural and enzymatic glycogens, with average chain lengths of 9-13 residues. Interior chain lengths tend to be shorter, with 3-4 glucose resides whereas exterior chains are longer at 6-8 resides. However, ESG has less variation in chain lengths than phytoglycogen, indicating that $\alpha(1\text{-}6)$ branches in phytoglycogen are more irregularly positioned due to trimming reactions during \textit{in vivo} synthesis.\textsuperscript{123}

Assessment of ESG toxicity revealed that these glycogen dendrimers are safe for use as food ingredients. ESG was orally fed to Sprague-Dawley rats and exhibited no acute toxicity at the highest dosage tested (2g/kg). In a sub-chronic toxicity study, animals were free fed AIN93M feed supplemented with up to 30% ESG for 13-weeks. All animals survived the experiment in good general health, with a no-observed-adverse-effect level of ESG established as 20g/kg body weight per day.\textsuperscript{145} While this low toxicity is encouraging for drug delivery applications, it is important to note that toxicity upon other administration routes must be investigated. High-dose fed animals had an increase in cecal content (material at the beginning of the large intestine) consistent with other high carbohydrate diets.\textsuperscript{145} This increase in cecal content was also observed in a study of rats fed ESG, but not with those fed natural phytoglycogen, sourced from mussels. This was attributed to the fact that phytoglycogen was more fully degraded and metabolized as
glucose, while only 80% of ESG mass was converted to glucose and the remaining 20% of the particle reaching the cecum as insoluble fiber.\textsuperscript{146}

In animal studies, ESG has also demonstrated to have an immunostimulating effect that promotes anti-tumor activity, inhibiting tumor growth.\textsuperscript{147} ESG treatment of macrophages has been shown to activate an antitumor response by the secretion of nitric oxide and the inflammatory cytokine tumor necrosis factor alpha.\textsuperscript{144,148} This response was dependent upon the degree of polymerization, as less branched α-glucans, such as cycloamylose and cyclic dextrin, did not activate macrophages, or inhibit tumor growth.\textsuperscript{147} This might be due to decreased recognition by the Toll-like receptor 2, which mediates glycogen recognition and activates the immune response.\textsuperscript{149} It is important to note that the modification of ESG for delivery purposes may also impact glycogen recognition, and as such, any complex designed would require independent toxicity studies.

\section*{1.5 Scope of the Thesis}

The literature above demonstrates some of the desirable features of glycogen-based dendrimeric particles that may prove advantageous in use of this material as a delivery vehicle for therapeutics. While state-of-the-art synthetic “designer” dendrimers have a high density of surface functionalities and chemical versatility, toxicity due to non-degradability, and limited drug release have prevented widespread clinical implementation.\textsuperscript{19} Naturally derived dendritic structures, such as ESG, may offer an alternative delivery platform.

The aim of our study focused on sub 50-\textsuperscript{nm} ESG and was based on the rationale that ESG-therapeutic conjugates could offer a biocompatible nanoparticle system resulting in low toxicity and sufficient release of cargo for delivery of therapeutics. The overall strategy to develop ESG as
a delivery system was to chemically functionalize the nanodendrimer for non-covalent encapsulation of anionic therapeutic species, including nucleic acids and small molecule therapeutic compounds. We investigated this hypothesis with the following chapters:

- **Chapter 2** investigated the chemical modification of ESG. Two different modification strategies were investigated to introduce stable quaternary ammonium groups into the particle.
- In **Chapter 3**, cationic ESG (cESG) was investigated for effectiveness as a plasmid and siRNA delivery vector in an *in vitro* ovarian cancer cell line model. The cellular fate of cESG was further characterized in **Chapter 4**, focusing on cellular uptake mechanisms and particle degradation.
- In order to demonstrate the versatility of cESG for delivery applications, **Chapter 5** shifted focus from genetic therapy to the manipulation of ESG for incorporation of a photodynamic small molecule therapeutics. This complex was then tested for therapeutic co-delivery of photosensitizer and siRNA in **Chapter 6**.

### 1.6 References


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CHAPTER 2.

CATIONIC MODIFICATION OF ESG FOR USE AS A DELIVERY VECTOR

2.1 Introduction to Cationic Delivery Vectors for Gene Therapy

Drug delivery systems are being investigated for not only the delivery of traditional therapeutic drugs, but also new genetic therapies. In 1998 RNA interference was discovered by Mello and Fire,\(^1\) in which the translation process is interrupted by short interfering RNA (siRNA) thus preventing the synthesis of functional proteins (protein knockdown). While this is an inherent mechanism for translational regulation, synthetic siRNAs may also be used to knockdown proteins of therapeutic interest.\(^2\) The high specificity of this gene silencing mechanism is desirable for clinical application, but efficient delivery of the 21-25 nucleotide sequences is a challenge to implementation.\(^3\) Naked siRNAs are easily degraded \textit{in vivo} by nucleases, quickly cleared by the body, and unable to traverse cellular membranes.\(^4\) In order to overcome these challenges siRNA delivery has been investigated with many of the drug delivery systems previously described including liposomes, micelles, and linear polymers, reviewed here.\(^4,5\) Delivery vectors for siRNA therapy are important for not only siRNA stability, but also cellular recognition, internalization, and trafficking into the cytoplasm (Figure 2.1).

As the genetic knowledge of disease has increased in the past three decades, so has the potential application of genetic therapy in treatment. In 2015, there were 2210 clinical trials worldwide investigating nucleic acids for gene therapy in general, 64% of which were for application in cancer. Nearly 400 of these clinical trials utilized naked or plasmid DNA injection. However, direct injection of DNA to the desired therapeutic site has limited applications as nucleases degrade nucleic acids during systemic delivery. The majority of clinical trials (66%) investigated viral vectors, such as adenovirus and retrovirus for delivery. Viral vectors have had high efficiency for both delivery and expression, but are confronted by public safety concerns and high manufacturing costs. As such, nonviral vectors have been investigated as nucleic acid delivery systems. Cationic polymers are a natural choice for such applications as they may electrostatically condense the negatively charged nucleic acids. Positively charged complexes can
be efficiently internalized by cells after binding to the negatively charged cell membrane and proteins on the cell surface, facilitating transport across the cell membrane.⁹

After internalization, cationic polymer-nucleic acid conjugates may be localized within vesicles, such as endosomes and lysosomes. In order for nucleic acid therapies to be effective, they must escape into the cytoplasm (for siRNA) or translocate into the nucleus (for plasmids). Cationic polymers may accomplish this endosomal escape via the proton-sponge effect. One polymer applied for this is branched cationic PEI (polyethylenimine) with pKa’s of primary and secondary amines of 4.6 and 6.7 respectively.¹⁰ In the acidified endosomal environment these amines become protonated, buffering the pH, resulting in an influx of protons followed by osmotic swelling and rupture as counterions enter the vesicle.¹¹ This contributes to high gene expression efficiency of PEI conjugates in vitro and in vivo.¹² Unfortunately, PEI also induces cytotoxicity due to high cationic charge densities. Initial PEI binding of proteoglycans disrupts the plasma membrane, resulting in necrotic like changes, followed within 24 hours by channel formation in the mitochondrial outer membrane that activates apoptosis.¹³ These effects may be compounded in vivo as neither linear nor branched PEI degrade in biological systems. As such, smaller, low molecular weight PEI, which undergoes clearance more readily, has been investigated to decrease toxicity.¹⁴

Another strategy to overcome toxicity of cationic polymers is to use biodegradable polymers such as chitosan and poly-L-lysine (PLL). PLL has lower cytotoxicity than PEI and sufficient cellular internalization, but it lacks a proficient mechanism for endosomal escape. For PLL, the amine (pKa 10)¹⁵ is already fully protonated at physiological conditions and therefore does not buffer compartmental pH. No proton sponge effect is observed, resulting in low gene expression levels.¹⁶,¹⁷ Chitosan is another biodegradable, naturally occurring cationic particle
explored for gene delivery. Chitosan based complexes exhibit effective, though slow, endosomal escape. But, the high charge density of native chitosan can result in similar toxicity issues as that observed with PEI. However, when the charge density or size were reduced by chemical modification, cytotoxicity could be remediated. The high charge density of inherently cationic polymers may disrupt mitochondrial membranes, resulting in cytotoxicity. When secondary or tertiary amine groups in PEI and a PAMAM dendrimer were replaced with quaternary ammonium groups (quats), there was a significant reduction in cytotoxicity. Modification with quats may also be harnessed to introduce stable, permanent (non pKa-dependent) positive charges into neutral particles with attractive physiochemical features. This strategy has been implemented with several different carbohydrate nanoparticles for gene therapy with mixed success. Quaternized dextran was designed for plasmid delivery, and while there was strong DNA binding, gene expression was low, likely due to low endosomal escape. Quat-modified cellulose also had efficient plasmid binding with better transfection efficiency and less toxicity than PEI.

As ESG is carbohydrate-based similar to these carbohydrate systems, we theorized that quaternization may produce cationic ESG suitable for gene delivery applications, without inducing unwanted toxicity. An indication that quaternization may produce effective siRNA delivery vectors has been demonstrated in vitro using nanoparticulate potato starch, which exhibited minimal toxicity and excellent uptake. Two different schemes were investigated in an effort to modify ESG with quats. First, a two-reaction synthesis was investigated, in which ESG was oxidized by sodium periodate followed by linkage with the quat-containing compound, Girard’s Reagent T (Girard’s). The second scheme was a one-pot synthesis in which ESG was modified via an epoxide ring opening reaction to introduce quats. In order for a reaction product to be explored
for delivery applications, the product should 1) not have considerably changed physical properties such as diameter and solubility and 2) have a charge high enough to facilitate nucleic acid binding and cellular entry.

2.2 Investigation of a Two-Step Cationic Modification of ESG

In this approach (Scheme 1), the well-established periodate oxidation of carbohydrates was investigated as a means to introduce reactive aldehyde groups into ESG. This versatile functionality may undergo a hydrazine linkage with Girard’s Reagent T, in order to introduce a permanent positive charge.
Scheme 2.1. A two-step cationic modification of ESG. Periodate oxidation of glucose to introduce aldehydes (blue) followed by hydrazine linkage with Girard's Reagent T containing quats (red)

2.2.1 Methods

2.2.1.1 Materials

All reagents were purchased from Sigma Aldrich unless otherwise specified. Reactions were performed using 18.2 MOhm-cm, RNAse free water.

2.2.1.2 Oxidation of ESG

Synthesis conditions for the oxidation of ESG were initially based upon those reported for dextran and sodium alginate. In order to conserve ESG stock, initial reactions were performed on phytoglycogen (PG). PG was dissolved in 20 mM sodium phosphate buffer, pH 5. Sodium periodate was added at various concentrations to investigate the influence of reactant ratios on oxidation (AGU:periodate 1:5, 1:1, 2:1, molar ratios). The reaction was carried out in a shaking incubator set at 30 °C for 6 h. The reaction was quenched by adding an excess of D-glucose. The product was purified by dialysis the reaction solution against 4 L of distilled water, with 3 volume changes, using 10k MWCO regenerated cellulose dialysis tubing (ThermoFisher). Following dialysis, samples were lyophilized and stored in a desiccator. Subsequent syntheses were based on the above protocol with the following changes; lower ratios AGU:periodate were explored (2:1, 4:1, and 10:1), the reaction was conducted at 4 °C overnight, and glucose were not used to quench the reaction. Low temperature syntheses were repeated according to this protocol with ESG.

2.2.1.3 Dynamic Light Scattering (DLS) and Zeta Potential

The efficacy of ESG modification was initially screened using DLS and zeta potential analysis. DLS measurements were performed on a Zetasizer Nano-ZS (Malvern Instruments) in 1 mM sodium phosphate buffer at approximately 5 mg/mL of sample. The particle diameter was
determined using the Zetasizer software according to the volume occupied calculation, based upon Mie theory. Mean particle diameter measurements were averaged over three measurements using 15 scans at 10 seconds each. The zeta potential (effective charge) of particles in solution was also determined using a Zetasizer Nano-ZS. Zeta potential measurements at 40 mV were averaged over three measurements of 60 scans each. All scans were performed at room temperature (25 °C).

2.2.1.4  *Fourier Transform Infrared Spectroscopy*

FTIR spectra of lyophilized samples were taken using a Bruker Tensor 27 spectrometer with a Pike Miracle single bounce attenuated total reflectance (ATR) attachment. Spectra were collected over a range of 800 – 4000 cm\(^{-1}\) and averaged over 32 scans at 4 cm\(^{-1}\) resolution.

2.2.1.5  *Hydroxylamine Hydrochloride Titration*

A hydroxylamine hydrochloride titration was performed to determine the amount of aldehyde formation. Oxidized ESG (oxESG) samples were dissolved at 10 mg/mL in distilled water. 1 mL of oxESG solution was reacted with 10 mL of 0.1 M hydroxylamine hydrochloride for three hours in a 37 °C shaking incubator. Control samples of 10 mL hydroxylamine hydrochloride with 1 mL of distilled water added were reacted in the same manner. After reaction, the pH of the control reactions were recorded as the endpoint for titration. Standardized 5 mM sodium hydroxide was used to titrate the sample to the endpoint pH. The moles of sodium hydroxide added to reach the endpoint pH (i.e. the control sample pH) is directly proportional (1:1) to the moles of aldehyde in the sample. The percent oxidation was calculated for three replicates of each sample using the formula:

\[
\frac{1}{2} \left( \frac{\text{moles sodium hydroxide added}}{\text{moles glucose reacted}} \right) \times 100\% = \text{Percent Oxidation}
\]
2.2.1.6 oxESG Reaction with Girard’s Reagent T

The aldehyde-hydrazine reaction of oxESG was based upon the reaction described by Hermanson\textsuperscript{28}. 10 mg/mL oxESG (6\% modified) was dissolved in 20 mM sodium phosphate buffer pH 6. A stock solution of Girard’s Reagent T was made in the same buffer at 10 mg/mL. Various reaction ratios were investigated, ranging from excess Girard’s to excess aldehydes (aldehyde:Girard’s 1:12, 1:2, 1:1, 2:1, and 10:1, molar ratios). Reactions were carried out in microcentrifuge tubes at 60 °C in a dry heat block. After a 6 hour reaction, the imine linkage was reduced using sodium cyanoborohydride to prevent reversal of the Schiff’s base linkage\textsuperscript{28}. 5 M sodium cyanoborohydride stock was prepared in 1 M sodium hydroxide until gas generation ceased, approximately one hour. Stock was added to the reaction solution to a final concentration of 0.05 M and incubated overnight at 4 °C. Samples were purified via dialysis against 4 L of distilled water, with 3 volume changes, in 10k MWCO regenerated cellulose dialysis tubing (ThermoFisher). In order to investigate the influence of pH on the modification, the reaction was also conducted at 1:12 molar ratio aldehyde:Girard’s in varying pH sodium phosphate buffer (pH 3, 6, and 13). Controls included oxESG in the absence of Girard’s Reagent T, and performing the reactions without reducing the imine bond.

2.2.2 Results and Discussion

2.2.2.1 Screening of Oxidation of ESG

Initially, phytoglycogen was oxidized using periodate at elevated temperature, 30 °C, for 6 h. PG has a similar size to ESG but a slightly higher polydispersity, with a diameter of 31.6 nm ± 8.11 nm. PG also has a slightly negative zeta potential of -4.06 ± 13.6 mV. Under these original
synthesis conditions, there was no notable change in the size or zeta potential of particles reacted with different reactant ratios (Table 2.1). FTIR analysis of lyophilized samples was obtained to confirm if successful oxidation occurred. Aldehydes have characteristic adsorption between 1740-1720 cm\(^{-1}\), corresponding to stretching vibrations of the carbonyl (C=O) bond. FTIR spectra of synthesized compounds (Figure 2.2) reveal that aldehydes, detected at 1730 cm\(^{-1}\), were incorporated into the sample synthesized at a 2:1 ratio but were not seen in the ratios with higher periodate.

Table 2.1. Phytoglycogen oxidation at 30°C. DLS and Zeta Potential

<table>
<thead>
<tr>
<th>Reaction Ratio (AGU:Periodate)</th>
<th>Size (nm) ± Standard Deviation</th>
<th>Zeta Potential (mV) ± Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:5</td>
<td>32.0 ± 8.9</td>
<td>-3.7 ± 15.0</td>
</tr>
<tr>
<td>1:1</td>
<td>28.3 ± 8.3</td>
<td>-7.9 ± 18.1</td>
</tr>
<tr>
<td>2:1</td>
<td>28.7 ± 7.6</td>
<td>-11.3 ± 16.1</td>
</tr>
<tr>
<td>Unmodified PG</td>
<td>31.6 ± 8.1</td>
<td>-4.06 ± 13.6</td>
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</tr>
<tr>
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<td>-11.3 ± 16.1</td>
</tr>
<tr>
<td>Unmodified PG</td>
<td>31.6 ± 8.1</td>
<td>-4.06 ± 13.6</td>
</tr>
</tbody>
</table>
Figure 2.2. FTIR spectra of initial periodate oxidation reaction of PG. The appearance of 1730 cm\(^{-1}\) peak indicates aldehyde formation in 2:1 AGU:periodate synthesis with 1640 cm\(^{-1}\) peak attributed to absorbed water.

To further explore the dependence of percent oxidation on relative periodate concentration, the synthesis was repeated at lower periodate concentrations. In order to ensure that the extent of reaction was limited by reactant rather than reaction time, reactions were conducted at 4°C overnight. As the reactions were intended to go to completion, there was no need for reaction quenching. FTIR spectra of oxPG synthesized in this manner, suggest that there was successful modification in AGU:periodate ratios 4:1 and 2:1 (Figure 2.3) Peak intensities, normalized to the peak area of the \(\alpha(1-4)\) glycosidic stretch at 925 cm\(^{-1}\), indicate that there are more aldehydes present with 2:1 rather than 4:1 AGU:periodate (0.09 and 0.04 respectively). However, there was no aldehyde evidence in the FTIR spectra for the 10:1 ratio. This suggests that periodate concentration must be carefully tuned, as no oxidation was observed at a 1:1 ratio, the highest degree of oxidation was achieved at 2:1 ratio with decreasing modification at higher AGU concentrations (4:1, 10:1).
Figure 2.3. FTIR spectra of low temperature periodate oxidation reaction of PG. The appearance of 1730 cm\(^{-1}\) peak indicates aldehyde formation in 4:1 and 2:1 AGU:periodate reactions with 1640 cm\(^{-1}\) peak attributed to absorbed water.

As the qualitative presence of aldehydes was shown, we then desired to quantify the extent of reaction. A hydroxylamine hydrochloride titration was used, in which free aldehydes react with hydroxylamine releasing hydrochloric acid (Scheme 2.2).

![Scheme 2.2 Hydroxylamine hydrochloride reaction with aldehydes resulting in acidification of the reaction solution](image)

The released protons are titrated using sodium hydroxide, allowing for the quantification of moles aldehyde in solution and calculation of percent modification. This determination was important for further analysis, as tuning the number of aldehydes may allow for more controlled downstream reactions. This titration confirmed the modification of all three samples at low temperature, with negligible change in size and charge (Table 2.2). Despite the absence of a 1730 cm\(^{-1}\) peak in the 10:1 AGU:periodate modified sample, the titration reveals that there was still a modification of 3.9% of the glucose residues. At lower modification levels, it is possible that the aldehyde content is below the limit of detection of the FTIR. Thus the absence of a 1730 cm\(^{-1}\) peak
should not be directly associated with lack of reaction. As the ratio of AGU:periodate approached 1:1 there was a drastic increase in modification with 19.8% of residues modified at 4:1 and 35.6% modification at a 2:1 ratio. This implies that periodate concentration is the limiting reagent at higher concentrations of AGU, as indicated by FTIR analysis.

Table 2.2. PG oxidation at 4 °C. DLS, Zeta potential, and Percent Modification

<table>
<thead>
<tr>
<th>Reaction Ratio (AGU:Periodate)</th>
<th>Percent Modification</th>
<th>Size (nm) ± Standard Deviation</th>
<th>Zeta Potential (mV) ± Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>10:1</td>
<td>3.9 ± 0.2</td>
<td>26.5 ± 8.2</td>
<td>-9.87 ± 14.7</td>
</tr>
<tr>
<td>4:1</td>
<td>19.8 ± 1.9</td>
<td>25.3 ± 7.6</td>
<td>-9.97 ± 12.8</td>
</tr>
<tr>
<td>2:1</td>
<td>35.6 ± 1.0</td>
<td>31.5 ± 10.0</td>
<td>-6.43 ± 15.0</td>
</tr>
<tr>
<td>Unmodified PG</td>
<td>0</td>
<td>31.6 ± 8.1</td>
<td>-4.06 ± 13.6</td>
</tr>
</tbody>
</table>

As the successful reaction of phytoglycogen had been demonstrated, we then tested the same modification on ESG. The low temperature oxidation protocol was utilized with ratios containing excess AGU, in hopes of tuning the degree of aldehyde modification. The hydroxylamine hydrochloride titration of oxidized ESG (oxESG) shows there is similarity with the percent modifications of oxPG (Table 2.3). The 4:1 modification resulted in a 19.8% ± 1.9% conjugation with PG and 15.3% ± 0.05% for ESG. Similarly, the 10:1 modification for PG was 3.9% ± 0.2% whereas in ESG it was 5.6% ± 0.6%. This degree of tunability was deemed reasonable to continue on with further modifications, and the 10:1 ratio with approximately 5% modification was used for further studies. For simplicity, this preparation will be referred to as oxESG for further discussion.
Table 2.3 ESG oxidation at 4 °C. DLS, Zeta potential, and Percent Modification

<table>
<thead>
<tr>
<th>Reaction Ratio (AGU:Periodate)</th>
<th>Percent Modification</th>
<th>Size (nm) ± Standard Deviation</th>
<th>Zeta Potential (mV) ± Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>20:1</td>
<td>2.3 ± 0.3</td>
<td>31.3 ± 0.9</td>
<td>-5.76 ± 22.1</td>
</tr>
<tr>
<td>10:1</td>
<td>5.6 ± 0.6</td>
<td>31.8 ± 1.6</td>
<td>-2.93 ± 20.9</td>
</tr>
<tr>
<td>4:1</td>
<td>15.3 ± 0.05</td>
<td>31.8 ± 2.2</td>
<td>-26.3 ± 13.9</td>
</tr>
<tr>
<td>Unmodified ESG</td>
<td>0</td>
<td>32.4 ± 1.0</td>
<td>-2.29 ± 73.3</td>
</tr>
</tbody>
</table>

2.2.2.2 Hydrazine linkage of Girard’s Reagent T with oxESG

In order to incorporate quats, oxESG (6% oxidized) was reacted with Girard’s Reagent T (Girard’s) to form a hydrazine linkage. Various ratios of reagents were used ranging from 12 molar excess aldehyde to 10 molar excess Girard’s Reagent T. Reactions were reduced and dialyzed for 24 h prior to measurement, in which the initial screening mechanism for determining the success of the reaction was the introduction of a positive zeta potential. However, this positive charge was modest, below 10 mV (Table 2.4).

Table 2.4 Investigation of Reaction Ratio on hydrazine linkage oxESG and Girard’s Reagent T

<table>
<thead>
<tr>
<th>Reaction Ratio (Aldehyde:Girard’s)</th>
<th>Size (nm) ± Standard Deviation</th>
<th>Zeta Potential (mV) ± Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:12</td>
<td>28.3 ± 0.0</td>
<td>2.9 ± 16.6</td>
</tr>
<tr>
<td>1:2</td>
<td>28.5 ± 0.9</td>
<td>8.4 ± 12.7</td>
</tr>
<tr>
<td>1:1</td>
<td>24.7 ± 0.6</td>
<td>1.3 ± 14.7</td>
</tr>
<tr>
<td>2:1</td>
<td>15.9 ± 6.3</td>
<td>4.6 ± 15.6</td>
</tr>
<tr>
<td>10:1</td>
<td>22.4 ± 1.3</td>
<td>0.2 ± 33.9</td>
</tr>
<tr>
<td>oxESG alone</td>
<td>19.4 ± 2.5</td>
<td>-1.6 ± 13.6</td>
</tr>
</tbody>
</table>

In an attempt to alter conjugation conditions to realize more consistent particles and higher zeta potential, we investigated the influence of reaction pH on particle size and charge. Sodium phosphate buffers of pH 2, 6, and 13 were investigated. Controls of oxESG in the absence of Girard’s were performed at each pH in order to determine if the heating process and reduction reaction were affecting size or charge. oxESG was highly unstable at pH 13, turning from a clear
to amber colored solution immediately upon heating. The instability of reducing sugars in alkali environments is well established.\textsuperscript{29} Under these harsh conditions, the aldehydes may react via aldol reactions – leading to crosslinking.\textsuperscript{30} DLS scans indicate that the solution formed aggregates of 350-400 nm. Conversely, control oxESG heat treated and reduced at pH 2 or 6, in the absence of Girard’s, retained their original size and negative charges (Table 2.5).

Conjugation reactions of oxESG with Girard’s at pH 2 and 6 were both successful, attaining positive zeta potentials of approximately 10 mV. However, oxESG-Girard’s synthesized at pH 2, decreased in particle size to 22.4 nm with 81.1 nm aggregates observed in approximately 10\% of particle volume distribution. Conjugates reacted at pH 6 retained their size and had a more uniform particle size distribution. oxESG-Girard’s conjugates synthesized at both pH 2 and 6 attained a zeta potential of approximately 10 mV.

Table 2.5 Influence of reaction pH on size and zeta potential of oxESG-Girard’s Conjugates

<table>
<thead>
<tr>
<th>Reaction Condition</th>
<th>Size (nm) ± Standard Deviation</th>
<th>Zeta (mV) ± Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control pH 2 – oxESG alone</td>
<td>33.1 ± 0.9</td>
<td>-3.37 ± 17.3</td>
</tr>
<tr>
<td>Control pH 6 – oxESG alone</td>
<td>31.5 ± 1.5</td>
<td>-4.06 ± 16.8</td>
</tr>
<tr>
<td>pH 2 – oxESG + Girard’s</td>
<td>22.4 ± 8.2 (90%)</td>
<td>11.8 ± 14.2</td>
</tr>
<tr>
<td></td>
<td>81.1 ± 19.7 (10%)</td>
<td></td>
</tr>
<tr>
<td>pH 6 – oxESG + Girard’s</td>
<td>28.2 ± 0.6</td>
<td>9.18 ± 16.4</td>
</tr>
</tbody>
</table>

We demonstrated that the oxidation of PG and ESG is tunable and may undergo further reaction with Girard’s Reagent T to incorporate a positive charge. The stability of oxESG at elevated pH or temperature is a potential concern. DLS studies of oxESG in slightly alkaline buffer resulted in particle degradation. As such, the one-step synthesis of cationic ESG, which was explored simultaneously, may serve as an alternative method for the introduction of a positive charge.
2.3 Investigation of GTMA Conjugation of ESG

In order to investigate one-pot functionalization of ESG with GTMA (Scheme 2.3), a range of synthesis conditions were tested. Reaction conditions were screened using DLS and zeta potential prior to the use of additional chemical analytical techniques, such as X-ray photoelectron spectroscopy (XPS), nuclear magnetic resonance (NMR) and elemental analysis ratio mass spectrometry (EA-IRMS).

![Scheme 2.3 Modification of glucose with GTMA modification of glycogen via an epoxide ring opening reaction](image)

2.3.1 Methods

2.3.1.1 Materials

All reagents were purchased from Sigma Aldrich unless otherwise specified. Reactions were performed using 18.2 MOhm-cm, RNAse free water.
2.3.1.2 *Synthesis of GTMA Conjugated ESG*

The initial synthesis conditions for cationic ESG via glycidyltrimethylammonium chloride modification were based upon the method for modification of dextran as described by Thomas et. al.\textsuperscript{31} Aqueous reactions of ESG (Glico Nutrition Co. Japan) with GTMA in alkaline buffer were investigated where initial studies surveyed the impact of buffer pH on modification using 0.1 M sodium phosphate buffer of pH 9.5, 10.5, 11.5, or 12.5. All reactions were carried out overnight at room temperature with a 1:10 molar ratio (AGU:GTMA). After reaction completion, samples were dialyzed in 10k MWCO regenerated cellulose dialysis tubing (ThermoFisher) against 4 L of 1 mM NaCl with three volume changes. Samples were then lyophilized using a Labconco Freezone 2.5 and resuspended in 1 mM sodium phosphate buffer pH 5.7 for further analysis. The same purification method and analysis was used to study the impact of temperature on reaction, with all reactions taking place in 0.1 M sodium phosphate buffer pH 12.5 overnight at a molar ratio of 1:10 (AGU:GTMA) at either 4 °C, 23 °C, 37 °C, or 60 °C. Similarly, a variety of reactant ratios (AGU:GTMA 1:10, 1:5, 1:1, 2:1, 10:1) were screened, with synthesis at room temperature, overnight in buffer of pH 12.5.

2.3.1.3 *Dynamic Light Scattering (DLS) and Zeta Potential*

The efficacy of GTMA modification was screened using DLS and zeta potential as described previously (section 2.2.1.3).

2.3.1.4 *X-Ray Photoelectron Spectroscopy (XPS)*

The elemental composition of ESG was probed using XPS. 100 µL of samples at a 5 mg/mL concentration in 1 mM sodium phosphate buffer were applied to an acetone cleaned silicon wafer and allowed to dry overnight. Thermo VG Scientific Theta Probe X-ray Photoelectron Spectroscopy (XPS) was used at 125 eV detector pass energy with a stepsize of 0.1 eV and a dwell
time of 50 ms. Each high resolution spectra was averaged over 30 scans. Degree of substitution (DS) calculations were performed from the molar C/N ratio using the equation:

\[
DS = \left( \frac{\text{mols C}}{\text{mols N}} - 6 \right)^{-1} 
\]

2.3.1.5 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra were collected as described previously over a range of 800 – 4000 cm\(^{-1}\), averaged over 32 scans at 4 cm\(^{-1}\) resolution.

2.3.1.6 Proton Nuclear Magnetic Resonance (NMR)

For cESG (1:10 AGU:GTMA ratio), NMR spectra were obtained of lyophilized sample dissolved in deuterium oxide (100 mg/mL) at room temperature using a 400 MHz Bruker Avance spectrometer with Topspin 2.1 software. Spectra were averaged over 64 scans at 400.13 MHz with a pulse of 1200 µs and a 1 s relaxation delay.

2.3.1.7 Elemental Analysis Isotope Ratio Mass Spectroscopy (EA-IRMS)

For cESG (1:10 AGU:GTMA ratio), EA-IRMS was conducted using a Carlo Erba NA 1500 elemental analyzer (Milano, IT) coupled to a VG Isochrom continuous flow IRMS (Isoprime Inc., Manchester, UK) and an integrated thermal conductivity detector (TCD) for the determination of carbon and nitrogen content. Analysis was performed by EcoCore Analytical Services (Colorado State University, Fort Collins CO).

2.3.1.8 Scanning Electron Microscopy (SEM)

For SEM preparation, approximately 0.5 µM ESG or cESG (1:10 AGU:GTMA ratio) complexes were prepared in 1 mM sodium phosphate buffer and 100 µL placed onto an acetone cleaned 1 cm\(^2\) silicon wafer. Samples were dried overnight under nitrogen. The samples were
analyzed on a LEO 1550 Scanning Electron Microscope at a 0.5 kV beam energy and 30 µm aperture. Fresh samples were used for each analysis.

2.3.1.9 Atomic Force Microscopy (AFM)

AFM specimens were prepared on freshly cleaved mica. 100 µL of ESG or cESG (0.15 µM in 1 mM sodium phosphate buffer) were added to a 1 cm² area of mica and dried overnight under nitrogen. The sample was analyzed by Dr. Vijay Jain using a Veeco Bioscope II AFM operating in tapping mode at 512 x 512 pixel resolution using a Bruker cantilever (model TAP525A) with 200 N/m force constant. Gwyddion analysis software was used to analyze the acquired AFM data.

2.3.1.10 Transmission Electron Microscopy (TEM)

TEM specimens were prepared on formvar coated gold grids using the drop method. Briefly, 10 µL of 0.1 µM solution of ESG or cESG (1:10 AGU:GTMA ratio) was added onto a grid for 30 seconds after which the solution being wicked off using a piece of filter paper. A 10 µL drop of methylamine tungstate dye (NanoW, Nanoprobes) was dropped on the grid and the specimen allowed to dry overnight under nitrogen. Images were taken on a JEOL 2010 LaB₆ transmission electron microscope operating at 200 kV in brightfield mode. ImageJ software was used to estimate particle dimensions. Particles were manually traced to determine circumference, which was used to estimate particle diameter, assuming spherical particles. The internal scale bar for each image was used to calibrate pixel length to physical length (nm).
2.3.2 Results and discussion

2.3.2.1 Synthesis conditions for GTMA modified cationic ESG (cESG)

The initial screening approach for determining success of ESG modification was size and zeta potential. Successful synthesis conditions were considered to be when the size of the particle remain similar to ESG with a high degree of uniformity, and a positive zeta potential. When screening for initial synthesis conditions, a ratio of 1:10 AGU:GTMA was used in an effort to avoid GTMA concentration being the limiting reagent. It was observed that when the synthesis was at a higher pH (11.5 and 12.5), the ESG particles took on a positive charge (Table 2.6). Syntheses taking place at lower pH (9.5 and 10.5) appeared less successful with little evidence of modification, based on the zeta potential values. The incorporation of nitrogen into the samples was confirmed using XPS elemental analysis. As there was no nitrogen present in unmodified ESG, any nitrogen detected in the sample was attributed to GTMA residues incorporated into cESG (Figure 2.4). Area under the peak was utilized to calculate the carbon to nitrogen ratio (C/N) of each sample. However for the lower pH synthesis, there was high noise and total nitrogen content was near the limit of detection for the system, requiring baselines for integration to be set manually. As such, C/N ratios for lower pH synthesis should be treated qualitatively rather than quantitatively. This technique corroborated the zeta potential results, showing that cESG synthesized at the highest pHs had the most relative nitrogen.

Table 2.6. Impact of pH on cESG synthesis - Size, Zeta potential, and Carbon/Nitrogen Ratio

<table>
<thead>
<tr>
<th>Synthesis pH</th>
<th>Size ± Standard Deviation</th>
<th>Zeta Potential ± Standard Deviation</th>
<th>Carbon/Nitrogen Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.5</td>
<td>32.4 ± 1.1</td>
<td>-4.1 ± 16.7</td>
<td>71</td>
</tr>
<tr>
<td>10.5</td>
<td>31.6 ± 0.3</td>
<td>-2.9 ± 17.5</td>
<td>75</td>
</tr>
<tr>
<td>11.5</td>
<td>30.2 ± 0.5</td>
<td>12.7 ± 15.9</td>
<td>65</td>
</tr>
<tr>
<td>12.5</td>
<td>28.9 ± 2.2</td>
<td>19.0 ± 16.6</td>
<td>44</td>
</tr>
</tbody>
</table>
Figure 2.4. XPS spectra of ESG (red) and cESG (blue) synthesized at a 1:10 AGU:GTMA molar ratio. Inset – High resolution nitrogen scan indicates incorporation of nitrogen into cESG.

The next synthesis parameter of interest was the impact of temperature on modification. The same AGU:GTMA ratio of 1:10 was used for synthesis, in pH 12.5 sodium phosphate buffer which was deemed the most promising for this and future synthesis. A positive zeta potential was observed in cESG synthesized at all temperature tested. XPS indicated more nitrogen was incorporated in samples synthesized above room temperature, 37 °C and 60 °C (Table 2.7). However, these samples were less stable, exhibiting a decrease in size and uniformity. As such, a room temperature reaction was deemed suitable for further syntheses.

<table>
<thead>
<tr>
<th>Synthesis Temperature (°C)</th>
<th>Size ± Standard Deviation</th>
<th>Zeta Potential ± Standard Deviation</th>
<th>Carbon/Nitrogen Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>30.6 ± 0.8</td>
<td>14.0 ± 18.5</td>
<td>108</td>
</tr>
<tr>
<td>23</td>
<td>30.7 ± 1.2</td>
<td>18.0 ± 20.5</td>
<td>72</td>
</tr>
<tr>
<td>37</td>
<td>23.9 ± 10.3</td>
<td>30.4 ± 16.7</td>
<td>49</td>
</tr>
<tr>
<td>60</td>
<td>19.5 ± 9.8</td>
<td>3.24 ± 13.6</td>
<td>45</td>
</tr>
</tbody>
</table>
The final parameter explored for tuning the reaction was the actual composition of the reaction, or reactant ratios. Ratios investigated ranged from a 1:10 to 10:1 AGU:GTMA. The relative concentrations of AGU to GTMA did not have a significant impact on particle size or uniformity, with all products having similar size (Table 2.8). Zeta potentials demonstrate a direct relationship between GTMA concentration and extent of modification. As the GTMA concentration increased, so did nitrogen concentration. The XPS data shows a difference in the amount of quaternary ammonium groups corresponding from one on every 11 glucose residues (10:1 ratio) up to a group on every 4.5 residues (1:10 ratio), according to degree of substitution calculations.

Table 2.8. Impact of Reactant Ratio on Synthesis of cESG: Size, Zeta Potential and Carbon/Nitrogen Ratio

<table>
<thead>
<tr>
<th>Reactant Ratio (AGU:GTMA)</th>
<th>Size ± Standard Deviation</th>
<th>Zeta Potential ± Standard Deviation</th>
<th>Carbon/Nitrogen Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:10</td>
<td>31.9 ± 2.1</td>
<td>20.2 ± 2.9</td>
<td>33</td>
</tr>
<tr>
<td>1:5</td>
<td>31.1 ± 2.1</td>
<td>9.5 ± 4.1</td>
<td>53</td>
</tr>
<tr>
<td>1:1</td>
<td>32.5 ± 2.3</td>
<td>6.6 ± 8.4</td>
<td>77</td>
</tr>
<tr>
<td>2:1</td>
<td>31.7 ± 1.3</td>
<td>0.7 ± 1.0</td>
<td>No detected Nitrogen</td>
</tr>
<tr>
<td>10:1</td>
<td>32.2 ± 0.8</td>
<td>-2.7 ± 0.9</td>
<td>73</td>
</tr>
</tbody>
</table>

ATR-FTIR analysis of cESG synthesized at various ratios was used to corroborate the XPS results that there was an increase in modification with increasing GTMA added. Spectra of cESG synthesized at various ratios reveals the appearance of a peak at 1475 cm⁻¹, which corresponds to the C-H stretching of methyl groups on the quaternary amine (Figure 2.5, full peak assignments Fig 2.6). This peak is clearly visible in the compounds synthesized at a 1:10 and 1:5 molar ratio, which is in agreement with XPS data indicating they have the highest degree of substitution (Table
2.8). As such, future cell studies were conducted using cESG synthesized at room temperature, overnight, in 0.1 M sodium phosphate buffer pH 12.5 at a 1:10 AGU:GTMA molar ratio. From this point on, unless otherwise specified, cESG refers to this synthesis condition.

Figure 2.5 FTIR spectra of cESG Compounds. Top (zoomed in region of interest) in which the 1475 cm\(^{-1}\) peak corresponds to the C-H stretch of methyl groups on the quaternary ammonium group.
2.3.2.2 Impact of Ionic Strength on Stability

An interesting phenomenon was observed over the course of investigating the cESG modification reaction, in that particles sporadically fragmented into smaller pieces. The size of unmodified ESG was stable in all conditions tested (data not shown). However, after modification with GTMA, breakage of the particles down to ~10 nm could be seen. In a study investigating reaction reproducibility, four identical synthesis reactions were conducted in parallel. Of these four reactions – one showed particle degradation to give 11 nm diameter particles, whereas the rest of the conditions retained the original size of ~35 nm diameter. We observed that the particles that
disintegrated had higher zeta potentials than the 30 nm particles, leading to the conclusion that charge was contributing to destabilization. Subsequent experiments performed in buffered solution and dialyzed against 1 mM sodium chloride, did not show any particle instability. We posit that counterions assist in stabilizing the particle by reducing charge effects. As such, standard practice for cESG usage in solution should be solvent with at least 1 mM salt, as lyophilized powder dissolved in distilled water also results in particle degradation.

2.3.2.3 Proton NMR and EA-IRMS

Once a synthesis approach for cESG was established, our next goal was to quantify the extent of modification in more detail. The first technique attempted to confirm XPS degree of substitution was proton NMR, however the large deuterium oxide solvent peak prevented accurate quantitative analysis. However, the appearance of a peak at 3.116 in cESG corresponding to the 9 hydrogens of \((\text{CH}_3)_3\)-N+ of the GTMA residue qualitatively confirm the presence of quaternary ammonium (Figure 2.7). As NMR was not sufficient to confirm the degree of substitution of cESG, elemental analysis isotope ratio mass spectroscopy (EA-IRMS) was used. This method is highly sensitive, using multiple internal standards to calculate nitrogen and carbon composition in independent runs. EA-IRMS indicated a 0.28 degree of substitution. This in line with the degree of substitution obtained from XPS, where three individual experiments gave a degree of substitution of 0.188 ± 0.027. This is a difference of one cationic amino group per 3.6 or 5.3 glucose residues respectively. This difference was expected as XPS is a surface sensitive measurement of a dried film but EA-IRMS measures the bulk material in solution. EA-IRMS has a higher sensitivity than XPS, which also may account for difference in calculated degree of substitution.
2.3.2.4 Metrology

A variety of metrology techniques were used to image the particle morphology and size. SEM was initially investigated for imaging. As we sought to estimate particle diameter, no metal sputtering was done prior to imaging. While at low beam energy (0.5 kV), the particle could be seen disintegrating after short beam dwell times. Images show that both unmodified and cESG are generally spherical and of a similar size (Figure 2.8). AFM was probed as an alternative method for determination of particle diameter (Figure 2.9). Micrographs reveal that both ESG and cESG are spherical and of a similar size 53 nm and 35 nm, respectively. TEM of the particles negatively stained with methylamine tungstate (Figure 2.10) indicated particles with a smaller diameter, 24.2 and 21.8 nm (Table 2.9) The values calculated with this method are considerably smaller than the hydrodynamic radius calculated by DLS, 24 nm vs 33 nm. As DLS measures the hydrodynamic radius of particles in solution, it is dependent on counterions in solution and water-induced swelling of the glycan chain. Considering this, the smaller size determination of TEM is not
unreasonable in comparison to the particle size determined in solution conditions. All techniques indicate that the particles are in the sub-50 nm range.

Figure 2.8. Scanning electron micrographs of unmodified ESG (left) and cESG synthesized at 1:10 ratio ESG:GTMA (right). Scale bar 100 nm

Figure 2.9 Atomic force micrographs of unmodified ESG (left) and cESG synthesized at 1:10 ratio ESG:GTMA (right). Scale bar 500 nm.
Figure 2.10 Transmission electron micrographs of unmodified ESG (A) and cESG synthesized at 1:10 ratio ESG:GTMA (B) stained with methylamine tungstate. Scale bar 50 nm.

Table 2.9 ESG and cESG particle diameter calculated using various metrology techniques. n=50-60 particles per metrology technique.

<table>
<thead>
<tr>
<th>Technique Used</th>
<th>ESG Diameter (nm)</th>
<th>cESG Diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEM</td>
<td>49.5 ± 15.8</td>
<td>43.9 ± 8.1</td>
</tr>
<tr>
<td>AFM</td>
<td>53.3 ± 14.6</td>
<td>35.6 ± 14.8</td>
</tr>
<tr>
<td>TEM</td>
<td>24.2 ± 3.0</td>
<td>21.8 ± 3.5</td>
</tr>
<tr>
<td>DLS</td>
<td>33.6 ± 10.2</td>
<td>37.0 ± 11.8</td>
</tr>
</tbody>
</table>

2.4 Conclusions

In this study, we demonstrated that ESG may be cationically modified by two different reaction schemes. First, the periodate oxidation of ESG was investigated with changing variables such as reaction temperature, time, and reagent ratios. oxESG was successfully produced, with the ability to tune oxidation of 2.5% to 35% of glucose residues. A hydrazine linkage of oxESG with Girard’s Reagent T was then explored to introduce quaternary ammonium groups. The resultant products suffered from poor solubility but the product did indicate incorporation of quaternary amine to give rise to a positive zeta potential around 10 mV. While this modification was not
investigated further, the controllable introduction of aldehydes into ESG could be utilized in the future for covalent conjugation with surface targeting functionalities or therapeutics via reaction with hydrazines, alkoxyamines or primary amines.

The second reaction scheme evaluated, a one-pot modification of ESG with quats, resulted in a higher zeta potential of up to 20 mV. This reaction product exhibited good solubility in aqueous media, high uniformity, and it was possible to tune the degree of substitution by varying GTMA concentration. cESG made via these means was further characterized using metrology techniques, corroborating that the size and shape of the particle was not significantly changed during modification. Based on these results, cESG synthesized via GTMA conjugation at a 1:10 molar ratio AGU:GTMA was used for future studies. Next, we explored the ability of cESG to bind and deliver nucleic acids in an *in vitro* ovarian clear cell carcinoma model.

### 2.5 References


and plasmid DNA have a neutral surface and gene delivery potency. *Bioconjugate Chem.* 14, 1214-1221.


CHAPTER 3.

INVESTIGATION OF cESG FOR NUCLEIC ACID DELIVERY

3.1 Introduction to Ovarian Cancer and Reactive Oxygen Species (ROS)

In order to investigate the application of cESG as an siRNA delivery vector, we focused on targeting the expression knockdown of antioxidant enzyme manganese superoxide dismutase (MnSOD, SOD2) in ovarian cancer. The rationale for using this model is highlighted below.

3.1.1 Ovarian Cancer Statistics, Histological Subtype & Diagnosis

While ovarian cancer accounts for only 5% of female cancers, it is the fifth leading cause of cancer deaths in women of all age groups. In 2016, there are projected to be 22,289 new cases with 14,240 deaths from the disease. The overall five year survival rate was 46% from 2005-2011, an increase of only ten percent survival in the past forty years.¹ When detected at an early stage, ovarian cancer has an excellent ten year survival rate of 73% (Table 3.1).² However, only 20% of cases are detected at such an early phase of progression, primarily due to the relatively asymptomatic nature of early stage disease. As the disease spreads throughout the abdominal cavity, prognosis drops drastically. New detection and screening methods along with more efficient treatments are necessary to improve these outcomes.

Table 3.1. Prognosis of Ovarian Cancer by Stage. Table modified from Jelovac et al.\textsuperscript{2}

<table>
<thead>
<tr>
<th>Stage*</th>
<th>Disease Spread</th>
<th>Distribution of Cases (%)</th>
<th>Ten Year Survival Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Confined to ovaries</td>
<td>20</td>
<td>73</td>
</tr>
<tr>
<td>II</td>
<td>Confined to pelvis</td>
<td>5</td>
<td>45</td>
</tr>
<tr>
<td>III</td>
<td>Present in upper abdominal cavity (liver surface, bowel and omentum)</td>
<td>58</td>
<td>21</td>
</tr>
<tr>
<td>IV</td>
<td>Metastasis beyond abdominal cavity</td>
<td>17</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

*as classified by the International Federation of Gynecology and Obstetrics.

Ovarian cancer is comprised of distinct histological subtypes, which vary in their genetic signatures and tissues of origin, yet these distinct diseases are classified under one umbrella due to their anatomical localization\textsuperscript{3} 90% of ovarian cancers are classified as epithelial ovarian cancer, as opposed to the less common germ cell cancers.\textsuperscript{4} Epithelial ovarian cancers are further divided into histological subtypes, as demonstrated in Table 3.2. High grade serous ovarian cancers originate on the ovary surface or distal fallopian tubes, whereas, clear cell carcinoma and endometriod cancers arise from the endometrium. Mucinous ovarian cancer is not thought to be derived from the reproductive organs, but rather to have metastasized to the ovary from the nearby gastrointestinal tract such as the appendix, stomach, or colon.\textsuperscript{5} Along with phenotypic characterization of the tumor there are also distinct genetic changes associated with the different histological subtypes, which may provide clues to disease etiology and for the development of treatment strategies (Table 3.2).
Table 3.2. Genetic mutations associated with histological subtypes of epithelial ovarian cancer. Modified From Engelberth et al.\(^6\)

<table>
<thead>
<tr>
<th>Histologic Subtype</th>
<th>Percentage of Epithelial Ovarian Cancer Cases</th>
<th>Genetic mutation (% of cases expressing)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serous – High Grade</td>
<td>70</td>
<td>TP53 (96%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BRCA1/BRCA2 (22%)</td>
</tr>
<tr>
<td>Serous – Low Grade</td>
<td>&lt;5</td>
<td>BRAF/KRAS (50%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ERBB2 (9%)</td>
</tr>
<tr>
<td>Clear Cell</td>
<td>10</td>
<td>ARIK1A (50%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PIK3CA (50%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PTEN (20%)</td>
</tr>
<tr>
<td>Endometrioid</td>
<td>10</td>
<td>CTNNB1 (40%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PIK3CA (20%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PTEN (20%)</td>
</tr>
<tr>
<td>Mucinous</td>
<td>&lt;5</td>
<td>KRAS (75%)</td>
</tr>
</tbody>
</table>

The incidence of ovarian cancer is age dependent, with only 1 in 100,000 women younger than 25 diagnosed with the disease. However, by 55 years of age, the incidence of ovarian cancer jumps to 32 in 100,000, and to 75 in 100,000 for women over 70.\(^7\) A major risk factor for developing clear cell and endometrioid carcinomas is endometriosis.\(^8\) Overall, ovarian cancer incidence is proportional to the number of lifetime ovulations. Therefore, risk is decreased by pregnancy, hysterectomy, contraceptive usage, or tubal ligation.\(^9\) Since the origin of serous ovarian cancer is fallopian tube epithelium rather than ovarian, it is postulated that during ovulation the fallopian tube fimbria is in close contact with ruptured ovarian surface epithelium, which plays a role in formation of precursor lesions on the ovary itself.\(^10\)

The diagnosis of ovarian cancer is complicated by a wide range of early symptoms. Often symptoms such as bloating, abdominal pain, and irregular menstrual bleeding are attributed to other ailments. This contributes to late stage diagnoses, in which most cases are not diagnosed until metastasis into or beyond the abdominal cavity has occurred (Table 3.2). Screening for
ovarian cancer in the general populace is minimal and not currently recommended by the American Medical Association.\textsuperscript{11} Manual pelvic exams conducted at annual gynecological appointments have a very low sensitivity for detecting ovarian malignancies, catching only 1 in 10,000 asymptomatic ovarian carcinomas. For women at special risk for developing ovarian cancer due to genetic risk factors, such as BRCA1 and BRCA2 mutations, transvaginal ultrasounds may be used to physically detect tumors. These are invasive, painful, and unable to distinguish between benign and malignant masses without further biopsy. An alternative screening modality looks at the expression of cancer antigen 125 (CA 125), an antigen overexpressed in 80\% of advanced stage ovarian carcinomas. However only 50\% of stage I disease overexpress CA125. As such, it is also a poor detection mechanism for early stage, asymptomatic disease.\textsuperscript{2}

New screening and diagnostic assays are on the horizon, with clinical trials having examined the use of CA125 in conjunction with other markers, such as human epididymal secretory protein (HE4) to decrease false negative results.\textsuperscript{12-14} In postmenopausal women, the Risk of Ovarian Malignancy Algorithm (ROMA) analysis of CA125 and HE4 levels has improved diagnosis of the disease over analysis of CA125 levels alone.\textsuperscript{15,16} While new biomarkers have been discovered including; increased protein glycosylation in serum;\textsuperscript{17-19} an overexpressed array of transthyretin, ApoA1, beta-2 microglobulin and transferrin;\textsuperscript{13,20} and the proteinases kallikrein and prostatin;\textsuperscript{21} early detection has not been improved.

\textit{3.1.2 Metastatic Spread}

In most cancer types, in order for a primary tumor to metastasize to a secondary site, it must invade either the circulatory or lymphatic system.\textsuperscript{9} Conversely, in ovarian cancer, the shedding of tumor cells from the primary tumor into the IP cavity (i.e. transcoelomic route) appears to be the most direct avenue for ovarian cancer to metastasize (Figure 3.1). However, it should be pointed
out that recent studies have also demonstrated the ability of ovarian cancer cells to utilize the hematological system to home to secondary sites.\textsuperscript{22-24} In ovarian cancer, tumor cells undergo an epithelial to mesenchymal transition, resulting in cells that detach from the primary tumor with an invasive phenotype and an ability to evade anchorage-independent cell death (anoikis).\textsuperscript{25,26} During transcoelomic metastasis cells are released into the intraperitoneal fluid (ascites). This shedding process occurs early, with even 30\% of stage I cancer patients presenting malignant cells in the ascites.\textsuperscript{9} While in this fluid, ovarian tumor cells have been shown to aggregate into spheroids, which may flow through the cavity until they bind to a new surface via extracellular matrix proteins. The first point of contact is the omental tissue lining the intraperitoneal cavity and abdominal organs. Here, the spheroid disaggregates, the cells proliferate and invade the mesothelium, leading to new tumor formation.\textsuperscript{27} In addition, metastatic cells in ascites access the lymphatic system through the diaphragm’s drainage ports, which eventually enters the circulatory system via the left subclavian vein.\textsuperscript{4}

3.1.3 Standard of Care and Novel Therapeutic Approaches

Once diagnosed, the primary treatment for ovarian cancer is cytoreductive surgery. In this procedure, tumors are removed from the intraperitoneal cavity (debulking). While surgery may remove a large portion of tumor mass, further treatment in the form of chemotherapy is still necessary, largely due to the vast metastatic spread and difficulty in removal of micrometastases in the peritoneum.\textsuperscript{28} Studies have shown that optimal debulking (removal of all or the majority of tumor mass) improves outcome and may improve the efficacy of subsequent treatment with chemotherapy.\textsuperscript{29} However, optimal cytoreduction remains difficult to achieve in some cases due to the unique transcoelomic metastasis of ovarian cancer.
Following debulking surgery, the main chemotherapeutic treatment for ovarian cancer is six to eight weeks intravenous (IV) platinum based treatments, such as cisplatin or carboplatin. These compounds induce apoptotic cell death by two major mechanisms. In the case of cisplatin, upon entering the cell, the drug becomes hydrolyzed and can react with both sulfhydryl groups on
proteins and nitrogen in nucleic acids. As such, it can bind purine residues on DNA, causing damage and blocking mitosis, eventually leading to apoptosis. Its second mechanism takes place at the mitochondria, where binding of the sulfhydryl group inhibits activity, leading to oxidative stress. Cisplatin also impacts a variety of other pathways including protein kinases, kinase receptors, and p53 protein.\textsuperscript{30} While over 70\% of cases respond to this initial treatment, it is also common for patients to relapse and develop resistance to platinum. In these cases, a secondary treatment is administered along with a taxane such as Paclitaxel or doxorubicin. The former inhibits microtubule depolymerization and thus cell division, resulting in death.\textsuperscript{31} The latter intercalates DNA and binds DNA-associated enzymes to result in apoptosis.\textsuperscript{32}

A number of new treatments for ovarian cancer are in clinical trials, most of which focus on genomic or signaling pathways of specific subtypes of the disease. One strategy for treatment is targeting the vascular endothelial growth factor (VEGF) receptor pathway to prevent angiogenesis. When used in combination therapy, Bevacizumab, an antibody that inhibits VEGF-A, has improved progression free survival, especially in platinum resistant disease.\textsuperscript{33,34} In combination therapy, Poly ADP ribose polymerase (PARP) inhibitors are being investigated to treat late stage serous ovarian cancers, which have defects in homologous recombination repair.\textsuperscript{35} MAP kinase inhibitors are of interest in early phase serous ovarian cancers due to their high degree of Ras protein mutations.\textsuperscript{36} For ovarian cancer cells which overexpress folate receptor, drugs inhibiting its activity such as Pemetrexed, Vintafolide, and Farletuzumab have shown promising results in clinical trials.\textsuperscript{37,38} Given the success of clinical trials in other tumor types and demonstration of efficacy in preclinical studies of ovarian cancer, the use of immunotherapy to target ovarian cancer is on the horizon.\textsuperscript{39}
As many cases of ovarian cancer are restricted to the abdominal cavity, clinicians have investigated the administration of chemotherapeutics directly into the intraperitoneal (IP) cavity. This IP administration, when used with both paclitaxel and cisplatin, increased progression free survival for women with stage III and IV disease significantly with a 17% increase in long term survival.\textsuperscript{41} While this route requires a certain degree of discomfort for the patient due to the implantation of an abdominal catheter and has been shown to induce localized toxicity, it is a promising route to increase local doses of therapy, while decreasing systemic side-effects. From a drug delivery standpoint, IP administration prevents the obstacle of clearance from the circulatory system prior to delivery to the tumor target. A long term goal of our research would be to explore this delivery route for therapeutics complexed with cESG.

cESG may also be explored for IV delivery in ovarian and other cancers, relying on passive targeting via the enhanced permeability and retention (EPR) effect. The EPR effect was first demonstrated in 1979 by Maeda et al\textsuperscript{42}, who observed that a polymer-bound anticancer protein (neocarzinostatin) had higher accumulation in tumors and longer plasma half-lives compared to free drug.\textsuperscript{43} This was demonstrated with a range of proteins that were larger than 40 kDa, in which there was an accumulation of 10-200 times more compound in tumor tissues compared to normal tissues.\textsuperscript{44} This theory is based upon the endothelial lining of blood vessels within tumors becoming more permeable than normal, allowing for particles in the 10 to 500 nm range to exit the vasculature (extravasate) and be released into the interstitial space. Leaky vasculature is a common occurrence in tumors due to abnormal development of vessels in response to aberrant VEGF stimulation, often as a consequence of the hypoxic (low oxygen) tumor environment. The EPR effect is further enhanced as particles accumulate at tumor sites due to poor lymphatic drainage.\textsuperscript{45} IV delivery of particles requires long-circulating times in the bloodstream, but has shown previous
success with liposomal nanocarriers such as Doxil, liposomal doxorubicin.\textsuperscript{46,47} This formulation has shown improved outcomes over free doxorubicin for breast cancer as well as ovarian cancer, as the free drug is released intracellularly rather than in the general bloodstream.\textsuperscript{45}

An interesting aspect of using carbohydrates as a delivery system in cancer is the modified role of glucose metabolism in the disease. In 1956, a German biochemist Otto Warburg, observed that cancer cells exhibit upregulated glycolysis, even in non-hypoxic environments.\textsuperscript{48} He hypothesized that oxidative phosphorylation is halted by mitochondrial dysfunction, and cancer cells look to the less efficient glycolysis to generate ATP.\textsuperscript{49} While mitochondrial dysfunction may play a role in some tumors, it has now been shown that cancer cells establish the Warburg effect by a variety of mechanisms, including transcriptional upregulation of glycolytic enzymes, switching of isoforms and phosphorylation activation of metabolic enzymes, as well as genomic aberrations contributing to metabolic reprogramming.\textsuperscript{50} Tumor cells also efficiently divert glucose to the pentose phosphate pathway to allow for the formation of nucleotides and NADPH, which provides reducing equivalents for biosynthesis reactions (lipids and amino acids) and the regeneration of reduced glutathione (GSH) important for reactive oxygen species (ROS) removal. This ability to scavenge ROS is integral for tumor cells to respond and survive to the ever-changing stress in the tumor microenvironment.\textsuperscript{49,51} Interestingly, an increased ability of tumor cells to breakdown glycogen stores for the shuttling of glucose into the above pathways, was recently demonstrated.\textsuperscript{52} We hypothesized that this increased metabolism of glucose in tumor cells may contribute to breakdown of cESG and subsequently enhance the release of its cargo for delivery. We investigated this further in the following chapter.
3.2 Role of Reactive Oxygen Species (ROS) and Mitochondrial Superoxide Dismutase (SOD2) in Cancer

3.2.1 Reactive Oxygen Species, Superoxide Dismutase, and Cancer

Reactive oxygen species (ROS) are a category of chemicals which are highly reactive byproducts of oxygen metabolism (Figure 3.2) The first ROS to be discovered was hydrogen peroxide, by Louis Thénard in 1818,\(^53\) followed by the hydroxyl radical, postulated by Fenton in 1894,\(^54,55\) and the superoxide radical discovered in 1934.\(^56\) Superoxide anion (O\(_2\)-\(^•\)) is the partially reduced form of oxygen and the first oxidant produced either at the level of mitochondria, where electrons are donated to oxygen through leakage at the electron transport chain, or via enzymatic activity by NADPH oxidases (NOX) at cellular membranes. The reactivity of ROS can be extremely high, such as the hydroxyl radical, which is the major ROS involved in DNA damage during the process of carcinogenesis. Others have lower reactivity, such as superoxide and hydrogen peroxide, the latter of which is not a radical species.\(^57\) Less reactive and species with longer half-lives, such as H\(_2\)O\(_2\) are now known to serve as molecules to regulate signaling of biological and physiological processes including inflammation, aging, metabolism, and proliferation.\(^58\) In cancer, ROS play a role in tumor initiation and progression. ROS are also produced at a higher rate in cancer relative to normal phenotypes. This in turn leads to a compensatory change in antioxidant enzyme expression.\(^59\) The study of ROS in cancer is actively growing and while a detailed review is outside of the scope of this dissertation, recent developments in the field are reviewed here.\(^57,60,61\)
In order to combat the toxic nature of ROS, or detoxify them, Thénard postulated that the body had some natural defense mechanism to neutralize these species. Enzymes and molecules that would later be known as antioxidants. These include enzymatic antioxidant and non-enzymatic antioxidant defenses, such as vitamin C, vitamin E, and β-carotene, which donate electrons to reduce free radicals. In 1900, there was a notable discovery of the first antioxidant enzyme, catalase, by Oscar Loew. This enzyme is integral for intracellular ROS regulation, and is responsible for the conversion (dismutation) of hydrogen peroxide into oxygen gas and water. In 1940, a protein with unknown specific function was discovered by Keilin and Mann. This copper containing compound was named hemocuprein (also known as erythrocuprein). In 1969, the enzymatic function of this protein was elucidated by Fridovich and McCord, as an antioxidant for the dismutation of superoxide to hydrogen peroxide, leading to its new name, superoxide dismutase (SOD). This was the first of three SOD isoforms to be discovered. SOD which contain copper and zinc cofactors include SOD1 (CuZn-SOD), a homodimer found intracellularly in the cytoplasm, and Sod3 (EC-SOD), a homotetramer located extracellularly in locations such as plasma, lymph and cerebrospinal fluid. Mitochondria localized SOD2 (Mn-SOD) is also a
homotetramer and has manganese as its metal cofactor (Figure 3.3).\textsuperscript{66} SOD has been shown to prevent superoxide-induced injuries related to a spectrum of diseases including heart attack, stroke, diabetes, ischemia, cancer, and neurodegenerative diseases.\textsuperscript{67} As our understanding of the complex nature of cancer increases, it is being realized that many cancer cells enhance their antioxidant status to promote cell survival and alter cellular redox balance to their benefit.

Fig 3.3. Structure of SOD isoforms with metal cofactors from Protein Data Bank in Europe. Conformation based on X-Ray Diffraction studies with at least 2.38 angstrom resolution from Ramilo et al,\textsuperscript{68} Merz et al,\textsuperscript{69} and Antonyuk et al\textsuperscript{70} respectively.
3.2.2 Mitochondrial Superoxide Dismutase (SOD2)

SOD2 specifically has been shown to have a unique role in cancers, with conflicting evidence that it serves as a tumor suppressor or an oncogene depending on cancer type and stage.\(^71\) In 1979, Oberley and Buettner postulated that SOD2 acted as a tumor suppressor, as decreased SOD2 expression was found in a variety of cancers when compared to normal tissues.\(^72\) This loss of expression has been linked to hypermethylation of the SOD2 promoter and possible instabilities of chromosome 6 during oncogenesis. However, in other cancer types this downregulation is not observed. Rather a correlation of increased SOD2 expression and metastatic progression or stage has been observed. This increase in expression in aggressive cancers has been attributed to increased ROS levels, and changes in transcription factors and cytokines that regulate the SOD2 gene promoter. Besides aiding in protection from endogenous surges in superoxide, this increased SOD2 expression may also serve a protective role from exogenous redox damage. High SOD2 levels have been demonstrated to aid in radio- and chemoresistance.\(^71\)

Previous work from our laboratory and that of Dr. Andres Melendez has demonstrated that SOD2 drives metastasis in a number of tumor models, which has been linked to both its ability to scavenge superoxide from the mitochondria\(^73\) and due to its role in manipulating the cellular steady state levels of $\text{H}_2\text{O}_2$.\(^74\) Increased SOD2 expression not only confers a survival advantage but also increases the metastatic phenotype of cancer cells by increasing Matrix Metalloproteinase (MMP) expression in a $\text{H}_2\text{O}_2$-dependent manner,\(^75\) which subsequently degrades the extracellular matrix, aiding in invasion through the basement membrane.\(^74\) In addition, it has been shown that $\text{H}_2\text{O}_2$-dependent oxidation of phosphatase such as PTEN can increase the angiogenic phenotype of cancer cells in a SOD2-dependent manner.\(^76\) More recently, we have shown that SOD2 also has a protumorogenic role in ovarian cancer. SOD2 was highly expressed in a number of ovarian cancer
cell lines, and was demonstrated to be specifically high in ovarian clear cell carcinomas (OCCC) when compared to other ovarian cancer histological subtypes. To demonstrate the role of SOD2 in ovarian cancer, SOD2 expression was reduced using stable transfection of shRNA constructs in an OCCC cell line, ES-2. A 70% reduction in protein expression resulted in significant decreases in cell proliferation and clonogenicity. In order to further study the role of SOD2 during tumorogenicity, a pseudo in vivo chorioallantoic membrane (CAM) tumor xenografts model was used. Tumor growth and migration and invasion of cells into the membrane were both decreased with reduced SOD2 expression, as was metastasis into the lungs and liver of the chick embryos (Figure 3.4). Mechanistically we showed that SOD2 not only protects ovarian clear cell carcinomas from superoxide build-up in mitochondria, thereby preserving high mitochondrial function, but that a concomitant SOD2-mediated shift towards higher steady state H$_2$O$_2$ also drives redox-dependent Akt signaling and migration of tumor cells (Figure 3.5).

Based on this work, it is our hypothesis that knockdown of SOD2 may serve as a therapeutic target for ovarian and other cancers. We anticipate that effectively decreasing SOD2 expression may have a two-fold effect. First, reducing SOD2 expression should decrease migration and metastasis of cancer cells, thereby prolonging the time for metastatic spread and ability to use this therapeutic as an adjuvant to standard of care therapies. Second, in addition to DNA intercalation and microtubule stabilization, chemotherapeutics such as Cisplatin and Taxol have also been shown to induce apoptosis via the generation of ROS. These agents may therefore become more effective, if the cell’s ability to detoxify superoxide is removed. Initial studies from our lab indicate that ES-2 cells with reduced SOD2 expression have increased chemosensitivity to cisplatin (Madhubhani Hemachandra, Ph.D., data not published).
Figure 3.4. CAM studies indicate SOD2 knockdown decreases tumor size and metastasis after a 7 day incubation of ES-2 cells with wild type (control) or shRNA-mediated knock-down of SOD2 expression (shSOD2). A) shRNA-SOD2-GFP-labelled transfected ES-2 cells display smaller tumor formation and decreased metastatic spread into the membrane B) Fewer tumor metastases are found in CAM and chick embryo liver and lungs with decreased SOD2 levels. C) Reduced SOD2 expression significantly inhibited CAM tumor size and weight (Student’s t-test **p<0.01). D) Images of n=11 tumors from CAM model. Data reproduced from Hemachandra et al.\textsuperscript{73}
In this chapter we investigated the use of cESG as a delivery vector for siRNA against SOD2 in ovarian cancer cell culture models. Initially, cESG was tested for nucleic acid binding using a GFP-expressing plasmid to characterize electrostatic condensation. The complex was then used as a delivery vector to ES-2 OCCC cells \textit{in vitro}. Subsequently, the binding and delivery of siRNA was investigated, measuring uptake and efficacy of SOD2 knockdown at a variety of particle-siRNA formulations. The cESG-siRNA-SOD2 complex was then studied for efficacy under various conditions, altering reagent storage time, incubation period, and cell type. MTT viability tests were used to assess the toxicity of the complex \textit{in vitro}.  

Figure 3.5 Proposed dual role of SOD2 in OCCC tumorigenesis and metastasis. High SOD2 expression provides efficient superoxide scavenging and maintenance of high mitochondrial function for increased cell proliferation. SOD2 shifts intracellular ROS balance to H$_2$O$_2$, diverging tumor cell migration and metastasis. Modified from Hemachandra et al. 73
3.3 Methods

3.3.1 Materials

McCoy’s 5A media, RPMI media, and Trypsin EDTA 1x were from ATCC. HyClone fetal bovine serum was from GE Healthcare. Dulbecco’s phosphate buffer saline 1x was from FisherScientific. pEGFP-N1 plasmid was from Clontech A 5’ fluorescein 6-FAM-labeled, previously validated73 siRNA targeting SOD2 was purchased from Dharmacon (On-Target Plus 5’-CAACAGGCCUUAAUCCACU-3’). A scramble oligonucleotide sequence was used as a non-targeting control (Dharmacon, OnTarget Plus Control siRNA Nontargeting siRNA #1). Antibodies were obtained from Cell Signaling Technology (Boston, MA) or Abcam (Cambridge, MA). Cell experiments were performed using sterile, nuclease free water (Qiagen). All other reagents were purchased from Sigma Aldrich unless otherwise specified.

3.3.2 cESG-Plasmid Condensation Reaction

Lyophilized cESG was dissolved in 1 mM NaPO₄ buffer pH 7.5 at a concentration of 5mg/mL (0.48 µM). pEGFP-N1 plasmid was prepared in distilled water at 100 ng/µL. The particle and plasmid were then mixed at various mass ratios (cESG:plasmid 50:1, 40:1, 30:1, 20:1) in distilled water. Solutions were vortexed and centrifuged for 15 seconds every 5 minutes, for a total 35 minute incubation period.

3.3.3 cESG-siRNA Condensation Reaction

Lyophilized cESG was dissolved in 1 mM NaPO₄ buffer pH 7.5 at a concentration of 3 µM ESG. 10 µM fluorescein-labeled siRNA with a SOD2-specific targeting sequence was prepared in distilled water. The particle and siRNA were then mixed at various mass ratios (1:2, 1:12.5, 1:25, 1:60, 1:125, 1:500), at a constant siRNA concentration of 1 µM, in distilled water. Solutions were vortexed and centrifuged for 15 seconds every 5 minutes, for a total 40 minute incubation period.
3.3.4 Agarose Gel Retardation Assay

A gel shift assay was performed using a 1% Tris-acetate-EDTA agarose gel. 2 µL of 10x gel loading dye was mixed with 10 µL of sample containing either 100 ng of plasmid or 60 ng of siRNA condensed with cESG. Electrophoresis performed in 1x Tris-acetate-EDTA (TAE) buffer at 100 V for 20-30 minutes. Ethidium bromide bath staining was conducted for 15 min, followed by imaging on a FluorChem E imager (Protein Simple).

3.3.5 DLS and Zeta Potential Analysis

Size and charge of particle were assessed using DLS and zeta potential analysis, as described previously (2.2.1.3).

3.3.6 Cell Culture

ES-2 clear cell ovarian carcinoma cells (purchased from ATCC) were maintained in McCoy’s 5A media supplemented with 10% fetal bovine serum, penicillin (100 U/L) and streptomycin (0.1 mg/L), and cultured at 37 °C under 5% CO₂. Nose007 normal ovarian surface epithelial cells and Ovca420 ovarian serous adenocarcinoma were provided by Dr. Susan K. Murphy of Duke University and maintained in RPMI media supplemented with 10% fetal bovine serum, penicillin (100 U/L) and streptomycin (0.1 mg/L), and cultured at 37 °C under 5% CO₂.

3.3.7 Analysis of cESG-Plasmid Transfection Efficiency

ES-2 cells were plated in a 24 well plate at a density of 20,000 cells per well and incubated overnight. The following day, cESG-plasmid complexes were made as described previously at a 50:1 mass ratio (cESG:plasmid). Cells were treated at a concentration of 500 ng plasmid per well (0.32 nM), which corresponds to 4.8 nM cESG per well. Lipofectamine2000 was prepared according to the manufacture’s direction as a positive control (ThermoFisher). Images were taken
on an AMG EVOS FL microscope with GFP LED light source filtercubes, using a 20x objective 24, 48, and 72 hours following treatment, to assess siRNA-particle uptake.

3.3.8 **MTT Viability Assay**

Toxicity was evaluated using an MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) cell viability assay developed by Mosmann. Cells were seeded at a density of 4,000 cells/well into 96-well plates. After 24 hrs the cells were incubated with ESG, cESG, cESG-plasmid, or cESG-siRNA complexes as indicated and incubated overnight. Subsequently, media was aspirated and replaced with 100 µL serum free media including 10 µL of 12 mM MTT stock solution. Cells were incubated for 4 hrs at 37 °C, at which time 85 µL of media was removed from each well and 50 µL of DMSO added for formazan solubilization. After a 10 min incubation at 37 °C, absorbance was read at 540 nm on a SpectraMax Paradigm plate reader. Absorbance values were corrected to the absorbance of the reactants in the absence of cells and normalized to an untreated control.

3.3.9 **Analysis of cESG-siRNA Uptake and Transfection Efficiency**

ES-2 cells were seeded into 6-well plates at a density of 80,000 cells per well. After 24 hrs cells were incubated with fresh cESG-siRNA complexes (100 nM siRNA/well) in growth media (2 mL). For fluorescent imaging, media was replaced with PBS after indicated treatment times and FAM-labeled siRNA-cESG within cells detected using an EVOS FL fluorescent microscope (AMG). Bright field images were taken simultaneously and the percentage of fluorescing to total number of cells quantified. Cells were washed with PBS and collected on ice in 50 µL RIPA buffer, containing proteinase inhibitors, for western blotting analysis. For comparison to Lipofectamine RNAiMAX, ES-2 cells were cultured as above and incubated for 72 hrs in growth media containing 100 nM siRNA freshly prepared as either cESG-siRNA or Lipofectamine RNAiMAX-
siRNA complexes (prepared according to the manufacturer’s directions). After 72 hrs cells were immediately replaced with PBS for fluorescence imaging and collected on ice in RIPA buffer for western blot analysis or allowed an additional 72 hr incubation in growth media prior to imaging and harvesting. Controls (including growth media alone, cESG and cESG-Scramble-siRNA) were treated in the same manner.

3.3.10 Western Blotting Protocol

Protein concentration was determined using a BCA assay (Pierce) and samples were loaded into a mini-Protean gel (BioRad) along with a Precision Plus Protein™ dual color standard ladder (BioRad), and run in 1x Tris-Glycine-SDS for 45 min at 160 V. Samples were transferred to a polyvinylidene fluoride (PVDF) membrane using a TurboBlot transfer unit (Bio-Rad) and blocked for 1 hr in 5% powdered milk in Tris-buffered saline-0.1% Tween 20 (TBST). The membrane was incubated with primary antibody to SOD2 or β-Actin control overnight and rinsed prior to a 1 hr incubation with secondary anti-mouse or anti-rabbit antibody respectively. West femto substrate (Thermo Scientific) was used for band visualization in a ChemiDoc MP imaging system (Bio-Rad). Band intensities were quantified using ImageJ software. Band intensities were standardized to the average band intensity of the blot, followed by normalization of the SOD2 band intensity to the corresponding β-Actin controls.

3.3.11 Statistical Analysis

All data presented are representative of at least three independent experiments and expressed as mean ± SEM, unless otherwise noted. Statistical data analysis were performed using OriginPro Software v8.5. Student’s T-Tests or ANOVA with Tukey’s post test were performed as indicated. In the case of two-way ANOVA analysis, the input variables (i.e. dosage, treatment type,
illumination time) are considered independent and the mean differences between groups are compared.

3.4 Results and Discussion

3.4.1 cESG-Nucleic Acid Binding

While the eventual goal of this research was to deliver therapeutic siRNA, initial studies of nucleic acid binding and delivery were conducted with a GFP expressing plasmid (pEGFP-N1). This was used in initial characterization studies as it is an efficient indicator of the capability of cESG as a nucleic acid carrier, as free plasmid will not traverse cellular membranes. Additionally, uptake can be readily visualized due to high expression efficiency of GFP in mammalian cells. A gel shift assay was utilized to determine if there was electrostatic binding of the plasmid with cESG. Binding of plasmid to cESG retains nucleic acids (stained with ethidium bromide) in the gel wells, while free plasmid readily migrates towards the cathode through the agarose gel. This is visualized in the first well of Figure 3.6, in which the 4700 base pair plasmid and supercoiled bands are clearly visible. Since cESG is too large to migrate through the gel, it is retained in the well and migration of any bound plasmid is therefore retarded or completely retained in the well. Ethidium bromide staining indicates that at a variety of ratios cESG:plasmid, the plasmid is sufficiently bound to cESG to prevent migration (Figure 3.6). In order to probe the impact of reaction time on binding, a 15:1 ratio cESG/plasmid reaction was performed for a range of reaction times. As seen in Figure 3.7, binding occurred in as little as 10 minutes. Complex left to react overnight at room temperature did not demonstrate visible disassociation of the nucleic acid from the cESG, as free plasmid was not detectable in the gel, suggestive of a strong condensation reaction. Although dissociation was not observed, storage of the nucleic acid at room temperature for prolonged periods of time was avoided to prevent nucleic acid degradation.
Figure 3.6. Gel shift assay of cESG-plasmid complexes confirms condensation of plasmid to cESG. Impeded migration (asterisks), indicative of condensation, is observed in ratios with at least 6 cESG per plasmid.

Figure 3.7. Gel shift assay of cESG-plasmid complexes at a 15:1 molar ratio cESG:plasmid confirms condensation of plasmid to cESG in as little as 10 min. Impeded migration (asterisks), indicative of condensation, is observed in all wells, with no apparent decrease in binding over the course of 24h.

Once binding of plasmid was successfully demonstrated, binding of smaller siRNA oligonucleotides were examined in a similar manner. Based on the time-course condensation studies (Figure 3.7), a 40 minute binding reaction was chosen for siRNA-cESG condensation. The upper limit of siRNA binding was visualized by agarose gel electrophoresis. Demonstrated in Figure 3.8, formulations reacted with 6000, 1200 and 500 siRNA molecules per cESG particle had excess free siRNA that was able to migrate into the agarose gel. At lower siRNA/cESG formulations complete association was demonstrated. Zeta potential analysis of these complexes
revealed that formulations demonstrating complete binding by electrophoresis (200 or fewer siRNA molecules /cESG) also had positive zeta potentials (Table 3.3). In ratios with higher concentrations of siRNA, the overall zeta potential was negative. This indicates that an excess anion concentration effectively neutralized the charge of cESG, demonstrating that while siRNA may be condensed to the particle there was still unbound siRNA, which migrated through the gel.

\[
\begin{array}{cccccccc}
\text{Calculated siRNA per cESG} & 6000 & 1200 & 500 & 200 & 40 & 20 & 8 & 4 \\
\end{array}
\]

Figure 3.8. Gel shift assay of cESG-siRNA complexes confirms condensation of siRNA to cESG. Migration of free siRNA (arrow) was observed in formulations with a higher numbers of siRNA per cESG. Impeded migration (asterisks), indicative of siRNA condensation, is observed in wells containing 200 siRNA per particle or less.

Table 3.3. Zeta potential of cESG-siRNA complexes formed at various ratios cESG to siRNA.\(^a\)

<table>
<thead>
<tr>
<th>Calculated siRNA per cESG</th>
<th>Zeta-Potential ± Standard Deviation (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>2.53 ± 17.8</td>
</tr>
<tr>
<td>8</td>
<td>2.62 ± 17.9</td>
</tr>
<tr>
<td>20</td>
<td>2.07 ± 17.7</td>
</tr>
<tr>
<td>40</td>
<td>1.57 ± 18.0</td>
</tr>
<tr>
<td>200</td>
<td>3.41 ± 18.0</td>
</tr>
<tr>
<td>500</td>
<td>-0.79 ± 17.6</td>
</tr>
<tr>
<td>1200</td>
<td>-5.78 ± 17.9</td>
</tr>
<tr>
<td>6000</td>
<td>-5.99 ± 16.9</td>
</tr>
</tbody>
</table>

\(^a\)siRNA concentration was held constant for all reactions. Measurements were conducted in 1mM SODium phosphate buffer pH 7.5. Results were averaged over three measurements, each consisting of 60 scans.
3.4.2  *cESG-pEGFP-N1 Transfection Efficiency and Toxicity*

Following successful electrostatic condensation of nucleic acids with cESG we sought to determine if cESG could serve as a plasmid delivery vector to ES-2 OCCC cells. Cells were treated with 0.32 nM plasmid, which was determined to induce bright GFP expression in as little as 24 hours using the commercial liposomal transfection reagent Lipofectamine2000. A 15 cESG per plasmid ratio was chosen, as we anticipated that more cESG would be more favorable to mediate cellular entry. Compared to the positive control Lipofectamine2000-plasmid transfection, no GFP expression could be seen in the cESG-plasmid treated cells after 24 h (Figure 3.9). Fluorescence was monitored again at 48 and 72 hours, with no fluorescence observed in the cESG-plasmid treated cells.

Two possible explanations for this unsuccessful transfection of the pEGFP-N1 plasmid by cESG may be insufficient cellular uptake or deficient release of the plasmid from cESG to enable transcription and translation of the GFP protein. Upon DLS analysis of the cESG-plasmid formulation used for treatment (15:1 ratio cESG:plasmid), the complex had a hydrodynamic radius of 374 nm ± 51 nm. This is tenfold higher than the diameter of cESG alone. This large size indicates that the complex may not have enough cESG particles to mediate entry into the cell. Due to the large size disparity between vector and gene it is likely that cESG were decorating the plasmid surface rather than acting as a carrier for the plasmid (Figure 3.10).
Figure 3.9. Expression of GFP due to plasmid pEGFP-B1 transfection. After 24h treatment of ES-2 clear cell carcinoma with lipofectamine2000-pEGFP-N1 transfection reagent GFP is clearly expressed and visible in the cytosol of ES-2 cells, however in cESG-plasmid there was no apparent expression.
Figure 3.10. Theoretical diagram of cESG (blue) decoration of the pEGFP-N1 surface (green) due to large size disparity between the particles. Figure to scale.

Another mechanism for failed transfection would be successful uptake of the particle but insufficient release of the plasmid from the particle. In the event that cESG-plasmid successfully traversed the membrane, the plasmid would need to enter the nucleus and dissociate from cESG in order for expression to occur. As this experiment did not include a mechanism to visualize cellular entry, we cannot comment further as to which step was unsuccessful. In future studies, labeling of the particle itself will allow for better visualization of cellular uptake of the complex. While these results were discouraging, we proceeded to test the use of cESG as a delivery vector for siRNA delivery, with the hypothesis that a smaller nucleotide molecule may be more efficiently incorporated into or onto cESG conjugated (Figure 3.11). As such, the siRNA of interest is composed of 19 base pairs, compared to the 4700 base pairs of pEGFP-N1.
The failure of cESG as a plasmid delivery system could not be attributed to toxic effects. In fact, the Lipofectamine2000 control demonstrated higher toxicity than our cESG formulation. When optimizing plasmid delivery using the reagent, according to the manufacturer’s directions, a range of particle concentrations were tested. The two Lipofectamine2000 concentrations that showed the most success, at constant plasmid concentration, were 1uL and 0.5uL per well (concentrations are unknowns as the formulation is proprietary). However, these exhibited significant toxicity (p<0.001) compared to the control treatment with no plasmid, decreasing viability to 48% and 67% respectively (Figure 3.12). Conversely, no statistically significant increases in toxicity were observed with increasing plasmid concentrations conjugated to the cESG between ESG, cESG, and cESG-plasmid treated ES-2 cells at all dosages tested (Figure 3.13). This suggests that cESG may provide a less toxic alternative to traditional liposomal transfection reagents, if plasmid conjugation and delivery can be effectively achieved with further optimization.
Figure 3.12. Lipofectamine2000 condensation with plasmid increases toxicity. MTT viability assay of optimization of Lipofectamine2000 concentration at four ratios reagent:plasmid shows no toxicity to ES-2 cells in the absence of plasmid. After plasmid condensation, the complex becomes toxic at larger DNA concentrations. (Data reported as µL. Lipofectamine2000. n=3 wells, One-way ANOVA with Tukey’s post-test *p<0.01 **p<0.001)

Figure 3.13. cESG modification and plasmid condensation does not significantly impact toxicity. MTT viability assay of ES2 cells treated for 72 hours in growth media with either ESG, cESG, or cESG-plasmid has no statistically significant difference in toxicity (n=3 wells, p<0.05, One-way ANOVA with Tukey’s post-test)
3.4.3 cESG-siRNA: Uptake, Transfection Efficiency, and Toxicity

In light of the inability of cESG to serve as a delivery vector for large plasmid DNA delivery, we began investigating its ability to act as a carrier for siRNA. Here, the target of the siRNA-mediated expression knockdown was the mitochondrial antioxidant enzyme SOD2. Again, ES-2 cells were chosen due to our previous work demonstrating high expression of endogenous SOD2 in this OCCC cell line, and the efficiency of the specific siRNA oligonucleotide used in efficiently knocking down expression of this protein. The sequence used was previously validated (5’-CAACAGGCCUUAUCCACU-3’), exhibiting excellent knockdown of SOD2 in a variety of cell lines (unpublished work, Hempel lab). In order to easily visualize the uptake of the complex a 5’ fluorescein dye (6-FAM), which has an excitation/emission spectrum of 495/520nm, was added to the sequence by the manufacturer (Dharmacon, ThermoFisher). Based on previous experiments, 100 nM siRNA concentration for treatment (approximately 7.5x10^8 siRNA per cell) was chosen, which had shown optimal SOD2 expression knockdown using the commercially available reagent Lipofectamine RNAiMax. This is also on par with concentrations used in other nanoparticle delivery formulations. For initial screening, molar ratios of siRNA/cESG, which showed complete siRNA binding in the gel shift assay (Figure 3.8) were used to treat ES-2 OCCC, ranging from one to 200 siRNA per particle. After a 48 hour incubation with the treatment, cells were fluorescently imaged in PBS to observe if there was any uptake of the complex. There was successful intracellular accumulation of the complex in formulations with 20 or fewer siRNA per particle, with the best uptake in the complexes with 1 or 4 siRNA per particle (Figure 3.14). Upon western blotting analysis of SOD2 expression, an inverse relationship between uptake and SOD2 expression was seen (Figure 3.15).
Figure 3.14. Uptake of fluorescently labeled cESG-siRNA by ES-2 ovarian clear cell carcinoma cells increases as siRNA per cESG particle decreases. Fluorescence images (right) and overlay bright field images (left) of ES-2 cells incubated for 72hrs with cESG-siRNA complexes of various formulations were visualized by microscopy. Increased uptake was seen in complexes with fewer siRNA per cESG particle. Scale bar 200 µm.
Figure 3.15. Uptake of cESG-siRNA correlates with SOD2 protein expression knockdown. A) Uptake of cESG-siRNA was quantified as the percentage of cells which display fluorescence signals (at least 50 cells/image, n=3 images, analyzed and counted in three independent experiments, data represent mean +/- SEM). B) SOD2 protein expression was determined by western blotting 72 hrs after treatment with cESG-siRNA complexes. Expression was quantified by densitometry and normalization to the loading control β-actin and untreated control cells (n=3). C) Representative western blot
It is important to note that since siRNA concentration was held constant in all wells, concentration of cESG were varied to alter the ratio between siRNA and particle. As the number of siRNA per particle decreased, there was a higher concentration of cESG in the well. For example, while there was the same concentration of siRNA, cells in figure 3.15 pane 3 (4siRNA/particle) had fivefold more particles than in fig 3.15 pane 1 (20siRNA/cESG). Therefore, the increased uptake and subsequent enhanced efficiency of SOD2 knockdown is likely due to not only effects from an optimal complex composition (siRNA per particle), but also due to exposure to a higher concentration of cESG.

Subsequently, the formulation of 4 siRNA/cESG, which showed significant SOD2 knockdown, was chosen to compare transfection and knockdown efficiency against the commercial reagent Lipofectamine RNAiMax. Cells were treated with the formulations for 72 hours and either harvested immediately (3 day) or incubated an additional 72 hour recovery period in growth media prior to harvesting for western blot analysis (6 day). After 3 days of treatment with the complex, cESG-siRNA treatment reduced SOD2 expression to 35% of that seen in the untreated control (Figure 3.16). Lipofectamine RNAiMax at this time point, elicited near complete knockdown of SOD2. However, after an additional 3 days of recovery in media, the commercial compound caused extensive cellular toxicity inhibiting our ability to assess SOD2 expression. In the cESG-siRNA treatment, cells did not suffer this toxicity, and maintained and further decreased SOD2 expression to 16% of control treated cells.
Figure 3.16. A) cESG-siRNA facilitates sustained protein expression knockdown. ES-2 cells were treated either with growth media (untreated control), cESG, cESG-siRNA-scramble, or cESG-siRNA-SOD2 for 72 hrs (3 Day) or allowed to recover in growth media for an additional 72hrs (6 Day) prior to assessment of SOD2 protein expression by western blotting. B) Western blot data was quantified by densitometric analysis and normalized to expression of the loading control β-Actin and expressed relative to SOD2 expression in untreated control cells. (n=3 independent experiments, mean ±SEM, * p<0.05, One-Way ANOVA with Tukey's post-test.)

Based on the observation that cESG-siRNA was less toxic than Lipofectamine RNAiMax, the cytotoxicity of the compounds at the concentrations used were probed using an MTT assay. Unmodified ESG, cESG, and cESG-siRNA were all significantly less toxic (p<0.05) than Lipofectamine RNAiMAX after 3 or 6 days of treatment (Figure 3.17). This suggests that the carbohydrate nanoparticle may serve as an effective delivery vehicle with less cytotoxicity than its liposomal counterparts.
An interesting effect was observed throughout treatments in which exposure to cESG alone increased SOD2 expression (Figure 3.16). This phenomena may be due to the complex relationship between sugar metabolism and oxidative stress. A synergy of SOD2 and glycolysis has been demonstrated in which increased SOD2 levels in cancer cells leads to \( \text{H}_2\text{O}_2 \) activation of AMP-kinase, switching metabolism to glycolysis.\(^{82}\) Unfortunately a relationship between the impact of glycolysis on SOD2 production is not discussed in the literature. One possible explanation of this trend is that cESG treated cells have an increased source of glucose increasing the rate of ES-2 cell glycolysis and Acetyl-CoA entering into the TCA cycle. The subsequent increased production of NADH provides necessary reducing equivalents for mitochondrial respiration and the potential increase in electron leakage at the level of electron chain complexes in mitochondria may yield higher superoxide production. The increase in SOD2 expression may be a compensatory mechanism to mitigate this increased ROS production. Alternatively, as we saw an increase in cell

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**Figure 3.17.** cESG-siRNA displays significantly less cytotoxicity than Lipofectamine RNAiMAX. Effects of cESG, cESG-siRNA and Lipofectamine RNAiMAX-siRNA complexes on ES-2 cell viability were assessed using a MTT Viability Assay. Treatments were conducted for 72 hrs (3 Day) or with an additional 72 hr recovery period in growth media (6 Day). MTT absorption values were normalized to control untreated cells. Representative data expressed as mean ± SEM; n=3 wells. *p<0.05 Controls compared to treatment with a Student’s T-Tests
viability with cESG, there may be an overall increase in protein biosynthesis relative to the loading control standard.

In order to further explore the transfection efficiency of cESG-siRNA, we explored the efficacy of the complex under a variety of conditions. First, the impact of treatment time was investigated. Previously, ES-2 cells were treated for 72 hours with the cESG-siRNA complex. In this study, ES-2 cells were treated for 24 h (at the same 100 nM concentration siRNA with the 4:1 molar ratio siRNA/cESG formulation). Treatment was followed by an incubation in growth media for an additional five days to allow for SOD2 knockdown to occur prior to analysis. When comparing this one day treatment to the previously described three day cESG-siRNA treatment, there was equal efficacy, expressing 26.0% and 26.4% of SOD2 respectively (compared to treatment with cESG alone) (Figure 3.18). This indicates that treatment periods with the particle may be decreased to one day without impacting efficacy, as long as there is an adequate period for protein knockdown to occur. In unpublished observations from the Hempel lab, it has been noted that the SOD2 protein is quite stable, requiring at least 72 hours for the endogenous protein to be degraded before the effects of siRNA mediated knockdown of newly synthesized mRNA can be observed.
Figure 3.18. A) cESG-siRNA is equally effective if incubated for 1 day or 3 days. ES-2 cells were treated either with cESG, cESG-siRNA-scramble, or cESG-siRNA-SOD2 for 24 or 72 hrs and then incubated in growth media until 6 days after initiating treatment. SOD2 protein expression was determined by western blotting. B) Quantification by densitometric analysis and normalized to expression of the loading control β-Actin and expressed relative to SOD2 expression in cESG treated control cells. (n=2 or n=3 independent experiments, mean ±SEM)

All complexes used for treatment up to this point were condensed immediately before treatment. In order to determine the effect of storage of cESG-siRNA on efficacy, a cESG-siRNA conjugation reaction was performed and particles stored in solution at 4°C for 3-4 weeks. Following this period, the formulations were diluted in fresh media and used to treat ES-2 cells for
72 hours prior to harvesting. This experiment revealed that when solutions were stored prior to use, the knockdown of SOD2 was less efficient (Figure 3.19). The stored solution was able to elicit only a 28% reduction in expression compared to the freshly made solutions which yielded a 72% decrease in SOD2 expression. This might be due to a variety of factors, including degradation of the siRNA itself at 4°C, as suggested storage conditions for nucleic acids is -20°C, or the siRNA becoming dissociated from the particle, resulting in a lower concentration of bound siRNA per particle being transfected.

Figure 3.19. A) cESG-siRNA is less effective after storage ES-2 cells were treated either with freshly conjugated cESG, cESG-siRNA-scramble, or cESG-siRNA-SOD2 (“fresh”) or the same complexes stored at 4°C for 4 weeks (“stored”) for 72 hrs prior to assessment of SOD2 protein expression by western blotting. B) Western blot data was quantified by densitometric analysis and normalized to expression of the loading control β-Actin and expressed relative to SOD2 expression in cESG treated control cells. (n=2 independent experiments, mean ±SEM)
As efficient knockdown has been demonstrated in ES-2 OCCC cells, we wanted to determine if the particle had similar efficacy in other cell types. Nose007 cells, normal ovarian surface epithelial cells, which are not cancerous, along with Ovca420 ovarian serous adenocarcinoma were chosen for comparison. SOD2 knockdown was not as efficient in the noncancerous Nose007, only decreasing expression by 20% (Figure 3.20). This could be due in part to high baseline levels of SOD2 in this cell type. In Ovca420 serous ovarian cancer, there was no evidence of SOD2 knockdown (Figure 3.20). This could be due to differences in uptake or degradative behavior within the cell.

Figure 3.20. A) cESG-siRNA efficacy varies by cell type. ES-2, Nose007 or Ovca420 cells were treated either with cESG, cESG-siRNA-scramble, or cESG-siRNA-SOD2 for 72 hrs prior to assessment of SOD2 protein expression by western blotting. B) Western blot data was quantified by densitometric analysis and normalized to expression of the loading control β-Actin and expressed relative to SOD2 expression in cESG treated control cells. (n=2 independent experiments, mean ±SEM)
3.5 Conclusions

cESG synthesized by a one-pot GTMA modification with quats was tested as a delivery vector for both plasmid and siRNA. cESG had a high enough zeta potential to electrostatically condense both nucleic acid systems. However, experiments utilizing the cESG-pEGFP-N1 plasmid complex did not lead to successful transfection in an ES-2 OCCC cell culture model. This may be attributed to the large size disparity between the plasmid (500 nm scale) and the cESG (30 nm scale). As such, cESG particles likely decorated the surface of the plasmid and were unable to mediate cellular entry. Based upon this study, cESG does not seem to be a feasible delivery vector for plasmids.

The cESG-siRNA delivery system exhibited binding of cESG at ratios with fewer siRNA per particle, in which the final complex retained its positive zeta potential. Uptake, visualized by a FAM dye on the complex, indicated successful cellular internalization of the complexes with a low number of siRNA per particle. SOD2 knockdown, assessed by western blot analysis, showed a direct correlation between knockdown and uptake, with increased knockdown seen in formulations with increased uptake (1 and 4 siRNA/cESG). This is attributed to both the formulation characteristics and the concentration of cESG used for treatment. As siRNA concentration was held constant, higher cESG concentrations were present in these complexes. In a comparison against the commercial transfection reagent Lipofectamine RNAiMax, cESG-siRNA was less efficient after a three day incubation, resulting in knock-down to 35% SOD2 protein expression compared to controls, while the commercial reagent yielded almost complete loss of SOD2 expression. Yet, after an additional 3 day recovery period in media, Lipofectamine RNAiMax was cytotoxic to cells whereas cESG-siRNA transfected cells remained viable, with a
sustained and even increased knockdown to 16% SOD2 protein expression, indicating our complex is more viable for \textit{in vivo} delivery.

cESG-siRNA treatment of ES-2 cells for 24 hours followed by an incubation in growth media for 5 days, showed identical knockdown behavior as the treatment for 3 days, indicating feasibility of shorter incubation periods. However, storage of the cESG-siRNA complex resulted in substantially decreased efficacy, calling into question the long term stability of the formulation. In the next chapter, in order to further investigate how the complex behaves \textit{in vitro}, we probed the uptake of the complex in ovarian cancer and normal epithelial cells, elucidating the mechanism of uptake, as well as investigate the enzymatic breakdown of the particle from a chemical standpoint.

3.6 References


CHAPTER 4.

CHARACTERIZATION OF cESG

4.1 Introduction

Cationically modified cESG has served as a non-toxic delivery vector for siRNA. While efficient at knocking down protein expression in ES-2 OCCE, the complex could not mediate decreases in protein expression in Ovca420 serous ovarian adenocarcinoma and demonstrated limited response in Nose007 normal ovarian surface epithelial cells. This prompted further characterization of the interactions between cells and the cESG complex. To explain the difference in efficacy, we compared the cellular uptake kinetics of cESG in the three different cell lines and interrogated potential cellular uptake mechanisms. In addition, cellular toxicity under varying conditions, and enzymatic breakdown of cESG was probed.

4.1.1 Cellular Uptake Mechanisms

A significant barrier to therapeutic delivery is passage across the plasma membrane. Once cargo, in this case a nanoscale therapeutic, reaches the cell it interacts with the membrane, undergoing recognition. This recognition may occur by receptor-specific binding or due to other material based factors such as charge and chemical composition. While there is still much to be learned about nanoparticle uptake, internalization of cargo is classified under 2 major routes, endocytic or non-endocytic uptake (Figure 4.1). Non-endocytic entry into the cell is less common and less investigated than endocytic pathways. Energy independent uptake may occur through the
lipids fusing to the cellular membrane or by the formation of holes in the cell membrane, in which particles ranging from 1-22 nm have been able to deform the lipid bilayer and obtain entry. This process is sensitive to hydrophobicity, charge, and surface ligands that can impart stress for pore formation or membrane destabilization.\(^1\) We will focus on endocytic uptake, the major mechanism of nanoparticle uptake, which is further subdivided into phagocytosis, pinocytosis, and receptor mediated endocytosis (Figure 4.2).

Figure 4.1. Endocytic and Non-Endocytic cellular uptake mechanisms
Figure 4.2. Endocytic uptake mechanisms of mammalian cells with intracellular trafficking to the lysosome. Clathrin mediated endocytosed and pinocytosed cargo are exposed to acidic pH whereas caveosomes are neutral pH.

In phagocytic uptake, solid particles are engulfed by the cell in order to remove senescent cells or foreign organisms. This mechanism serves as a defensive immune response in cells such as phagocytes, fibroblasts, and natural killer cells. The formation of large phagosome vesicles of
1 μm or larger, may be triggered by receptor ligands or soluble factors that indicate the presence of a foreign agent.\textsuperscript{3,4} Frequently, a foreign particle may be bound by opsonins in the circulatory system, including antibodies, collagen I, fibronectin, laminin, and the complement system. These opsonins are recognized by receptors on the surface of a macrophage, which in turn triggers phagocytosis. The most integral receptors for phagocytosis are the Fc receptor family, which recognize IgG antibodies, complement receptors (CR1, CR3, CR4) and the integrin α5β1.\textsuperscript{5} Other receptors that may also trigger phagocytosis include mannose and scavenger receptors.\textsuperscript{4,6} For nanoparticle therapies, the goal is often to avoid phagocytic uptake by macrophages during systemic administration, which may prematurely clear therapies from the body. Strategies to decrease opsonin binding include altering size, zeta potential, shape, or surface decoration with PEG.\textsuperscript{7} The binding of nanoparticles by proteins within the blood, forming a protein corona, also significantly impacts phagocytic response and can both suppress and stimulate an immune response.\textsuperscript{8}

Pinocytosis is a mechanism wherein fluid is taken up nonspecifically by the cell. Unlike phagocytosis, pinocytosis is triggered by the cellular environment rather than cargo. This process is signal dependent and may be initiated by the presence of a variety of factors such as colony-stimulating factor-1 (CSF-1), epidermal or platelet-derived growth factor (EGF) or tumor promoting factors.\textsuperscript{9-11} In macropinocytosis, actin-rich membrane protrusions form in response to these factors, eventually collapsing and fusing with the plasma membrane.\textsuperscript{6} These uncoated vesicles range from 500 nm to 5 μm in size and intracellularly fuse with the lysosome to release its cargo.\textsuperscript{4,12} In contrast, micropinocytosis forms much smaller 100 nm vesicles which may be either actin-dependent or and actin-independent (microtubule-dependent) processes.\textsuperscript{13-15}
The most common receptor mediated endocytosis pathways are clathrin- and caveolin-mediated endocytosis. In caveolin-mediated endocytosis, caveosome vesicles form from the invagination of a cholesterol sphingolipid, and are flask shaped with a base diameter of 50-80 nm and a neck of 10-50 nm.\(^{16}\) As with other endocytosis mechanisms, this receptor mediated process may also be triggered by signaling ligands like epidermal growth factor and insulin.\(^{17-19}\) Actin polymerization assists in the internalization of the caveosome, which is GTPase dynamin dependent.\(^{20}\) Unlike the endosome, the caveosome is not acidic and may allow for delivery to the Golgi or endoplasmic reticulum rather than lysosomal degradation.\(^{21}\) This uptake method is most common in endothelial cells and smooth muscle.\(^{3,4}\)

In clathrin mediated endocytosis (CME), the ordering of clathrins on the cytosolic membrane surface deforms the membrane, creating a coated pit. Clathrin rearrangement continues until the pit is invaginated, at which point the GTPase dynamin induces fission of a 100-150 nm vesicle.\(^{22}\) The size of this vesicle can vary based on cellular species as well as the size of the cargo, with an upper limit of approximately 200 nm.\(^ {23}\) Clathrin itself does not bind to the plasma membrane, but rather is recruited by adaptor (AP2) and accessory proteins (AP180, epsin) during budding. AP2 is the major hub for interactions in CME, acting specifically at the plasma membrane binding both transmembrane receptors and cargo (via accessory adaptor proteins).\(^ {23}\) This vesicle is intracellularly trafficked to the lysosome via the late endosome or recycled to the plasma membrane.\(^ {24}\) CME is the major mechanism for recycling of surface proteins within the cell and uptake for sub-100 nm particles.\(^ {6,23}\) Cationic nanoparticles are also commonly taken up through this mechanism, which may be attributed to the electrostatic interaction with the negative cell membrane.\(^ {4}\) Surface charge may have a large impact on uptake mechanism. For example, in a comparative study, cationic PLA nanoparticles efficiently underwent clathrin mediate endocytosis,
whereas their negatively charged analogs had poor internalization via a clathrin-independent mechanism.\textsuperscript{25} Due to its charge modification and size, cESG would be an excellent candidate for uptake \textit{via} this mechanism. In the work described below we interrogate potential uptake mechanisms of cESG in ovarian cancer cell line models.

\subsection*{4.1.2 Enzymes in Carbohydrate Breakdown}

Once cESG-siRNA has entered the cell, one possible mechanism for the release of siRNA from cESG is the partial breakdown of the carbohydrate chains of the dendrimer by enzymes responsible for glycogen metabolism. \textit{In vivo} several different enzymes are responsible for cytosolic glycogen breakdown. Glycogen phosphorylase (GP) is responsible for hydrolysis of $\alpha(1\rightarrow4)$ bonds, releasing glucose-1-phosphate from the non-reducing end of carbohydrates, the rate limiting step of glycogen breakdown. There are three different isoforms; liver (PYGL), muscle (PYGM) and brain (PYGB) which are present in a variety of tissue types. When GP is four glucose units from an $\alpha(1\rightarrow6)$ branching point, glycogen debranching enzyme (AGL) transfers three glucose units to the end of another glucose chain and hydrolyzes the $\alpha(1\rightarrow6)$ linkage.\textsuperscript{26} PYGL overexpression may be induced in cancer cells by prolonged exposure to the hypoxic tumor microenvironment. This increase of glycogen metabolism in cancer is largely compensation for decreased nutrients in the tumor environment.\textsuperscript{27} However, recent studies suggest that PYGL overexpression may help prevent apoptosis and reduce stress-response\textsuperscript{28} as well as regulate the activity of AMP-activated protein kinase, an important intracellular homeostasis regulator.\textsuperscript{29}

This is of interest to our work, as chemical modifications may alter the enzymatic breakdown of ESG and cESG within cells. To further analyze the degradation mechanisms of our
particle, we focused on two enzymes which are typical in carbohydrate chemistry for the characterization of oligosaccharides, pullulanase and alpha amylase (Figure 4.3).

![Enzymatic degradation of glycogen](image)

Figure 4.3. Enzymatic degradation of glycogen. α-amylase degrades α(1→4) linkages (red) at least 3 glucose residues from an α(1→6) branch (blue). Pullulanase degrades α(1→6) linkages separated by at least 3 glucose residues.

Pullulanase, an enzyme originally extracted from *Aerobacter aerogenes* in 1961 was so named as it fully degrades the sugar pullulan, which is composed of α-maltotriose trisaccharide units joined by α (1→6) bonds. Pullulanase functions by hydrolyzing the α-1,6 bonds in an “endo” fashion, in which it is not directionally limited to the reducing or non-reducing end. This releases sugars of various lengths bound by α-1,4 linkages. However, pullulanase can only hydrolyze an α-1,6 linkage where the chains have at least two glucose residues. While this may fully degrade pullulan, it has decreased activity in carbohydrates with higher α-1,6 branching densities, such as glycogen or amylopectin. As such, both naturally occurring phytoglycogen and enzymatically synthesized glycogens are resistant to pullulanase, which only degrades exterior linkages.
on this resistance we anticipated that pullulanase would have limited efficacy at degrading cESG, due to its high level of carbohydrate branching and density. However, if a change in degradation behavior was observed, this would indicate alterations in structure. Another enzyme used in carbohydrate analysis which cleaves α-1,6 bonds is isoamylase. This can more fully hydrolyze starches, resulting in linear α-1,4 bonded oligosaccharides. Also an endo-acting enzyme, isoamylase active sites must bind to both chains involved in a α-1,6 bond in order to be catalytically active. Thus more complex three-dimensional structures, such as helices and crystalline regions, may prevent hydrolysis by isoamylase.

For the specific degradation of α-1,4 glycosidic bonds, α-amylase may be used. This enzyme hydrolyzes linkages that are at least 3 to 4 glucose residues from an α-1,6 branching locus. This leads to varying rates of starch digestion, influenced by a variety of structural factors at both the macro scale and micro (granule) scale. This is most likely due to the steric interaction of the enzyme with the carbohydrate binding site. In order for hydrolysis to occur, α-amylase must adsorb onto the surface of the substrate. This adsorption is highly dependent upon conformation such as surface area, density of chains, and exposed amorphous regions of the sugar. A higher catalytic efficiency is observed in amorphous rather than crystalline samples, as well as granules with smaller diameter (higher surface area per unit weight). It is important to note that in both phytoglycogen and enzymatically synthesized glycogen, the interior chain lengths are on the order of 3-4 residues, which would not allow for complete degradation of the inner core. In vivo α-amylase is a major enzyme in the upper gastrointestinal tract responsible for carbohydrate digestion, allowing for glucose absorption into the blood.

In comparing the α-amylase hydrolysis profiles of enzymatically synthesized glycogen and natural phytoglycogen, Takata et al., discovered that phytoglycogen is broken up into several α-
macrodextrin cores dispersed throughout the particle whereas ESG has one large α-macrodextrin core (Figure 4.4). This difference is attributed to the multiple synthesis-degradation events in vivo, which are not present in vitro. In ESG, the particle exterior has a more regular distribution of α-1,6 bonds, separated by at least 3 glucose residues, than observed in phytoglycogen. As such the products of α-amylase degradation of ESG were small linear chains. In natural glycogen, the irregular distribution of α-1,6 bonds leads to both linear products and branched oligosaccharides, with smaller macrodextrin cores. Through enzymatic studies with α-amylase, we probed whether cESG had a similar degradation profile.

Figure 4.4. Alpha amylase degrades phytoglycogen (A) and ESG (B) into different components. Phytoglycogen is degraded into several macrodextrins and branched oligosaccharides whereas ESG is degraded into one large macrodextrin core and small saccharide units. Figure reproduced from Takata et al.,\textsuperscript{37} used with permission from Elsevier.

4.2 Methods

4.2.1 Materials

McCoy’s 5A media, RPMI media, and Trypsin EDTA 1x were from ATCC. Hyclone fetal bovine serum was from GE Healthcare. Dulbecco’s phosphate buffer saline 1x was from
FisherScientific. Cell experiments were performed using sterile, nuclease free water (Qiagen). All other reagents were purchased from Sigma Aldrich unless otherwise specified.

4.2.2 *cESG-Lucifer Yellow Reaction*

For visualization of cESG uptake into cells, 4.2 mM stock Lucifer yellow dipotassium salt (LY) was added at a 1:1 volume ratio to cESG, that was dissolved in 20 mM sodium phosphate buffer pH 7 at a concentration of 5 mg/mL (7.3:1 molar ratio AGU:LY). Reactions were stirred overnight at room temperature, protected from light. Reactions were dialyzed against 4 L of 1 mM sodium chloride in 10k MWCO regenerated cellulose dialysis tubing, with three volume changes. The cESG-LY product was filter sterilized using a 0.45 µm PES filter.

4.2.3 *Cell Culture*

Cell culture was performed as previously described (3.3.6)

4.2.4 *cESG-LY Uptake Timecourse*

ES-2, Nose007, or Ovca420 cells were plated either on glass coverslips in a 6-well plate or MatTek glass bottom dishes at a density of 80,000 cells per well. The following day cells were treated in growth media with 7.2nM cESG:LY or cESG-siRNA-SOD2-08FAM. Treatments were removed at a variety of time points (30min, 2h, 4h and 24h) and imaged in warm PBS using an AMG EVOS® microscope with LED lightsource and GFP filtercube, using a 20x and 100x oil immersion objective. Following live cell imaging, selected samples were fluorescently labeled for DNA and Actin. Nucleic acids were visualized using Hoescht stain (5 µg/mL in PBS) by incubating cells for 5-10 minutes, followed by a 10 minute PBS wash. F-actin fibers were stained with AlexaFluor 488- phalloidin for 20 minutes at room temperature, followed by two 5 minute PBS rinses. Cells were fixed in 4% formaldehyde in PBS for 10 minutes followed by three 5-
minute PBS washings. Cells were imaged using a 100x oil immersion objective on an EVOS® microscope (AMG) with an LED light source and either a GFP, Texas Red, or DAPI dye filtercube.

4.2.5 cESG-LY Mechanistic Inhibitor Studies

ES-2 and Nose007 cells were plated either on glass coverslips in a 6-well plate or MatTek glass bottom dishes at a density of 80,000 cells per well. The following day cells were pretreated with serum free media containing sodium azide (50 mM, general active uptake inhibitor)\(^{38-41}\), chlorpromazine (10 µg/mL, clathrin mediated endocytosis inhibitor)\(^{42-44}\), indomethacin (400 µM, caveolin mediated endocytosis inhibitor)\(^{45-47}\), or media alone. Cells were incubated for a 1 hour pretreatment. After this period, cells were treated with 7.2 nM cESG-LY in fresh serum free media containing inhibitor. After 6 hours, media was aspirated and replaced with 37 °C PBS for imaging on an EVOS® scope (AMG), as described above.

4.2.6 cESG-LY Uptake in Spheroids

To test the ability of cESG to penetrate freely suspended cellular spheroid aggregates, a common feature of metastatic ovarian cancer, ES-2 cells were plated in a 96-well ultra-low attachment (ULA) plate at a density of 4,000 cells per well and incubated overnight to form spheroids. The following day, media was replaced with 7.2 nM cESG-LY or cESG-siRNA-SOD2-FAM in growth media. Cells were incubated for 72 hours. Media was removed and replaced with 37 °C PBS for fluorescent imaging on an EVOS® scope (AMG). For confocal microscopy, spheroids were placed on a glass slide and allowed to dry immediately before imaging on a Leica TCS SP5 II confocal microscope, with 428 nm excitation and 520–560 nm emission range. Image acquisition and processing was performed using Leica Application Suite v2.1.2 software.
4.2.7 Uptake in Chorioallantoic Membrane (CAM) Tumor Model

The CAM method, modified from Deryugina et al., was used to interrogate uptake of cESG in a pseudo in vivo tumor model. cESG-siRNA-SOD2-FAM was conjugated immediately before inoculation of the CAM with tumor cells. Ten days post fertilization, a small opening was cut into the shell, the membrane dropped and each CAM inoculated with 500,000 ES-2 cells stably expressing scramble-shRNA-RFP in 65 μL of 1 mM sodium phosphate buffer containing either cESG alone (1.6 μM) or cESG-siRNA (1.6 μM cESG, 0.94 μM siRNA scramble or SOD2-FAM). RFP labeled cells were used to enable visualization of tumor cells on the CAM at completion of the experiment. Tumors were allowed to form for 7 days prior to sacrificing the chick embryo and harvesting tumors. Fluorescent microscopy of tumors on the CAM was conducted on an EVOS scope (AMG) with an LED light source and either a GFP, Texas Red, or DAPI filtercube. Tumors then dissected from the CAM,. Tumor mass was measured and volume measure with calipers.

4.2.8 Toxicity of ESG, cESG, and oxESG

To assess any cellular toxicity of our constructs, Nose007, ES-2 or Ovca420 cells were plated in a 96-well plate at a density of 4,000 cells per well and cell viability determined using an MTT assay. ESG, cESG, and oxESG were dissolved in 1 mM sodium phosphate buffer and filtered using a 0.2 μm PES filter. The following day, serial dilutions of particles were made in serum free or growth media (1000, 100, 10, 1, 0.1, 0.01 nM). Cells were incubated with 100 μL of solution overnight. The following day, an MTT viability assay was performed as previously described (3.3.8).

4.2.9 Enzymatic Breakdown Behavior of ESG and cESG

In vitro enzymatic assays were carried out with recombinant α-amylase and pullulanase to assess the degradation of the ESG. ESG or cESG was dissolved at 5 mg/mL in 20 mM sodium
phosphate buffer pH 5.7. Alpha amylase stock was added at a concentration of 300 units per gram substrate (U/g) and the reaction allowed to proceed overnight at room temperature. Change in size over the course of the reaction was measured by DLS in a Malvern Nanosizer in a quartz cuvette. A size scan was taken every 20 minutes, consisting of 10 averaged 10 second scans. Pullulanase degradation was monitored at a concentration of 200 U/g at 60 °C for 2 hours. A DLS size scan was taken every 4 minutes, consisting of 12 averaged 10 second scans.

4.2.10 Real-time polymerase chain reaction (qPCR)

To assess expression levels of glycogen breakdown enzymes in ovarian cancer cells used in our study, semi quantitative real time RT-PCR for the glycogen degradation enzymes PYGL and AGL was conducted. These experiments were carried out with the help of Dr. Dong Hui Shin in Dr. Nadine Hempel’s lab at the Penn State College of Medicine Department of Pharmacology. PCR primers were designed as described by Kondaveeti et al.\textsuperscript{49} RNA was extracted from ES-2, Nose007, and Ovca420 cells using an RNeasy Mini Kit (Qiagen) and complementary DNA (cDNA) was synthesized using 1 µg of RNA with iScript cDNA synthesis (Bio-Rad). Real Time PCR was carried out on CFX96 Real-Time PCR machine (Bio-Rad) using SYBR Green Supermix (Bio-Rad) and with the following primer pairs: Glycogen phosphorylase, liver (PYGL) forward: 5'-AATTCATGCTGCTGTGCAC-3'; Reverse 5’-AGGACCACCTTGAATGTCAATCC-3’ and Amylo-alpha-1,6-glucosidase, 4-alpha-glucanotransferase (AGL) forward: 5’-GGATGGGTAATGGAGATGA-3’; Reverse 5’- TAACACTGTCTCCCAAGCAGA-3’. Gene expression from each sample were normalized to β-actin and data expressed relative to mRNA levels of normal ovarian epithelial cells (Nose007).
4.2.11 *Statistical Analysis*

All data presented are representative of at least three independent experiments and expressed as mean ± SEM, unless otherwise noted. Statistical data analysis were performed using OriginPro Software v8.5. Student’s T-Tests or ANOVA with Tukey’s post test were performed as indicated. In the case of two-way ANOVA analysis, the input variables (i.e. dosage, treatment type, illumination time) are considered independent and the mean differences between groups are compared.

4.3 *Results and Discussion*

4.3.1 *Time-dependent Cellular Uptake of cESG-LY*

To further characterize the cellular fate of cESG, experiments were carried out to interrogate cellular uptake kinetics and mechanisms of the particle. For visualization of particle within cells, lucifer yellow dye (LY, 428/536 ex/em) was chosen. This non-cell permeable anionic dye binds cESG via a charge condensation, similar to the interaction of the cESG-siRNA complex. An overnight conjugation reaction was performed followed by dialysis against 1 mM sodium chloride, and sterile filtration. The resultant complex retained its size, 40.3 ± 2.5 nm, as well as a slightly positive zeta potential (3.6 ± 17.5 mV). Bound LY concentration after purification were determined by fluorescence measurements using a SpectraMax Paradigm plate reader, indicating there were an average of 660 LY bound per cESG particle.

The kinetics of cESG-LY complex uptake was investigated in three different cell lines: Nose007 normal ovarian surface epithelial cells, ES-2 ovarian clear cell carcinoma (OCCC), and Ovca420 ovarian serous adenocarcinoma. Experiments were conducted in growth media, in order to mimic the same conditions used for cESG-siRNA transfection experiments of Chapter 3. The
uptake behavior as a function of time was consistent in all cell types. No significant uptake was apparent after a 30 minute incubation with cESG-LY (Figure 4.5). At this short timepoint, cells repeatedly exhibited morphological changes, indicative of cellular stress. Two hours after treatment, cells returned to normal morphology with vesicle-localized uptake of cESG-LY, with a slight increase in uptake at the 4 hour mark. After an overnight incubation, a large increase in uptake was observed. This was marked by punctate staining throughout the cytoplasm, indicative of vesicular accumulation, with no fluorescence signals observed in areas of the nucleus. Corresponding 20x magnification images show the same trend in uptake in the whole cell population, with highest uptake observed at 24 hrs (Figure 4.6). Upon fluorescence intensity quantification, there was no statistically significant difference in uptake between cell types at the p=0.05 level (Figure 4.7). While an extensive time-dependent uptake study with FAM labeled cESG-siRNA was cost prohibitive, uptake with the complex was also visualized for comparison. After a six hour incubation, the cellular uptake profile of cESG-siRNA was highly similar to cESG-LY in both ES-2 and Nose007 cells. The particle was localized in punctate vesicles, located in the cytoplasm, with no apparent nuclear uptake (Figure 4.8).
Figure 4.5. cESG-LY uptake over time is similar in Nose007, ES-2, and Ovca420. No uptake is seen after 30 minutes with treatment, with increasing vesicle-localized uptake over time. 100x magnification. Scale bar 10 µm.
Figure 4.6. Percentage of cell population with cESG-LY uptake increases over time in Nose007, ES-2, and Ovca420 cells. 20x magnification. Scale bar 200 µm.
Figure 4.7 Quantification of percent cell population fluorescing as a function of incubation duration is not statistically different (p=0.05) in Nose007, ES-2 or Ovca420 cells. (Mean ± standard deviation, n=3 images, 20-60 cells per image. One-way ANOVA with Tukey’s post-test)

Figure 4.8. Uptake of cESG-siRNA-SOD2-FAM (green) after 6 hours is not localized in the nucleus in A) Nose007 and B) ES-2 cells. F-Actin co-stain (Phaloidin, red) and nuclear stain (Hoechst, blue). Scale bar 10 µm.
4.3.2 *Mechanisms of cESG-LY Cellular Uptake*

In order to further characterize the mechanism of cESG uptake, common uptake pathways were interrogated using pharmacological inhibition. Prior to cESG exposure, cells were pretreated for one hour with an inhibitor that specifically prevents uptake *via* the mechanisms outlined in Table 4.1. The cells were then incubated with cESG-LY for 6 hours in serum free media containing the inhibitor. In both ES-2 and Nose007 cells, inhibition of general energy dependent uptake using sodium azide shows that uptake of cESG requires ATP, and is most likely endocytic (Figure 4.9). Uptake in Ovca420 cells also indicates uptake is energy dependent, but does exhibit some energy-independent uptake. Inhibitors against clathrin and caveolin mediated endocytosis did not appear to reduce uptake in any cell type. However, quantification was not feasible using this imaging method. Preliminary studies investigating nystatin suggest the role of micropinocytosis in cESG uptake is minor. However further experiments need to be carried out to find optimal treatment doses and times to verify these findings.

Table 4.1. Uptake inhibitors with mechanism of action

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Uptake Subtype</th>
<th>Mechanism of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Azide</td>
<td>Endocytosis</td>
<td>ATPase inhibitor</td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>Clathrin Mediated Endocytosis</td>
<td>Dissociates clathrin from the plasma membrane</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>Caveolin Mediated Endocytosis</td>
<td>Prevents internalization of caveolae</td>
</tr>
<tr>
<td>Amiloride</td>
<td>Macropinocytosis</td>
<td>Inhibits sodium-proton exchange in plasma membrane</td>
</tr>
<tr>
<td>Nystatin</td>
<td>Micropinocytosis</td>
<td>Binds cholesterol in the plasma membrane</td>
</tr>
</tbody>
</table>
Figure 4.9. cESG-LY uptake is an energy-dependent process, but not clathrin- or caveolin-mediated endocytosis in Nose007, ES-2 cells, and Ovca420 cells. Cells pretreated for 1 h in serum free media with 50 mM sodium azide (energy dependent inhibitor), 10 µg/mL chlorpromazine (CME inhibitor) or 400 µM indomethacin (caveolin mediated endocytosis inhibitor) followed by 6 h treatment with cESG-LY and inhibitor. Scale bar 10 µm.
4.3.3 Ability of cESG to Penetrate Three-dimensional Cell Culture Models

Once the uptake of cESG had been characterized in traditional cell culture conditions, we sought to determine if the complex could infiltrate cells in more realistic microenvironments, including growth as 3D anchorage independent spheroids and \textit{in vivo} grown tumors. This is integral in determining whether cESG is a physiologically relevant delivery vector. In ovarian cancer, a common metastatic route is the dissemination via the peritoneal ascites fluid (transcoeleomic metastasis). Cells detach from the primary tumor and are suspended in ascites, often aggregating to form spheroids. Therefore, testing the uptake of therapeutics in non-vascularized spheroids is of clinical relevance. ES-2 OCCC readily form compact, uniform spheroids when plated in an ultra-low-attachment (ULA) coated plate. Following overnight spheroid formation in a 96-well ULA plate, spheroids were treated in growth media containing FAM labeled cESG-siRNA and incubated for 3 days. Lipofectamine RNAiMax-siRNA was used as a control to compare uptake efficiency to this liposomal vector. cESG-siRNA treated cells displayed strong fluorescence, indicating significant uptake at the spheroid exterior (Figure 4.10A). This fluorescent signal was far greater than in the commercial Lipofectamine RNAiMax transfected cells, which exhibit limited uptake (Figure 4.10B) This is not attributed to imaging artifacts as control cESG treated spheroids do not exhibit green fluorescence (Figure 4.10C). In order to determine if this uptake was confined to the surface of the spheroid or penetrated into the core, confocal microscopy was employed. Spheroids were dried on a glass slide immediately before imaging. An overlayed z-stack of cESG:LY fluorescence within the spheroid indicates that particle uptake occurs throughout the spheroid (Figure 4.11). However, some of this fluorescence may be attributed to changes in spheroid morphology upon drying. In future studies, confocal images in a gel matrix could more accurately quantify the extent of cESG penetration into the
spheroid. These data suggest that cESG may be advantageous over liposomal vectors in delivering cargo to three dimensional cell culture models.

Figure 4.10. Uptake in ES-2 OCCC spheroids of treatment for 72 hours in A) cESG-siRNA-SOD2-FAM B) Lipofectamine RNAiMax-siRNA-SOD2-FAM C) cESG alone. Images overlay of phase contrast and fluorescent images. Fluorescence intensity of cESG-siRNA treatment is statistically different from media control p<0.05. Scale bar 200 µm.

Figure 4.11. Confocal compiled z-stack of uptake of cESG-siRNA (A) occurs throughout the spheroid, not observed in cESG treatment alone (B). Scale bar 100 µm.
A final uptake study was conducted using the pseudo in vivo chorioallantoic membrane (CAM) chick egg model. In this model, tumor cells are seeded onto the CAM of a fertilized chicken egg, leading to tumor formation and vascularization by the membrane. Immediately prior to inoculation of the membrane, RFP expressing ES-2 cells were mixed with FAM labeled cESG-siRNA-SOD2. Following a one week incubation, tumors were harvested and fluorescently imaged. Lower 4x magnification images of a large portion of the tumor (Figure 4.12, left) indicate that there is strong green fluorescence from cESG-siRNA in the tumor that is not present on the surrounding membrane. Higher 10x magnification images of the tumor itself (Figure 4.12, right) clearly show the uptake of cESG-siRNA in individual cells within the tumor. As cESG-siRNA was inoculated with the cells prior to tumor formation, this model is more representative of uptake during tumor formation than penetration into an existing tumor. Treatment with cESG-siRNA did not impact tumor size in a statistically significant manner compared to control treated tumors, a preliminary indication that cESG-siRNA treatment did not induce toxicity (Figure 4.13).
Figure 4.12. cESG-siRNA-SOD2-FAM is taken up in individual tumor cells within an RFP expressing CAM tumor. 4x magnification of tumor and membrane (left) 10x magnification of tumor mass (right). Scale bar 400 µm.
4.3.4 Toxicity Dose Curves of ESG, cESG, and oxESG

Another important characteristic for consideration in the future implementation of ESG based particles for treatment is dose-limiting toxicity. In previous studies, cESG was used for treatment at concentrations of 15 nM or less. The toxicity of ESG, cESG, and oxESG at a wider range of dosages was investigated in three different ovarian-derived lines in both serum free and growth media. All ESG formulations (ESG, cESG and oxESG) were well tolerated up to 100 nM particle concentration. At 1 μM concentration oxESG was most toxic, with viability dropping to 20-40%, and 1 μM cESG resulting in a decrease to 60% viability. Regardless of particle concentration it was noted that ES-2 cells were most sensitive to serum deprivation (Figure 4.14E) exhibiting an overall 20% decrease in viability compared to treatments conducted in growth media (Figure 4.14B). These data suggest that ESG formulations do not induce toxicity in ovarian cancer cells up to 100 nM concentrations.
Figure 4.14. Dose curves of 24 hour treatment with ESG, cESG, or oxESG in growth media (left) and serum free media (right) in Nose007 (top), ES-2 (middle), or Ovca420 cells (bottom). Toxicity exhibited in all cells and treatment types at a 1000nM dosage. (Mean ± Standard Deviation, n=3 wells.)
4.3.5 **cESG Enzymatic Degradation**

One possible mechanism for intracellular siRNA release from the cESG particle is enzymatic degradation of the carbohydrate. Therefore we sought to characterize the degradation of cESG *ex vivo*. α-amylase, an endo enzyme which breaks α(1-4) bonds up to 3 glucose residues away from an α(1-6) branching point, can partially degrade ESG and cESG within 30 minutes of enzyme addition (Figure 4.15A). However, there was a statistically significant difference (p<0.05) in α-amylase degradation of ESG, which dropped to 63% of its original volume, and cESG, which retained 89% of its original mass (Figure 4.16). We attribute this difference in behavior to either steric limitations or charge interference induced by GTMA residues which may prevent binding of the active site of the enzyme. Natural phytoglycogen and ESG are resistant to degradation by the enzyme pullulanase, which hydrolyzes α(1-6) glycolytic bonds separated by at least 3 glucose units. cESG exhibited similar behavior over time, retaining its original particle diameter (Figure 4.15B)

Figure 4.15. Representative degradation of ESG and cESG as a function of time after the addition of A) α-amylase 300 Units/g substrate or B) Pullulanase 200 Units/g substrate
Figure 4.16. Percent of particle remaining after enzymatic degradation with pullulanase and α-amylase. n=3 independent experiments. Mean ± Standard deviation. *p<0.05 determined by One-way ANOVA with Tukey’s Post-Test

This change in enzymatic specificity, while not unanticipated, is of concern. Intracellularly glycogen debranching enzyme (AGL) and glycogen phosphorylase (PYGL, PYGM, PYGB) work in combination to debranch glycogen and release glucose.\(^{26}\) If cESG modification impedes degradation \textit{via} these routes, the biodegradability and eventual clearance of the complex are threatened. To better understand the role of glycogen breakdown in our cell lines, we probed AGL and PYGL expression levels with semi-quantitative real time Reverse transcriptase polymerase chain reaction (sqRT-PCR). When normalized to expression in Nose007 normal ovarian surface epithelial cells, both AGL and PYGL are more highly expressed in Ovca420 than ES-2 cells. PYGL is downregulated in ES-2 OCCC (Figure 4.17). PYGM levels were undetectable by sqRT-PCR and PYGB levels were similar in all cell lines tested. This indicates that Ovca420 cells may have a higher capacity to breakdown cESG than ES-2 cells. If enzymatic breakdown of cESG-siRNA is the primary mechanism governing siRNA release and efficacy, then we would expect cESG-siRNA to have higher efficiency in Ovca420 cells than ES-2. However, this is not the case
as Ovca420 cells had no protein knockdown with cESG-siRNA treatment and ES-2 cells showed significant knockdown. To further investigate this we would need to assess enzyme protein expression and activity in these cells and relate this to cESG cargo release. It is also possible that enzyme expression did not correlate to the efficiency of cESG-siRNA treatment due to insufficient endosomal escape in Nose007 and Ovca420 cell lines, in which the complex would not be exposed to enzymes. In future studies, we aim to manipulate PYGL and AGL expression levels in ES-2 and Ovca420 cells to further interrogate a potential role in breakdown of ESG.

Figure 4.17. AGL and PYGL display higher expression in Ovca420 high grade serous adenocarcinoma cells compared to ES-2 OCCC cells. Fold change in expression relative to Nose007 cells. Mean ± Standard Deviation.
4.4 Conclusions

This chapter sought to characterize the cellular behavior of cESG in order to better understand its mechanism of action. cESG mediated delivery of siRNA for SOD2 knockdown had varying degrees of efficacy. Excellent knockdown was seen in ES-2 OCCC, with limited knockdown in normal ovarian epithelium (Nose007) and no response in ovarian serous adenocarcinoma (Ovca420). Possible explanations for this include differences in cellular uptake or release of the complex. However, upon analyzing the uptake behavior of the complexes, all cell types exhibited gradual uptake of the complex 2 to 4 hours after treatment, with nearly 100% of the cell populations taking up cESG after 24 hours. This agrees well with our observation that in ES-2 OCCC, cESG-siRNA was equally effective following a 24 h or 72 h incubation with treatment.

Cationic modification did not significantly impact toxicity, being well tolerated up to 100 nM dosages, it does induce changes in the enzymatic breakdown of the particle. cESG was no longer a suitable substrate for α-amylase degradation, only exhibiting minor degradation. However, modification did not change the activity of pullulanase in the complex. These results are not conclusive to demonstrate that cESG is resistant to enzymatic degradation, but rather suggests that
the specificity of the enzyme-substrate complex is impacted by GTMA incorporation. Therefore, the enzymatic behavior of each enzyme of interest should be investigated individually to determine response or further studies of cESG-enzyme binding structure conducted using x-ray crystallography or similar methodologies.

4.5 References


CHAPTER 5.

INVESTIGATION OF cESG FOR PHOTODYNAMIC THERAPY

5.1 Introduction to Photodynamic Therapy (PDT)

Photodynamic therapy (PDT) is a treatment modality that uses light (photo-) in conjunction with a photosensitizer (PS) to combat disease. The first observation of PDT was in 1900 when Oscar Raab discovered that the compound acridine, when irradiated with light, resulted in death of paramecia.¹ These findings were expanded upon in 1903 by Herman von Tappeiner, who discovered that oxygen played a key role in inducing cellular death when irradiating topically applied eosin on basal cell carcinoma, coining the term photodynamic action.² Over a century later, the physical mechanism for this cellular death is attributed to the production of reactive oxygen species (ROS). Incident light of a specific wavelength excites a PS to an excited singlet state, which is of higher energy. Energetically, the PS desires to return to its ground state. It does this through either collisions, heat, fluorescent emission or relaxation to a triplet state (Figure 5.1). This triplet, also not energetically stable, can either loose energy via photoemission (phosphoresce) or through some other means to return to the ground singlet state. Two principal mechanisms of energy transfer to other chemical species are possible and may occur simultaneously. In a type I mechanism, an electron or hydrogen atom transfer occurs with oxygen to create a variety of different ROS (H₂O₂, O₂⁻). In a type II mechanism, an energy transfer with ground state oxygen (O₂) results in the formation of singlet oxygen (¹O₂) specifically.³
This scheme has been harnessed for the treatment of a wide variety of skin conditions, such as acne vulgaris and actinic keratosis, as well as a variety of cancers, in which photosensitizers are preferentially accumulated in tumor tissues. Upon illumination, direct oxidative stress induces cellular death. For tumors, indirect cell damage also occurs due to the destruction of vascularization. After initial tumor ablation, a secondary immune response is also triggered due to the release of inflammatory mediators, which may assist in long-term tumor control.\(^4\)

![Diagram of photochemical reactions](image)

**Figure 5.1.** Generation of free radicals from photosensitizers upon illumination by incident light by a type I (general ROS generating) or type II (singlet oxygen generating) mechanism

From a medicinal perspective, a number of factors are of critical importance in the design of a PS. As PDT seeks to decrease the unwanted toxicity to normal tissues, a photosensitizing agent should not be inherently toxic in the absence of light (dark toxicity). In order to be efficient, a photosensitizer agent should have a high quantum yield of radicals produced upon illumination, preferably excited by a 600-800 nm light which has better tissue penetration than lower wavelengths.\(^5\) Compounds must be chemically pure and stable in aqueous media, with limited
aggregation in the presence of serum proteins. To minimize unwanted toxicity, a photosensitizer should be preferentially retained in the target tissue and rapidly cleared from the body.\textsuperscript{6}

In 1993, Canada approved the first photosensitizer for clinical use, Photofrin\textsuperscript{®}, to treat bladder cancer metastasis.\textsuperscript{7} PDT application has expanded to a wide variety of cancers (Table 5.1) including prostate,\textsuperscript{8} head and neck,\textsuperscript{9-12} gastrointestinal,\textsuperscript{13} pancreatic,\textsuperscript{14} lung,\textsuperscript{15} and nonmelanoma skin cancer.\textsuperscript{16-19} This method is also used for non-malignant conditions such as macular degeneration\textsuperscript{20,21} and psoriasis\textsuperscript{22}. Popular compounds for photosensitizers are porphyrins and its analogs, nearly a dozen of which are FDA (Food and Drug Administration) approved for use in cancer and other diseases.

One possible side effect of PDT is skin sensitivity to light. As PS are often IV administered, residual drug can be retained in healthy tissues for up to six weeks following treatment, and direct sun exposure must be limited.\textsuperscript{23} In intraperitoneal (IP) PDT, adverse events such as bowel perforations and intestinal wall destruction have been observed when using red light for illumination. These perforations may be prevented by using green light for excitation, which decreases phototoxicity. Similar toxicity was seen using red light with Foscan (mTHPC), leading to inflammation and change in liver and kidney function. However these complications occur in a minority of patients, with far fewer side effects than chemotherapeutic regimens overall.\textsuperscript{24}
Table 5.1. Clinical indications of photosensitizers used in United States clinical trials

<table>
<thead>
<tr>
<th>Photosensitizer</th>
<th>Trade Name (Company)</th>
<th>Indications Tested in Clinical Trials*</th>
<th>Number of Clinical Trials*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porifimer sodium</td>
<td>Photofrin® (Axcan Pharm Inc.)</td>
<td>Bile duct cancer, bladder cancer, breast cancer, esophageal carcinoma, gallbladder cancer, head and neck cancer, lung cancer, mesothelioma</td>
<td>43</td>
</tr>
<tr>
<td>Talaporphin sodium</td>
<td>Litx™, Aptocine (Light Sciences Oncology)</td>
<td>Benign prostatic hyperplasia, bladder cancer, colorectal neoplasms, head and neck cancer, liver cancer, macular degeneration, mouth cancer, neurofibroma, pelvic cancer, port wine stains (birthmark), rectal cancer, sarcoma</td>
<td>13</td>
</tr>
<tr>
<td>5-Aminolevulinic acid (5-ALA)</td>
<td>Levulan® (Dusa Pharma Inc.)</td>
<td>Acne vulgaris, actinic keratosis, basal and squamous cell carcinoma, Bowen’s disease, cervical dysplasia, colon cancer, head and neck cancer, lymphocytic leukemia, oral leukoplakia, rosacea,</td>
<td>102</td>
</tr>
<tr>
<td>Methyl aminolevulinate (5-ALA-methylester) (MAOP)</td>
<td>Metvix® (Galderma)</td>
<td>Acne vulgaris, actinic keratosis, basal cell carcinoma, cervical dysplasia, rosacea</td>
<td>56</td>
</tr>
<tr>
<td>Hexyl aminolevulinate (5-ALA-hexylester)</td>
<td>Hexvix®, Cysview® (Photocure)</td>
<td>Actinic keratosis, bladder cancer, cervical intraepithelial neoplasia, colorectal cancer</td>
<td>23</td>
</tr>
<tr>
<td>Verteporfin</td>
<td>Visudyne® (Novartis AG)</td>
<td>Choroidal neovascularization, macular degeneration, port wine stains (birthmark), pleural effusion</td>
<td>92</td>
</tr>
<tr>
<td>5,10,15,20-Tetrakis(m-hydroxyphenyl)chlorin (m-THPC) (Temoporfin)</td>
<td>Foscan (BioLitec Pharma)</td>
<td>Head and neck cancer, lip an oral carcinoma, non-small cell lung carcinoma</td>
<td>7</td>
</tr>
<tr>
<td>2-(1-Hexyloxyethyl)-2-devinyl pyropheophorbide-a (HPPH)</td>
<td>Photochlor (Roswell Park Cancer Institute)</td>
<td>Head and neck cancer, lung cancer, oral cavity carcinoma</td>
<td>3</td>
</tr>
<tr>
<td>Motexafin lutetium</td>
<td>Antrin®, Lutex, Lutrin (Pharmacyclics Inc.)</td>
<td>Abdominal cancer, cervical intraepithelial neoplasia, non-small cell lung cancer, recurrent prostate cancer</td>
<td>3</td>
</tr>
<tr>
<td>Silicon phthalocyanine 4 (Pc-4)</td>
<td>Pc-4 (Case Western Reserve University)</td>
<td>Breast cancer, head and neck cancer, lymphoma, non-melanomatous skin cancer</td>
<td>3</td>
</tr>
</tbody>
</table>

*US NIH clinical trials on clinicaltrials.gov as of April 8, 2016
For PDT of skin cancers and other surface ailments, treatment is fairly straightforward. A photosensitizer, such as Levulan® or Metvix®, is applied topically to the area. After a brief absorption period, the area is irradiated with light corresponding to the excitation wavelength of the photosensitizer. For treatment of internal cancers, however, treatment is far more complex. Photosensitizers, like Photofrin®, are injected intravenously into a patient. After an incubation period for the agent to accumulate in the tumor, normally one to three days, the area is irradiated. Yet for our disease model of interest, ovarian cancer, finding an efficient, non-invasive, irradiation mechanism for the internal IP cavity is a major hurdle to the clinical implementation of PDT.

In PDT for ovarian cancer, the entire peritoneal cavity must be treated to ablate secondary tumors. The IP cavity has a large area, ranging from 15,000-24,000 cm² depending on the patient size.25 IP phototherapy without surgical intervention involves the implantation of a quartz fiber using an 18-gauge needle. This illumination system, modified with a light diffusing tip, was tested using Photofrin II® to treat 21 women with a variety of gynecological malignancies. After treatment, 7 patients with cutaneous lesions had complete response. However only 4 of 11 patients with cervical or vaginal recurrent disease responded to treatment (36%), with the remaining 7 having disease progression.26

Strides are being made to improve PDT through a two pronged approach of improving light dosage as well as through new design and delivery of photosensitizers.27 One strategy for better PS delivery is the encapsulation and targeting of existing PS. Active targeting to overexpressed receptors, such as folate receptor alpha (FRα) and human epidermal growth factor receptor 2 (HER2), have been used to improve preferential uptake of PS. A folate-modified porphyrin photosensitizer showed a tenfold increase in uptake compared to the unmodified PS.28 A similar response was seen with anti-HER2 monoclonal antibody targeting, which improved PS
accumulation and photodynamic efficiency in an in vivo mouse study.\textsuperscript{29} The encapsulation of photosensitizers can also improve delivery, especially for hydrophobic PS, such as hypericin, which are not stable in aqueous media. Using passive targeting from the enhanced permeability and retention (EPR) effect, hypericin loaded polymeric PLA (polylactic acid) nanoparticles increased PS localization into malignant ovarian micrometastases in Fisher 344 rats for both PDT and imaging.\textsuperscript{30,31} Despite these improvements in PS delivery, the major criticism for the implementation of PDT in IP localized cancers is still light delivery systems.

Several different strategies have worked towards improving light delivery and demonstrating clinical feasibility. One strategy to improve this response is implementing PDT illumination as the final step in debulking surgery. In a phase II clinical trial of Photofrin\textsuperscript{®} for the treatment of ovarian cancer, there were high recurrence rates, in which only two patients (6\%) had no evidence of disease recurrence.\textsuperscript{32,33} In an effort to improve this response, DeLaney et al. designed a light diffusing devise to illuminate the peritoneal or pleural cavity. Used in conjunction with a lipid emulsion, the flexible device was capable of illuminating hard to reach areas such as the diaphragm and liver in phase I studies.\textsuperscript{25} In a study using this device for Photofrin II\textsuperscript{®} PDT treatment for 13 ovarian cancer patients, two patients remained free of recurrence (15\%), a slightly improved response rates than traditional illumination.\textsuperscript{34} These results from human trials are in stark contrast with the preclinical studies conducted by Tochner et al. in mice with ovarian embryonal carcinoma using Photofrin\textsuperscript{®} in which there was a 100\% response rate with 40\% to 85\% of animals remaining disease free.\textsuperscript{35,36}

This limited response in human trials highlights the challenge in scaling up PDT to achieve complete illumination of the IP cavity. New light sources in preclinical studies include flexible textile-based light diffusers,\textsuperscript{37} blood vessel catheters,\textsuperscript{38-41} and endoscopes to improve the placement
of light sources without surgical intervention.\textsuperscript{42} The process of fractionated illumination has also been investigated as a modality of improving light response. As ROS are generated from photosensitizers, oxygen is depleted from the region as it undergoes conversion. Pulsing the light during treatment allows for reoxygenation of the environment during the unilluminated dark period. In Fischer 433 rat models, 5-ALA-hexylester (Hexvix\textsuperscript{®}) was IP injected for the treatment of ovarian adenocarcinoma followed by phototreatment using an optical fiber inserted intraperitoneally.\textsuperscript{43} When the light was pulsed, with 60 s dark intervals at 30 J/cm\textsuperscript{2} there was significantly deeper necrosis and more tumor destruction than continuously illuminated PDT treatment at 45 J/cm\textsuperscript{2}.\textsuperscript{44,45}

As the above improvements in light delivery mechanisms have made PDT for ovarian cancer more feasible, we chose to investigate the improved delivery of existing photosensitizers using ESG and cESG. The size (30 nm) should allow for enhanced accumulation (passive delivery) within tumors due to the EPR effect, decreasing unwanted photosensitization in surrounding tissues. In this chapter, we explored ESG and cESG encapsulation to improve the solubility properties of the PS THPP (tetra(hydroxyphenyl)porphyrin), which is hydrophobic. The nanodendrimers were also investigated as a delivery vector for anionic TPPS (Meso-tetra(4-sulfonatophenyl)porphyrin), in order to decrease undesirable neurotoxicity which results from administration without a carrier.

THPP is a highly hydrophobic photosensitizer (Figure 5.2) that was thoroughly investigated in 1986 for \textit{in vivo} PDT by Winfield et al.\textsuperscript{46} Three isomers of THPP (ortho- meta- and para-) were characterized for PDT using red light. All three were more potent photosensitizers than Photofrin\textsuperscript{®}, the clinical gold standard, and showed excellent efficacy in treating brain tumors. However o-THPP exhibited too much skin sensitization for usage and was ruled out for use as a
therapeutic. Upon illumination of THPP with visible light, THPP exhibits a type II mechanism, generating primarily singlet oxygen. After excitation, THPP undergoes photomodification to give a new chromophore, rather than true photobleaching in which the product is broken into small fragments. In the case of m-THPP this photoproduct appears to be a dimer of two covalently linked units. After prolonged illumination (225 minutes), trimeric and tetrameric structures may be formed. Encapsulation of THPP may prevent the formation of these complexes.

![Figure 5.2. Structure of p-THPP (5,10,15,20-Tetrakis(4-hydroxyphenyl)-21H,23H-porphine)](image)

THPP is readily soluble in both ethanol and DMSO, but has a low solubility in water, which increases slightly at higher pH. The aggregation of THPP in solution is highly solvent dependent with unstable linear or stacking aggregates formed due to changes in temperature, pH, and ionic strength. Aggregation changes the spectroscopic properties of the solution (which may exhibit a red or blue shift), and may also have implications for cellular uptake. In order to overcome these aggregation effects and improve the delivery and efficacy of THPP, several formulation approaches have been investigated. Allemann et al. worked toward delivering p-THPP in PLGA
particles and demonstrated an increased therapeutic efficiency for the loaded compound versus the free drug in vitro.\textsuperscript{50,51} Further testing using the chorioallantoic membrane (CAM) pseudo-in vivo tumor model, revealed that encapsulated THPP had longer retention in the vasculature and enhanced photodynamic efficiency compared to free THPP.\textsuperscript{52} Smaller THPP-loaded PLGA (poly(lactic-co-glycolic acid) nanoparticles exhibited more photodynamic activity than larger, likely due to enhanced cellular uptake via the EPR effect.\textsuperscript{53} Encapsulation of THPP has also been shown using the carbohydrate cyclodextrin (both modified and unmodified) by inclusion complexation of THPP due to hydrophobic interactions.\textsuperscript{54} As cyclodextrin is composed of $\alpha$(1-4) linked glucose residues, we hypothesized that THPP would have a similar affinity for encapsulation using ESG and cESG.

The second photosensitizer of interest is TPPS (Figure 5.3). This hydrophilic PS has an overall anion charge, which may electrostatically bind to cESG. TPPS was first explored as a photosensitizer in the 1960s, and showed very high tumor localization. This was postulated to be caused by the binding of albumin increasing circulation times, leading to accumulation in stromal cells. Less polar PS tend to bind cholesterol and have shorter circulation.\textsuperscript{55} Due to this enhanced accumulation, the photodynamic efficiency of TPPS in vivo was much higher than suggested by in vitro studies.\textsuperscript{56}

However, these in vivo studies also revealed that TPPS had neurotoxic effects. Regardless of dosage tested (5-150 mg/kg body weight) animals exhibited nerve fiber degeneration as well as cytoskeletal abnormalities. This was attributed to the interaction of TPPS with tubulin, a cellular component necessary for mitotic division.\textsuperscript{57} One month after intravenous (IV) injection of TPPS, there were morphological changes in peripheral nerves, attributed to metabolic disturbances.
Similar functional damage was observed with injection of Photofrin® and Levulan®, but this damage was reversible once they had cleared circulation. The increased circulation time of TPPS, due to albumin binding, led to increased irreversible damage resulting in structural changes in the peripheral nervous system.\textsuperscript{58} Given this unwanted toxicity, TPPS formulation has, so far, not moved from the benchtop to the clinic.

The pH of TPPS is important when formulating it. Dimers and trimers of the molecule are formed at neutral and basic pH. In acidic solution larger, stable cyclical aggregates of 25 TPPS are formed, which may reorganize into tubes or rods at higher (mM) concentrations.\textsuperscript{59} Free TPPS accumulates lysosomally within the cell, and following illumination, escapes into the cytoplasm.\textsuperscript{60,61} At the decreased pH of the lysosome, there are few changes in the light responsiveness.\textsuperscript{62} However, in strong acidic solutions the protonation of the 4-sulfonatophenyl groups can occur, impacting efficacy.\textsuperscript{63} This lysosomal escape is most pronounced in cells irradiated during their growth phase, in which TPPS eventually redistributes into the nucleus.\textsuperscript{64}
This growth phase is more pronounced in cancer cells, providing another explanation for TPPS enhanced efficacy.\textsuperscript{64,65}

In order to overcome toxicity and harness this potent PS, new nanoformulations have been investigated. It is of paramount importance that encapsulated TPPS retain its high photodynamic efficiency. The electrostatic condensation of anionic TPPS with cationic amphiphilic cyclodextrin has been shown to preserve light responsiveness. In tuning this complex, it was observed that formulations with more TPPS per nanoparticle exhibited a self-quenching reaction, decreasing light responsiveness. However, the loading ratios could be tuned, and maintain photodynamic efficiency in an \textit{in vitro} HELA cell system.\textsuperscript{66} TPPS photobehavior also remained consistent when electrostatically condensed to lysine residues in a coiled peptide assembly.\textsuperscript{67} This indicates that electrostatic condensation of TPPS to compounds does not negatively impact photodynamic efficiency. TPPS can be quenched by other compounds, such as trinitrotoluene, so care must be taken that any formulation does not interfere with excitation.\textsuperscript{68} Based on these previous studies, we anticipated that electrostatic condensation of TPPS using cESG could be feasible without negatively impacting photoresponse.

Important factors in determining the success of a PS delivery system are 1) having an efficient, stable encapsulation 2) exhibiting low dark toxicity to cells and 3) seeing significant light response upon illumination. We anticipated that, as ESG and cESG had low cytotoxicity, encapsulation with the complexes would not notably increase dark toxicity. It was our hypothesis that since cESG had efficient cellular uptake it could improve the internalization of both THPP and TPPS.
5.2 Methods

5.2.1 Materials

McCoy’s 5A media, RPMI media, and Trypsin EDTA 1x were from ATCC. HyClone fetal bovine serum was from GE Healthcare. Dulbecco’s phosphate buffer saline 1x was from FisherScientific. meso-tetra(hydroxyphenyl)porphyrin (THPP) and meso-tetra(4-sulfonatophenyl)porphyrin (TPPS) were obtained from Frontier Scientific. Cell experiments were performed using sterile, nuclease free water (Qiagen). All other reagents were purchased from Sigma Aldrich unless otherwise specified.

5.2.2 ESG- and cESG-THPP Loading

Lyophilized ESG or cESG (20 mV, 1 GTMA residue per 3.6-5.2 glucose residues) were dissolved in 1 mM sodium phosphate buffer pH 7 at concentrations ranging from 50-1000 mM. Stock 2.06 mM THPP in 95% ethanol was added to a final concentration of 100 µm THPP. Solutions were magnetically stirred overnight in glass vials at room temperature. In order to remove precipitated THPP, solutions were filtered using 0.2 µm sterile PES filter. Products were diluted 1:50 in 20 mM sodium phosphate buffer pH 6 for absorbance measurement. Absorbance at 424 nm was measured in a quartz cuvette using a Cary 50 Scan UV-vis spectrophotometer (Varian) or in a 96-well half area plate on a SpectraMax Paradigm plate reader (Molecular Devices). Concentration THPP encapsulated was calculated using a standard curve (424 nm) obtained on the corresponding spectrophotometer used for measurement.

5.2.3 Cell Culture

Cell culture was performed as previously described (3.3.6).
5.2.4 Dark Toxicity of THPP Compounds

ES-2 and Nose007 cells were plated at a density of 4,000 cells per well in 96 well plates. The following day, cells were treated with THPP in serum free media at concentrations ranging from 0.3 μM to 3.9 μM. The treatments were composed of either free THPP diluted from ethanol stock, ESG-THPP or control ESG particle alone. For the ESG control, the concentration ESG added corresponded to particle concentrations used for ESG-THPP treatments. After 6 hour incubation at 37 ºC, the solutions were removed and the cells allowed to incubate in growth media overnight. 24 h after initiating treatment, an MTT assay was performed as previously described (3.3.8) to determine cell viability.

5.2.5 Impact of Light Dosage and THPP treatment

Solutions with low dark toxicity in the previous experiment were chosen for exploration with light treatment (0.3, 0.6 and 0.9 μM). Treatments were conducted as described for dark toxicity experiment with one exception. After incubation with THPP complexes and replacement of the treatments with growth media, the cells were illuminated using a 630 nm 8 mW/cm² LED lamp for either 0, 2, 5, 10, or 15 minutes. Solutions were then incubated overnight and an MTT assay performed as previously described (3.3.8). Experiments were repeated using cESG-THPP complexes with light illumination times of 0, 5, or 10 minutes.

5.2.6 TPPS Condensation of ESG- and cESG-siRNA

Lyophilized ESG or cESG were dissolved in 1 mM NaPO₄ buffer pH 7.5 at concentrations ranging from 6000 to 25 nM (6000, 3000, 1500, 750, 400, 200, 100, 50, 25 nM). Stock 10 mM TPPS was added to the particles at a final concentration of 102 μm. Solutions were magnetically stirred overnight in glass vials at room temperature. Products were dialyzed in 10 kDa MWCO regenerated cellulose membrane (ThermoScientific) for 24 h against 4 L of distilled water to
remove free TPPS. Samples were then diluted 1:50 in 20 mM sodium phosphate buffer pH 9 for absorbance measurements. Absorbance at 417 nm was read in a 96-well half area plate on a SpectraMax Paradigm plate reader (Molecular Devices). Absorbance spectra were also collected from 350-500 nm with a 1 nm step size. A TPPS standard curve (0 to 5 µm) was generated using the same instrumentation and used to calculate TPPS concentration. Additional syntheses were performed using the above protocol, with cESG concentration held constant (0.78 µm, 8 mg/mL) and TPPS added at a range of concentrations (100, 50, 25, 12.5 and 6.25 µm).

5.2.7 DLS and Zeta Potential Analysis

DLS and zeta potential analysis were performed as described previously (2.2.1.3)

5.2.8 Gel permeation chromatography (GPC)

100 µL of ESG-TPPS or cESG-TPPS was loaded into a Sephadex G-25 crosslinked dextran desalting column with 38-235 µm particle size and 2.5 mL void volume (GE Healthcare). 20 mM sodium phosphate buffer pH 9 was added up to a 2 mL volume. Eight 250 µL fractions were collected followed by an additional collection of four 500 µL fractions. Absorbance of all fractions was read in triplicate on a SpectraMax Paradigm plate reader (Molecular Devices) in a 96-well half area plate. Free TPPS was bound to the column and did not elute. Absorbance (417 nm) of particle-bound TPPS was used to calculate the TPPS concentration of each fraction using the standard curve (Equation 5.1). The concentration of bound TPPS (µM) in the original sample was then determined by equation 5.2.

Equation 5.1. \[ \text{Concentration (µM)} = \left(\frac{417\text{nm Absorbance}-0.0261}{0.0991}\right) \]

Equation 5.2. \[ \frac{\sum \left(\frac{\text{µM Concen}tration\cdot\text{Fraction Volume}}{\text{Initial Sample Volume}}\right)}{\text{Initial Sample Volume}} = \text{µM TPPS Encapsulated} \]
5.2.9 Testing Efficacy of cESG-TPPS compounds for PDT

ES-2 and Nose007 cells were plated at a density of 4,000 cells per well in 96 well plates. The following day, cells were treated with cESG-TPPS in either serum free or growth media with 0.7 µM TPPS encapsulated in cESG-TPPS (100 TPPS per cESG). Treatments were conducted for either 4 or 24 h, prior to being aspirated, replaced with fresh growth media, and illuminated with a 630nm 8mW/cm² LED lamp for 5 minutes or left unilluminated as a dark control. An MTT cell viability assay was performed as previously described (3.3.8) to determine cell viability. Subsequent experiments were performed using various formulations of cESG-TPPS at either a constant TPPS concentration (0.6 µM) or constant cESG concentration (17 nM), while varying the other component (cESG or TPPS). Treatments were conducted in growth media for 1 day and 3 days respectively.

5.2.10 Statistical Analysis

All data presented are representative of at least three independent experiments and expressed as mean ± SEM, unless otherwise noted. Statistical data analysis were performed using OriginPro Software v8.5. Student’s T-Tests or ANOVA with Tukey’s post test were performed as indicated. In the case of two-way ANOVA analysis, the input variables (i.e. dosage, treatment type, illumination time) are considered independent and the mean differences between groups are compared.
5.3 Results and Discussion

5.3.1 Exploration of ESG-THPP and cESG-THPP for PDT

The encapsulation of THPP was investigated by incubating ESG-THPP formulations at room temperature overnight. Lyophilized ESG was dissolved at a range of concentrations (mM) and THPP added to a final concentration of 102 µM. Due to its low solubility, unencapsulated THPP precipitated out of solution and could be removed by filtration. As the aqueous solubility of THPP is low, remaining THPP measured by absorbance was assumed to be ESG bound (ESG-THPP). This measurement would also include any intrinsically soluble THPP. A slight increase in the amount THPP bound to ESG was observed as the ESG concentration increased, plateauing at approximately 50 µM of THPP encapsulated (Figure 5.4). This represent a rather low binding capacity, as there is a one thousand fold higher concentration of ESG particles compared to THPP. Repeating this procedure using cESG, gave similar results, with slightly higher loading of THPP of up to 80 µM THPP encapsulated (Figure 5.5). However, it is of note that there was higher variation in loading between batches when using cESG.

![Figure 5.4. Encapsulation of THPP (µM) in ESG (mM) plateaus at 50 µM THPP. Reactions stirred overnight and purified by filtration. Concentration measured by absorbance at 424 nm, mean ± standard deviation. n=2 independent syntheses.](image-url)
Figure 5.5. Encapsulation of THPP (µM) in cESG (mM) plateaus at 80 µM. Reactions stirred overnight and purified by filtration. Concentration measured by absorbance at 424 nm, mean ± standard deviation. n=2 independent syntheses.

ESG-THPP synthesized using 370 mM ESG was chosen in order to investigate the efficiency of ESG-THPP for PDT in an *in vitro* cell culture model. An initial screening of dark toxicity of the compound alone was conducted on both ES-2 OCCC and Nose007 normal ovarian surface epithelial cells. As the concentration of THPP increased from 0.3 to 3.9 µM, there was a large increase in dark toxicity in the ESG-THPP encapsulated compounds (Figure 5.6). This toxicity was not seen in the THPP control alone or in the vehicle control (free ESG). This increased toxicity due to encapsulation was attributed to increased intracellular uptake of encapsulated THPP versus that of the free THPP. Dosages of 0.3, 0.6 and 0.9 µM were chosen for further investigation of the impact of light treatment on cellular viability, as they had favorably low dark toxicity.
Figure 5.6. Dark toxicity of THPP, ESG-THPP, or ESG (concentration equivalent to ESG-THPP dosage) treatment of Nose007 normal ovarian surface epithelial cells (A) and ES2 OCCC (B) treated for 6 h in serum free media. Viability was assessed 24 h after initiating treatment using an MTT assay. n=3 independent experiments. Dark toxicity of ESG-THPP is statistically different than THPP or ESG treatment, *p<0.05 determined by two-way ANOVA with Tukey’s post-test.

In order to observe the cytotoxic behavior of ESG-THPP upon illumination, a range of light dosages were tested (Figure 5.7). The illumination of ESG controls alone did not contribute to a statistically significant increase in cellular death (p>0.05, Figure 5.7C, 5.7F). Treatment using free THPP resulted in cellular death upon illumination of as little as 2 minutes, with less than 20% cell viability after a 15 minute illumination at all THPP dosages tested (Figure 5.7A, 5.7D). This death was statistically significant from the unilluminated control (p<0.05 ES-2 cells, p<0.001 Nose007 cells). All illumination times were statistically similar, though a trend of decreasing viability with increasing illumination times could be discerned at 0.3 µM THPP. Encapsulated ESG-THPP follows the same trends, with light illumination decreasing cell viability in both cell types (Figure 5.7B, 5.7E). However, there is no statistically significant difference with treatment of free THPP or ESG-THPP. While ESG-THPP showed an increase in dark toxicity with increasing dosages not seen with free THPP, a sufficient concentration of free THPP is taken up
by the cells to induce cellular damage. ESG encapsulation does not appear to either improve or inhibit photodynamic efficiency of THPP.

In order to explore if cESG-THPP may improve cellular uptake due to its positive charge, several formulations of cESG-THPP were used for PDT in ES-2 and Nose007 cells. The same THPP dosages were tested (0.3, 0.6, 0.9 µM) encapsulated in 3 different formulations of cESG. To prevent toxicity due to charge effects, lower concentrations of cESG were used compared to ESG-THPP tested. Light treatments were limited to either a 5 min or 10 min treatment. All cESG-THPP formulations as well as free THPP had a statistically significant cell death as a response to light treatment compared to unilluminated control (p<0.001). As the concentration of cESG increased (number of THPP per particle decreased), an increase in in dark toxicity was observed (Figure 5.8). While there were statistically significant differences between the particle formulations (p<0.05), there was no correlation between cESG concentration and light response observed. In Nose007 cells, the encapsulation of THPP in cESG resulted in increased cell death compared to free THPP upon illumination in all dosages tested (Figure 5.8 left pane). Yet, there is also an increase in dark toxicity with these compounds which contributes to this lower viability. In ES-2 OCCC cells, no improvement in efficiency is observed with cESG encapsulation (Figure 5.8 right pane). This may be due to reduced uptake of the free THPP formulation in Nose007 cells relative to the cancerous ES-2 OCCC. While cESG appear to provide slightly better delivery for THPP than ESG, the formulations tested were not deemed promising for continued investigation.
Figure 5.7. ESG encapsulation does not improve PDT response of THPP in Nose007 normal ovarian surface epithelial cells (left) or ES-2 OCCC (right) measured by MTT viability. Treatments for 6 h in serum free media with THPP (top), ESG-THPP (middle) or ESG (bottom) treated, followed by light treatment in growth media. Viability was assessed 24 h after initiating treatment. n=3 independent experiments, ESG and ESG-THPP dark controls are statistically different than illuminated treatments. * p<0.05 **p<0.01 ***p<0.001 determined by two-way ANOVA.
Figure 5.8. cESG encapsulation does not improve PDT response of THPP in Nose007 normal ovarian surface epithelial cells (left) or ES-2 OCCC (right) measured by MTT viability. Treatments for 6 h in serum free media at THPP concentration: 0.3 µM (top), 0.6 µM (middle) or 0.9 µM (bottom), followed by light treatment in growth media. Viability was assessed 24 h after initiating treatment. n=3 wells, Dark controls are statistically different than illuminated treatments. * p<0.05 ***p<0.001 determined by two-way ANOVA.
5.3.2 *ESG and cESG Encapsulation of TPPS*

The charge based encapsulation of TPPS using ESG and cESG was investigated using a similar method as with THPP. Lyophilized ESG or cESG were dissolved in 1 mM sodium phosphate buffer at a variety of concentrations and reacted with TPPS, 102 µM final concentration. As TPPS is highly water soluble, dialysis was attempted to remove unbound TPPS and UV-Vis absorbance used to determine concentration. However at neutral pH, TPPS stacking occurred in solution. These aggregates were larger than the pores of dialysis tubing, resulting in unbound TPPS retained in solution. As such, gel permeation chromatography was used to determine the concentration of TPPS bound. In this method, anionic free TPPS bound to the dextran column matrix and was unable to elute. ESG or cESG with bound TPPS eluted in the void volume and was detected in early fractions using UV-Vis absorbance (Figure 5.9).

![Figure 5.9](image)

Figure 5.9. Representative GPC elution profile of ESG-TPPS (A) and cESG-TPPS (B). Absorbance measured at 417 nm. n=3 wells.

After dialysis but prior to GPC absorbance measurements, the TPPS concentration was similar for the ESG and cESG reactions. However, the GPC elution profile indicates no bound TPPS in ESG formulations (Figure 5.9A). This suggests that either no TPPS was bound to ESG or
that the interactions were weak and the TPPS was removed from the complex by binding to the dextran GPC column. A weak interaction is not surprising, as TPPS is highly water soluble and the ESG lack positive charge, thus there is little driving force for encapsulation. The cationic cESG on the other hand is capable of binding TPPS (Figure 5.9B), with a direct correlation between the concentration of cESG and the amount of TPPS bound. At a neutral reaction pH, free TPPS is a bright green solution. Over the course of encapsulation reaction with ESG or cESG, the solution turns to light pink solution (Figure 5.10). This green to pink transition in ESG formulations indicates that there might be some interaction between ESG and TPPS. After storage in solution at room temperature for several weeks, ESG-TPPS solutions prepared at lower concentrations of ESG returned to their initial green coloration, evidence that TPPS is no longer associated with ESG. This color change is not observed in cESG-TPPS formulations or in ESG-TPPS solutions which were filtered and stored at 4 °C for cell culture experiments.

<table>
<thead>
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<th>TPPS/Particle</th>
<th>250</th>
<th>500</th>
<th>1000</th>
<th>2000</th>
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<tr>
<td>Stored at 23C</td>
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<tr>
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</tr>
<tr>
<td>Stored at 23C</td>
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</tbody>
</table>

Figure 5.10. Coloration of ESG-TPPS stored at room temperature for several weeks (top) differs from ESG-TPPS filtered and stored at 4 °C (middle) and cESG-TPPS stored at room temperature (bottom) at increasing TPPS concentrations.
In order to have better control of the TPPS per cESG particle, conjugations were performed at constant cESG concentration (0.78 µM) and decreasing TPPS concentrations (6 to 100 µM). Upon GPC analysis of TPPS encapsulated in cESG a direct linear relationship is seen with TPPS addition and the amount of TPPS encapsulated (Figure 5.11), resulting in complexes with a differing number of TPPS per cESG. At lower concentrations of TPPS, 6 µM and 12 µM, there is complete encapsulation of TPPS in solution. As more TPPS was added, loading efficiency (percent TPPS bound) decreased to 76% and 66% at 25 µM and 50 µM respectively. It is of note that encapsulation in this system is much more efficient than that of THPP, with µm concentrations of TPPS being encapsulated in 0.78 µM cESG. Size and zeta potential analysis of the complexes indicate the particles are still sub-50 nm with a positive zeta potential, with slight variation between batches of synthesis (Table 5.2).

Figure 5.11 Concentration of cESG encapsulated TPPS determined using UV-Vis after GPC purification. Concentrations calculated using equation 5.2. n=3 independent synthesis. y = 0.5852x + 4.2874 $R^2 = 0.9964$
Table 5.2. Size and Zeta potential of cESG-TPPS. n=3 independent synthesis measured in triplicate.

<table>
<thead>
<tr>
<th>Average TPPS Encapsulated (μM) in cESG</th>
<th>Size ± Standard Deviation</th>
<th>Zeta Potential ± Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>37.0 ± 11.8</td>
<td>20.2 ± 2.9</td>
</tr>
<tr>
<td>7</td>
<td>41.4 ± 8.5</td>
<td>4.4 ± 4.2</td>
</tr>
<tr>
<td>12</td>
<td>44.5 ± 10.8</td>
<td>5.2 ± 1.5</td>
</tr>
<tr>
<td>19</td>
<td>35.7 ± 0.4</td>
<td>4.6 ± 3.8</td>
</tr>
<tr>
<td>33</td>
<td>45.6 ± 10.9</td>
<td>4.4 ± 4.0</td>
</tr>
</tbody>
</table>

In order for bound TPPS to retain functionality as a photosensitizer, it should have a similar absorbance and fluorescence profile to free TPPS. The absorbance spectrum of free TPPS (Figure 5.12A) has a Soret band (maximum absorbance) at 413 nm. The shoulder seen is attributed to both the absorption of monomeric free-base TPPS species (412 nm) and free base species aggregated in face-to-face dimer stacks (406 nm). In cESG-TPPS complexes, this maxima is red shifted slightly to 420 nm, with no change in absorbance intensity (Figure 5.12B). cESG-TPPS exhibits a corresponding redshift in the Q-bands located between 500-700 nm (Figure 5.12C) in which the 581 nm peak shifts to 586 nm and the 623 nm peak shifts to 632 nm. A Soret band redshift to 440 nm absorbance is observed for TPPS J-aggregates (edge-by-edge or side-to-side stacks) formed at lower pH. It is possible that this shift in cESG-TPPS is due to a similar mechanism, in which the close conformation of the TPPS causes an absorbance change. This shift is not attributed to cESG itself, as the particle alone does not exhibit absorbance in this region (Figure 5.12B). This redshift may also be attributed to differing conformation of the aromatic rings on the porphyrin exterior, which extends the pi electron system.
Figure 5.12. Absorbance (left) and fluorescence emission spectra at 420nm excitation (right) of TPPS and cESG-TPPS formulations (3 µM TPPS). Spectra are stacked for visualization (A, B, D, and E) overlay for comparison (C, F). cESG encapsulated TPPS exhibits a red shift in maximum absorbance of the Soret band B as well as in Q band region at 500-600 nm (C). A distinct fluorescence peak is present in cESG-TPPS at 568 nm (D) accompanied by an increase in fluorescence intensity (F). n=3 independent synthesis, measured in triplicate.
The fluorescence emission profile of cESG-TPPS also exhibits minor changes. The fluorescence maxima of TPPS alone has a right shoulder which is redshifted to a distinct secondary peak at 568 nm in all cESG-TPPS formulations (Figure 5.12E), consistent with a change in TPPS conformation. There is an increase in the fluorescence intensity of cESG-TPPS complexes over free TPPS, with the highest signal in formulations with the most TPPS per cESG (Figure 5.12F). This indicates that complexation does not induce self-quenching effects, but rather may decrease self-quenching by preventing the formation of dimers seen in the free compound.

5.3.3 Exploration of cESG-TPPS for PDT

Initial screening reactions investigating the impact of both incubation time with treatment (4 or 24 hours) and treatment media type (growth or serum free media) were conducted using cESG-TPPS containing 100 TPPS per particle (0.7 µM TPPS). As the behavior of ES-2 and Nose007 cells were not statistically different in studies using THPP, only ES-2 cells were investigated. Unencapsulated TPPS did not induce cellular death following illumination. (Figure 5.13). cESG-TPPS treatment in SF media for either 4 or 24 hours resulted in almost total cell death, but there was undesirable dark toxicity of 60-70% viability remaining. Treatment in growth media for 4 hours exhibited similar dark toxicity and a decrease in efficacy of light treatment, with only a 20% decrease in viability after a 5 minute illumination. Treatment overnight in growth media prevented this dark toxicity and had similar cell killing upon illumination. Although treatment in serum free media had a better photodynamic efficiency than treatment in growth media, it is less physiologically relevant. Since clinical PDT treatment often necessitates an incubation period of 2-3 days for PS accumulation, investigation of at least an overnight treatment in growth media was deemed appropriate for further investigations.
Figure 5.13. MTT viability of cESG-TPPS treated or TPPS control treated ES-2 OCCC at a constant 0.7 µM TPPS. Cells were treated either 4 or 24 hours in serum free (SF) or growth media, followed by light treatment in growth media. Viability was assessed 48 h after initiating treatment. 4 h treatments n=3 independent experiments, 24 h treatments n=1 representative experiment.

Treatment with cESG-TPPS of various formulations was investigated at a constant 0.6 µM TPPS dosage. These formulations were incubated overnight in growth media prior to light illumination. Again, free TPPS did not have a light response at this dosage (Figure 5.14). In contrast, all cESG TPPS treatments had statistically significant response to light at both 5 min and 10 min illumination (p<0.01). It is important to note that some dark toxicity was observed in cESG-TPPS treatments, with approximately 60% cell viability prior to light treatment. While no statistically significant difference between formulations was present, there was a slight decrease in light response in the highest concentration cESG, 48 nM. We attribute this to uptake rates of the cESG particles. The 48 nM cESG treatment had the fewest number of TPPS molecules per particle, so given a similar rate of particle uptake in all formulations, less TPPS would be taken up intracellularly in this formulation.
Treatment with cESG-TPPS was then investigated at a constant concentration of cESG (17 nM), with variable TPPS concentrations. In this study treatment was conducted for 3 days in growth media in an effort to increase recovery time and decrease dark toxicity effects. cESG-TPPS treatment under these conditions had low dark toxicity, with violabilities greater than 95% (Figure 5.15). A statistically significant light response was seen with all formulations tested after a ten minute illumination compared to the dark control (p<0.001). A direct correlation was seen with increasing TPPS dosage and increasing photodynamic efficiency, with 15% viability in 0.6 µM treated cells. This TPPS control, corresponding to the total TPPS concentration in the well, also responded with statistically significant toxicity (p<0.05). But, the overall viability only decreased to 90%, compared to 15% in the cESG-TPPS formulation. No other TPPS controls exhibited statistically significant toxicity, likely due to poor uptake of the complex. Illumination of free TPPS up to 16 µM did not reduce viability below 90% in our system (data not shown). But at 0.3
μM, cESG-TPPS had decreased cell viability to 50%. This demonstrates that the encapsulation of TPPS in cESG significantly increases killing efficiency over free formulation.

Figure 5.15. MTT viability of cESG-TPPS treated (A) or TPPS control treated (B) ES-2 OCCC at constant concentration cESG (17 nM). Cells were treated 3 days in growth media, followed by light treatment in growth media. TPPS control values are set to the total TPPS concentration in treatment, which is higher than encapsulated cESG-TPPS. Viability was assessed 48 h after initiating treatment. n=4 independent experiments * p<0.05 ***p<0.001 determined by 2-way ANOVA with Tukey’s post-test.

5.4 Conclusions

In this chapter we investigated the use of dendritic glycogen complexes for the encapsulation of photosensitizers for PDT. A hydrophobic encapsulation of THPP was accomplished with both ESG and cESG. There was a poor encapsulation efficiency with both particles, with 1 THPP encapsulated for every 8,400 ESG or 7,000 cESG particles in solution. Nonetheless, ESG-THPP formulations tested at dosages over 1 μM THPP resulted in significant dark toxicity. When tested at lower concentrations, this effect was attenuated but there was no improvement in light response over that achieved with free THPP in either Nose007 or ES-2 in vitro models. Various formulations of cESG-THPP also failed to improve photodynamic efficiency in ES-2 cells. For Nose007 cells,
a minor increase in light response was observed with cESG-THPP over free photosensitizer. The failure of this delivery system likely stems from the low concentration of THPP encapsulated. Compared to cyclodextrin, there was a weak interaction between ESG or cESG and THPP.

The charge based condensation of TPPS with cESG was a far more effective mechanism for photosensitizer incorporation, loading up to 100 TPPS per cESG particle. ESG did not form a stable complex with TPPS, due to its neutral charge. cESG-TPPS was effective at improving photodynamic efficiency in both serum free and growth media in ES-2 cells. As the concentration of TPPS was increased, toxicity upon illumination also increased. At constant TPPS concentration, there was no direct correlation between increasing cESG concentrations for cell treatment and efficiency. In the literature TPPS encapsulated in cyclodextrin for the treatment of HeLa cells resulted in killing of 60% of the cell population after a 1 µM treatment and 30 minute illumination. In our complex we achieved better cell killing (90% cell death) at lower concentrations of TPPS (0.6 µM) for shorter illumination periods (10 minutes). This indicates that cESG may have application for photosensitizer delivery. In the next chapter, we will probe the versatility of cESG for co-delivery of TPPS.

5.5 References

(1) Raab, O., 1900. Ueber die Wirkung fluorescirender Stoffe auf Infusorien


CHAPTER 6.

INVESTIGATION OF cESG FOR CODELIVERY OF siRNA AND TPPS

6.1 Combination of Traditional and Genetic Therapies for Cancer Treatment

In order to improve therapeutic efficacy, combinatorial therapy may be used where multiple treatments are conducted in tandem. The ultimate goal of combinatorial therapy is to achieve a synergistic effect, in which the combined total efficacy is greater than the sum of individual treatments. For cancer therapy, tumors may have intrinsic drug resistance or acquire drug resistance over the course of treatment, such as platinum resistance that occurs in ovarian cancer, which decreases therapeutic response. Advances in genomics have elucidated a myriad of gene targets that may be manipulated to negate the effects of such resistance. Therapeutics that inhibit specific genetic pathways in cancer signaling networks are not efficient as stand-alone treatments and can fail over time due to mutations in other oncogenes. As such, combining inhibitors can improve efficacy, inhibiting multiple pathways to prevent tumor proliferation, or can act as adjuvants to decrease chemoresistance. This strategy is of great interest, with hundreds of clinical trials currently investigating genetic targets in conjunction with chemotherapeutics.

For the treatment of ovarian cancer, genetic-chemotherapy combination treatments have reached human trials. A recent stage III clinical trial investigated the use of Bevacizumab, a monoclonal antibody therapy targeting vascular endothelial growth factor (VEGF-A), in conjunction with chemotherapy for advanced platinum-resistant ovarian cancer. The addition of
Bevacizumab to the treatment doubled progression-free survival from 3.4 months to 6.7 months. Yet, overall survival was not statistically significantly different, increasing from 13.3 to 16.6 months. In a xenograft murine model, siRNA against overexpressed CD44 improved Paclitaxel efficiency. Treatment with a polypropyleneimine dendrimer complex containing siRNA, paclitaxel, and a luteinizing hormone releasing hormone (LHRH) targeting moiety resulted in a slight tumor reduction 30 days after treatment, whereas all other control treatments had significant tumor growth.

The combination of gene therapy with PDT is an emerging field. In PDT, while initial tumor ablation is efficient, there is a significant recurrence rate due to incomplete death of resistant populations. Recurrent tumor populations after PDT, oftentimes located in the hypoxic tumor core, exhibit genetic aberrations allowing for increased survival (Figure 6.1).

Figure 6.1. Mechanism of tumor recurrence after PDT. Direct killing of tumor cells along with destruction of tumor vascularization occurs after illumination. ROS-resistant tumor population in the hypoxic core survives, leading to tumor relapse. From Milla Sanabria et al. Used with permission from Elsevier.
These abnormalities provide a myriad of genetic targets to improve PDT response. For example, the codelivery of siRNA against Sod1 for PDT has shown efficacy in an in vivo mouse model. Lipid-modified gold nanoechinus, a gold nanostructured photosensitizer that can induce singlet oxygen formation at near-infrared (NIR) wavelengths, was complexed with siRNA against Sod1. When irradiated, this complex resulted in complete tumor destruction, greater than that seen in photosensitizer treatment alone. Promising results have also been observed for the siRNA-mediated PDT treatment of ovarian cancer using more traditional photosensitizers by Taratula et al. The knockdown of DJ-1, an upregulated protein in ovarian cancer that helps prevent ROS-mediated apoptosis, was the genetic target. Codelivery of siRNA for the knockdown of DJ-1 and the photosensitizer phthalocyanine (Pc) was accomplished by loading of a PEG modified polypropyleneimine dendrimer. In an in vivo mouse model, there was a substantial increase in anticancer activity of PDT with siRNA treatment, with an 85% increase in intracellular ROS. Animals treated with siRNA-PDT remained disease free for the 25 day follow up period, whereas animals treated solely with PDT had disease recurrence by day 16.

An advantage to utilizing gene therapy in conjunction with existing therapeutics, such as PDT, is the ability to tune the treatment to accommodate the genetic profile of the disease. For example, in head and neck cancer, siRNA targeted at both HIF1α (hypoxia-inducible factor 1-alpha) and VEGF-A (vascular endothelial growth factor A) have demonstrated that they are promising gene targets for improving photodynamic efficacy. HIF1α, which regulates apoptosis and cell cycle progression, was knocked down using a lipid-calcium-phosphate delivery system for siRNA, followed by photodynamic therapy using Photosan®. When tested in a xenograft mouse model, treatment with this modality increased apoptosis and decreased tumor size, with no tumor growth 10 days after treatment. PDT treatment alone exhibited a similar decrease in tumor volume,
but reinitiated tumor growth 4 days after treatment, a characteristic recurrence of resistant population.\(^9\) The same siRNA delivery and PDT schemes were investigated using siRNA against VEGF, an angiogenesis regulator overexpressed in malignant tumors, which demonstrated no disease recurrence in siRNA-PDT treated tumors after 12 days.\(^10\)

For the treatment of ovarian cancer, there are a variety of potential genetic targets. Highly invasive ovarian cancers are glutamine dependent, an indicator for poor patient prognosis. Blocking the entry of glutamine into the citric acid cycle or inhibiting glutamine synthesis may decrease invasiveness and increase therapeutic response.\(^11\) Another genetic target of interest is EGFR (epidermal growth factor receptor), which plays an important role in cell cycle progression and proliferation, with high expression correlated to increased metastatic behavior.\(^12\) When EGFR was inhibited by a monoclonal antibody (C225) in a murine model followed by PDT with benzoporphyrin derivative (BPD), there was a significant decrease in tumor burden compared to PDT or C225 treatment alone. There was a corresponding increase in median survival to 80 days in PDT-C225 treated mice, a significant increase from 36 days survival (PDT alone) and 26 days survival (C225 alone).\(^13\) Following PDT, EGFR has been shown to accumulate within the nucleus due to phosphorylation, which confers a survival advantage to cancer cells.\(^14\) Improvement in PDT treatment by targeting disease-specific genes has also been demonstrated \textit{in vitro} for urothelial cancer,\(^15\) breast cancer,\(^16-18\) and head and neck cancer.\(^9,19\) Preclinical studies of nanoformulations combining PDT and chemotherapy have also been investigated as a strategy to combat drug resistance.\(^20-26\) However, an in-depth discussion of this modality is outside the scope of this chapter.

In this chapter, we investigated, the use of cESG for co-delivery of a PDT agent (TPPS) and siRNA (SOD2) for improved treatment efficacy. As discussed in chapter 3, SOD2 is a mitochondrially located enzyme essential for the dismutation of superoxide. Our lab has previously
demonstrated that the knockdown of SOD2 in ovarian cancer can improve chemosensitivity. It was our hypothesis that a similar increase in efficiency can be observed in photodynamic treatment by increasing susceptibility to superoxide-induced toxicity. This may induce a synergistic effect, in which lower dosages of TPPS may be used for therapeutic treatment (Figure 6.2). To investigate this hypothesis, we employed cESG for simultaneous delivery of siRNA and TPPS.

![Figure 6.2. Treatment scheme of cESG-mediated siRNA and TPPS combination therapy for increased photodynamic efficiency](image)

### 6.2 Methods

#### 6.2.1 Materials

McCoy’s 5A media, RPMI media, and Trypsin EDTA 1x were from ATCC. Hyclone fetal bovine serum was from GE Healthcare. Dulbecco’s phosphate buffer saline 1x was from FisherScientific. 5,10,15,20-tetrakis(4-sulfonatophenyl)-21H,23H-porphyrin (TPPS) was purchased from Frontier Scientific. A 5’ fluorescein 6-FAM-labeled, previously validated siRNA targeting SOD2 was purchased from Dharmaco (On-Target Plus 5’-CAACAGGCCCUCUAUCCACU-3’). A scramble oligonucleotide sequence was used as a non-targeting control (Dharmacon, OnTarget Plus Control siRNA Nontargeting siRNA #1). Antibodies were obtained from Cell Signaling Technology (Boston, MA) or Abcam (Cambridge, MA). Cell
experiments were performed using sterile, nuclease free water (Qiagen). All other reagents were purchased from Sigma Aldrich unless otherwise specified.

6.2.2 cESG-TPPS-siRNA Condensation Reaction

100 ng of siRNA was reacted with cESG-TPPS at either a 1:1, 4:1 or 8:1 molar ratio siRNA:cESG-TPPS and the diluted to a final volume of 14 µL using 1 mM sodium phosphate buffer. Formulations had either 24 or 42 TPPS per particle, with cESG concentrations of 340, 170, and 40 nM respectively. Reactions were conducted for 40 min, vortexing every 5 min to facilitate mixing and siRNA incorporation. Then, 10 µL of reaction was mixed with 2 µL of 10x loading buffer and loaded into a 1% agarose gel containing ethidium bromide. The gel was run in TAE buffer at 100 V for approximately 20 min, followed by ethidium bromide staining for an additional 10-15 min. Gels were imaged on a FluorChem E imager (Protein Simple).

6.2.3 DLS and Zeta Potential Analysis

DLS and zeta potential analysis were performed as described previously (2.2.1.3).

6.2.4 Cell Culture

Cell culture was performed as previously described (3.3.6).

6.2.5 Ability of cESG-TPPS-siRNA to Knockdown SOD2

cESG-TPPS-siRNA complexes were condensed immediately prior to treatment. Lyophilized cESG-TPPS was dissolved in 1 mM NaPO₄ buffer pH 7.5 at a concentration of 0.75 µM cESG. 10 µM siRNA (fluorescein-labeled with a SOD2-specific targeting sequence or scramble control) were prepared in distilled water. The particle and siRNA were then mixed at a 1:4 molar ratio (cESG-TPPS:siRNA). Solutions were vortexed and centrifuged for 15 seconds every 5 minutes, for a total 40 minute incubation period. Controls included cESG alone, cESG-TPPS, and cESG-TPPS-siRNA-scramble. Treatments were conducted in growth media for 3 days.
(10 nM siRNA, 15.2 nM cESG) followed by imaging and western blot analysis, as described previously (3.3.10). Uptake of the complex was assessed by fluorescence of the particle using an AMG EVOS flats microscope with Texas Red LED light source filtercube, using a 20x objective. Bright field images were taken simultaneously and the percentage of cell population fluorescing quantified using ImageJ.

6.2.6 Efficacy of cESG-TPPS-siRNA for PDT

ES-2 cells were plated in a 96 well plate at a density of 4,000 cells/well. The following day, cESG-TPPS of varying TPPS concentrations were condensed with SOD2 targeted siRNA at a 1:4 molar ratio, as described above. Complexes were diluted in growth media to a constant final concentration of 10 nM siRNA and 15.2 nM cESG. Final TPPS concentrations for treatment were 1.1, 0.46, 0.32, and 0.17 µM. Treatments using cESG-TPPS without siRNA were performed simultaneously for comparison. Cells were incubated with 100 µL of treatment solution for 3 days at 37 ºC. Then solutions were replaced with fresh growth media and illuminated with a 630 nm 8 mW/cm² LED lamp for 0 or 10 min. The following day, an MTT assay was conducted as previously described (3.3.8) to determine cell viability.

6.2.7 Investigation of Radical Generation Mechanism of TPPS in ES2 Cells

ES-2 cells were plated in a 96 well plate at a cell density of 4,000 cells/well. The following day, the cells were treated in growth media with 1 µM cESG-TPPS or free TPPS. After a 24 h incubation with compounds, the treatments were removed and replaced with either 5 mM sodium azide (a singlet oxygen scavenger) or growth media and incubated for 2 h, based on the protocol from Hirohara et al.27 Wells were then illuminated for 0 or 10 min using a 630 nm 8 mW/cm² LED lamp. Following a 30min incubation, treatments were removed and replaced with fresh growth
The next day, an MTT assay was conducted as previously described (3.3.8) to determine cell viability.

6.2.8 Statistical Analysis

All data presented are representative of at least three independent experiments and expressed as mean ± SEM, unless otherwise noted. Statistical data analysis were performed using OriginPro Software v8.5. ANOVA with Tukey’s post test were performed as indicated. In the case of two-way ANOVA analysis, the input variables (i.e. dosage, treatment type, illumination time) are considered independent and the mean differences between groups are compared.

6.3 Results and Discussion

6.3.1 cESG-TPPS siRNA Conjugation

We have shown that cESG having a 20mV zeta potential could successfully bind and facilitate siRNA and TPPS delivery. Previous studies indicate that storage of the cESG-siRNA complex for 3-4 weeks at 4 °C had an adverse impact on siRNA efficacy. Therefore, cESG-TPPS charge-condensation was conducted first followed by incubation with siRNA (Figure 6.3). cESG-TPPS formulations synthesized with variable concentrations of TPPS, all retained a positive zeta potential of approximately 5 mV.

![Figure 6.3. Conjugation scheme of cESG encapsulation of TPPS followed by siRNA condensation.](image-url)
In order to determine if this charge was sufficient for binding siRNA, a gel shift assay was performed. As previously described, in this assay, bound nucleic acid is too large to migrate through an electrophoresis gel and is retained in the well. The binding of siRNA with cESG-TPPS was investigated at molar ratios that exhibited SOD2 knockdown in previous cESG-siRNA cell experiments (1, 4, or 8 siRNA per particle). Following a 40 minute room temperature condensation reaction, complete binding occurred at all ratios tested (Figure 6.4). Two different cESG-TPPS formulations (24 or 42 TPPS per cESG) were tested, with no apparent difference in binding with increased TPPS counterion concentration. This indicates that the higher anionic TPPS species does not prevent siRNA binding, at the concentrations tested. Size and zeta potential analysis of the particles indicate that there is no major aggregation induced by siRNA condensation, with zeta potentials shifting toward neutral (Table 6.1).

![Figure 6.4. Gel shift assay of cESG-TPPS-siRNA complexes at various molar ratios cESG-TPPS-siRNA confirms condensation with two different formulations of cESG-TPPS. Impeded migration (asterisks), indicative of condensation, is observed in all wells, with no apparent decrease in binding in the presence of higher TPPS counterion concentration.](image)
Table 6.1. Size and zeta potential of cESG-TPPS-siRNA complexes with varying TPPS concentration.\(^a\)

<table>
<thead>
<tr>
<th>cESG-TPPS-siRNA TPPS per particle</th>
<th>Size ± Standard Deviation (nm)</th>
<th>Zeta-Potential ± Standard Deviation (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>51.2 ± 5.5</td>
<td>0.03 ± 15.1</td>
</tr>
<tr>
<td>16</td>
<td>56.4 ± 3.3</td>
<td>3.6 ± 32.1</td>
</tr>
<tr>
<td>24</td>
<td>36.1 ± 4.1</td>
<td>0.2 ± 14.3</td>
</tr>
<tr>
<td>42</td>
<td>37.7 ± 3.0</td>
<td>2.0 ± 22.3</td>
</tr>
</tbody>
</table>

\(^a\) cESG and siRNA concentration held constant, 4 siRNA per particle. Results were averaged over three measurements.

6.3.2 Efficacy of cESG-TPPS-siRNA Co-delivery Treatment

Once the ability of cESG-TPPS to bind siRNA was confirmed, experiments to determine if the complex had similar cellular uptake and gene knockdown to cESG-siRNA were performed. cESG-TPPS-siRNA-SOD2 treatment was investigated in ES-2 OCCC using cESG-TPPS with 42 TPPS per particle. siRNA condensation was conducted immediately before treatment and cellular uptake in growth media assessed by the red fluorescent signal of TPPS after a three day incubation. Nearly 100% of the cell population internalized cESG-TPPS and cESG-TPPS-siRNA formulations (scramble and SOD2 targeted, Figure 6.5). This is a slight increase in uptake over that seen with cESG-siRNA-SOD2, which was taken up by 90% of cell population under the same conditions. SOD2 knockdown was assessed using western blotting and quantified. There was successful SOD2 knockdown using cESG-TPPS-siRNA-SOD2, with SOD2 expression decreased to 35% (normalized to cESG treated control). This is on par with cESG-siRNA-SOD2 treatment which resulted in 25% protein expression (Figure 6.6). This result demonstrates that cESG-TPPS can be used as a siRNA delivery vector for ES-2 cells and that it is comparable to cESG-siRNA alone.
Figure 6.5. cESG-TPPS and cESG-TPPS-siRNA are internalized by nearly 100% of the ES-2 cell population after a 72 h incubation with complexes. Fluorescence images (right) overlayed with phase contrast images (left). Quantification of the percent of cell population which displayed fluorescent signal (bottom) (n=2 independent experiments, n=3 images at least 50 cells/image. Mean ± Standard deviation. Scale bar 200 µm)
Figure 6.6. A) cESG-TPPS-siRNA facilitates knockdown of SOD2 protein expression similarly to cESG-siRNA in ES-2 OCCC. Cells treated for 72 hrs prior to assessment of SOD2 protein expression by western blotting. B) Western blot data was quantified by densitometric analysis and normalized to expression of the loading control β-Actin and expressed relative to SOD2 expression in vector control. (Mean ± standard deviation. n=2 experiments for cESG-TPPS-siRNA or n=3 experiments for cESG-siRNA)
A PDT experiment was performed to investigate if there was a synergistic effect between SOD2 knockdown and light treatment. Again, siRNA condensation was conducted immediately prior to the treatment of the cells in growth media. All treatments were conducted at a constant 17 nM cESG and 10 nM siRNA, with varying TPPS concentrations. Cells were incubated for 3 days with the particle to allow for SOD2 knockdown. Following this incubation, cells were treated with 630 nm light for 10 minutes in fresh growth media. The next day, viability was assessed using an MTT assay. Both cESG-TPPS and cESG-TPPS-siRNA exhibited minimal dark toxicity (Figure 6.7). Upon illumination, TPPS was effective at inducing cellular death, with increasing response at increasing TPPS dosage. Cells with siRNA-mediated SOD2 knockdown showed a slightly increased efficacy compared to cESG-TPPS treatment alone, but there was no statistically significant difference between treatments (p>0.05).
Figure 6.7. cESG-TPPS-siRNA shows no statistically significant effect in an *in vitro* PDT model. ES-2 ovarian clear cell carcinoma cells treated for 72 hours with cESG-TPPS or cESG-TPPS-siRNA prior to 10 minute illumination with light, show no statistically significant difference with the inclusion of siRNA against SOD2. (mean ± standard error of the mean, n=4 independent experiments, *p<0.01, Two-Way ANOVA with Tukey’s post-test).

In order to further investigate why SOD2 knockdown did not have a synergistic effect with PDT, the mechanism of TPPS radical generation was probed. TPPS may generate radicals via a combination of type I (ROS) and type II (singlet oxygen) mechanisms. SOD2 is specific for the dismutation of superoxide, so in order for knockdown to have the most substantial effect, a type I radical generation would need to feed into the SOD2 pathway. The generation mechanism of...
photosensitizers is influenced by their surroundings, and therefore must be characterized in different cellular environments.\textsuperscript{29} In order to investigate whether the TPPS radical generation in cells are type I or type II, a singlet oxygen scavenger, sodium azide, was used.\textsuperscript{27} In this assay, cells are pretreated with sodium azide for 2 hours prior to illumination. During the course of illumination, singlet oxygen which is generated is quenched by sodium azide, preventing cellular damage. Other ROS, like superoxide, are not quenched via this pathway, and would still result in cell death. Control cells treated with cESG-TPPS in the absence of scavenger responded to light treatment as expected, exhibiting increasing toxicity with increasing TPPS concentrations (Figure 6.8). Corresponding treatment with the quencher present, had a substantial increase in viability, indicating that the production of singlet oxygen rather than superoxide is the principal mechanism involved in TPPS radical generation and cell toxicity. Considering these results, SOD2 is not a suitable genetic target for combination therapy using TPPS as a photosensitizer.
Figure 6.8. Quenching of singlet oxygen radicals reduce the toxicity from PDT using cESG-TPPS in ES-2 OCCC. Cells treated overnight with cESG-TPPS complexes show toxicity upon illumination. Pretreatment with sodium azide prior to illumination increases viability (mean ± standard deviation, n=2 independent experiments).

6.4 Conclusions

In this chapter we investigated the use of cESG for the codelivery of therapeutic siRNA against SOD2 and the photosensitizer TPPS. Loading of cESG with both moieties was accomplished via sequential charge condensation reactions. At all formulations tested, siRNA was able to bind cESG, even though the overall cESG charge was reduced due to TPPS binding. In an in vitro ES-2 OCCC model, the codelivery system was successful for both the delivery of siRNA, as seen by SOD2 knockdown, and TPPS, demonstrated by effective phototoxicity upon illumination. This treatment modality was not synergistic, as TPPS in the system generated singlet oxygen rather than superoxide, but codelivery using cESG was demonstrated.

This verifies the flexibility of cESG for delivery, as both individual components were successful. In future experiments, siRNA against a different genetic target should be explored. As
previously discussed, knockdown of EGFR, HIF1α, or VEGF-A could be targeted to improve PDT response in resistant tumor populations. Alternately, a type I (superoxide) generating photosensitizer could be employed in conjunction with SOD2 knockdown. The versatility of the complex could also be probed for other co-delivery strategies, incorporating other anionic therapeutics or contrast imaging agents.

6.5 References


CHAPTER 7.

CONCLUSION AND FUTURE DIRECTIONS

7.1 Summary

The motivating hypothesis of this research was that ESG is a promising material for drug delivery applications. Investigation into the application of chemically modified ESG for therapeutic siRNA and photosensitizer demonstrated both benefits and caveats in the future development of this system as a delivery vehicle.

7.1.1 Chemical modification of ESG

The cationic modification of ESG was investigated via two different reaction schemes. Desired product characteristics were: diameter of approximately 30 nm, high uniformity, a positive zeta potential, and stability under physiological conditions. Periodate oxidation of ESG was tunable, controlling the percent oxidation of the particle. In order to incorporate positive quaternary ammonium groups (quats) into the complex, aldehydes were reacted with Girard’s Reagent T. The resultant complex had a slightly positive zeta potential but was polydisperse, exhibiting breakage at alkaline conditions. As such, the modification was not explored further.

A one-pot modification of ESG via an epoxide ring opening reaction with glycidyltrimethylammonium chloride (GTMA) was more successful. An overnight room temperature modification in pH 12.5 sodium phosphate buffer resulted in the incorporation of 1 quat per 3.6 to 5.3 glucose residues (measured by EA-IRMS and XPS respectively). This
corresponded to a 20 mV zeta potential. Metrology and DLS indicated minimal agglomeration, with retention of its sub-50 nm diameter.

7.1.2 Application of cESG for Nucleic Acid Delivery

cESG was then tested for application as a nucleic acid delivery system. The cationic particle was capable of condensing both a 4700 base pair plasmid and 21 base pair siRNA. However, transfection of mammalian ES-2 OCCC cells using the cESG-plasmid conjugate for delivery was unsuccessful. This was attributed to either a lack of cESG-mediated cell entry or the inability of the system to dissociate intracellularly. cESG-siRNA was able to mediate protein expression knock-down of the target transcript in ES-2 cells. A 74% knockdown of the SOD2 target protein was accomplished by either 1 day or 3 day incubations with the complex. This protein knockdown was sustained for at least 3 days following treatment. Unfortunately, no protein knockdown was observed with cESG-siRNA treated serous ovarian adenocarcinoma (Ovca420) and only a 20% protein reduction was obtained in noncancerous epithelium (Nose007). Storage of the cESG-siRNA conjugate reduced efficacy in ES-2 cells.

In order to further characterize the cellular behavior of cESG, mechanistic uptake studies were performed. Fluorescently labeled cESG was internalized by all cell lines at similar rates, with 75% of cell populations internalizing the complex in 4 hours and nearly 100% of the populations exhibiting fluorescence after 24 hours of treatment. cESG uptake was energy dependent and localized intracellularly within vesicles. When tested in three dimensional ES-2 spheroid and CAM models, cESG was still internalized within individual cells. The expression of glycogen breakdown enzymes was also probed, but showed no correlation with siRNA efficiency in mediating protein expression decreases. Modification of ESG made cESG resistant to enzymatic degradation by alpha amylase, but did not change pullulanase degradation.
7.1.3 Application of cESG for Photosensitizer Delivery

cESG was also investigated for the delivery of photosensitizers for photodynamic therapy. Hydrophobic encapsulation of THPP was accomplished using ESG and cESG, but was inefficient with only 1 bound for every 7-8,000 carrier molecules. As such, encapsulation of THPP did not improve illumination response over that of free THPP. The charge-based condensation of TPPS using cESG was far more efficient, binding up to 100 TPPS per particle. Photodynamic therapy using various formulations of cESG-TPPS markedly improved cell killing with the complex at low dosages compared to free photosensitizer.

The co-delivery of TPPS and siRNA against SOD2 was then investigated. Delivery of both compounds simultaneously resulted in successful siRNA knockdown and, upon illumination, cell death. The therapeutics both satisfied their individual roles but the therapies did not synergize. While SOD2 knockdown increased susceptibility to superoxide toxicity, TPPS induced toxicity by generation of singlet oxygen rather than superoxide.

7.2 Critical Assessment and Recommended Future Directions

The studies contained in this dissertation indicate that cESG may be a viable delivery vehicle. However, future studies should focus on better characterizing the complex prior to any advancement to in vivo applications. One of the greatest concerns for the use of cESG as a siRNA delivery vector is the widely different efficacies in ovarian-derived cell lines. The cause of decreased transfection in cell lines is unknown. Possible explanations for this may be endosomal entrapment, preventing cESG-siRNA release into the cytosol, and/or a lack of dissociation of the siRNA from the cESG complex, both resulting in a lack of protein knockdown of the siRNA target. Other cationic polymers, such as PLL and chitosan, suffer from low efficacy as they have poor endosomal escape.\(^1\)\(^-\)\(^3\) In order to determine if insufficient escape contributes to cESG-siRNA
efficacy, the intracellular tracking of cESG should be assessed. This may be achieved through the use of various fluorescent organelle stains, such as Lysotracker (ThermoFisher) or dextran conjugates. In order to trace the intracellular fate of cESG in real time, co-localization with pH responsive dyes could be used, which change fluorescent intensity as pH decreases from the early endosome to late endosome and finally lysosomal compartments (pHrodo™ ThermoFisher). One strategy to overcome endosomal entrapment, which has been successful with chitosan compounds and may be applicable in our system, is induction of the proton sponge effect by chemical modification with buffering moieties, such as PEI,4,5 polypropyl acrylic acid6 or histidine.7 These moieties contain pKₐ responsive amines that buffer endosomal pH, which leads to an influx of protons and eventual rupture of the vesicle.8 As quaternary ammonium groups are not pKₐ dependent, they will not undergo escape via this mechanism.

It was our hypothesis that the inherent biodegradability of glycogen would provide a mechanism for targeted siRNA release. Moreover, the reported increased expression of glycogen breakdown enzymes, such as PYGL in certain cancers, could potentiate the breakdown of the particle and release of cargo in cancer cells. Overexpression of PYGL has been observed in glioblastoma, breast and colon cancers to compensate for decreased nutrients in the tumor microenvironment.9 However, in studies of expression of glycogen degradative enzymes in our model, enzyme overexpression did not correlate with siRNA efficacy. There was no observable siRNA activity in cESG-siRNA treated Ovca420 cells, which express relatively high levels of the enzymes responsible for hydrolysis of α(1-4) and α(1-6) linkages. cESG-siRNA was actually most effective in ES-2 OCCC, which had the lowest relative enzyme expression. However, future studies should confirm enzyme expression at the protein level. This indicates that siRNA release
and efficacy may not be largely impacted by enzymatic involvement, or as previously mentioned the complex was not released into the cytosol.

The decrease in cESG-siRNA efficacy after storage for 1 month at 4 °C indicates that there is some natural degradation or dissociation of the complex in the absence of enzymes. As such, future studies must probe the mechanism and timecourse of release of cargo from cESG. The activity of physiologically relevant glycogen breakdown enzymes (AGL, PYGL) toward cESG should be probed ex vivo to determine what role they may play in release of cargo. In vitro experiments may also be conducted to analyze this relationship, in which glycogen degradation enzyme expressions could be knocked-down by RNAi, followed by an assessment of cESG-siRNA efficacy. If the cause of differing efficacy can still not be determined, the use of cESG-siRNA in an array of cell types of varying origin may assist in observing trends in transfection efficiency. Unfortunately, it is difficult to tune release properties of non-covalent charge condensed cargo. One strategy to improve release using cESG would be to utilize a less-modified cESG with a lower zeta potential, such as the 9.5 ± 4.1 mV cESG produced at a 1:5 ratio ESG:GTMA. This weaker charge interaction might more readily allow for particle dissociation, but also could adversely impact particle uptake.

At this stage, application of cESG as a delivery vehicle for photosensitizers is more viable than siRNA transfection, as it does not require release of cargo specifically into the cytoplasm. In previous studies, illumination of TPPS was shown to induce escape from lysosomes due to membrane disruptions, a phenomena which may assist in compartmental escape for cESG-TPPS. Particle encapsulation may be an effective way to overcome the major hurdle that has prevented the clinical implementation of TPPS. In the 1980s, it was demonstrated that unencapsulated TPPS caused neurotoxicity that was further exacerbated by increased circulation
time from albumin binding. Since cESG encapsulation of TPPS improved the efficacy of the photosensitizer at low dosages, this could allow for decreasing dosages used for treatment in order to combat toxicity. We also anticipate that the encapsulation of TPPS should lower neurotoxicity due to its larger size and a lowering of the affinity of albumin for the complex.

In current literature, studies have characterized the photochemical response of TPPS encapsulated in dextrin, PAMAM dendrimers, and nanoporous silicon. Investigations reveal that while encapsulation prevents aggregation, it can introduce changes in the triplet states following excitation. While TPPS light response was retained in our cESG-TPPS formulations as evidenced by the cell death response, future studies analyzing the triplet state decay profile in conjunction with quantifying singlet oxygen generation of cESG-TPPS complexes may determine which TPPS loading density (TPPS per cESG) has optimal ROS generation.

Cell killing upon illumination with cESG-TPPS was enhanced compared to not only free TPPS, but also other TPPS nanoformulations (Table 7.1). Direct comparison of systems is difficult due to the many variables impacting treatment, including TPPS dose, light dosage, excitation wavelength, and cell type. It is of note that for nanovehicles resulting in equivalent or improved cell death response compared to cESG, TPPS dosages were approximately an order of magnitude higher (7.5-15 times). This indicates that our system may decrease TPPS dosage below that of other nanovehicles in order to combat neurotoxicity and serve as a feasible photosensitizer for clinical use. While our disease model of interest was ovarian cancer, TPPS may have application as a photosensitizer for not only other cancers but also other diseases including macular degeneration, acne vulgaris and actinic keratosis.
Table 7.1 Efficacy of various TPPS-nanoformulations for photodynamic therapy in vitro

<table>
<thead>
<tr>
<th>Material</th>
<th>Cell Type</th>
<th>TPPS Dosage</th>
<th>Light Dosage</th>
<th>Cell Viability After Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>porous silicon nanoparticles(^{19})</td>
<td>Human Breast Cancer (MCF-7)</td>
<td>0.3 µM</td>
<td>40 min 650 nm</td>
<td>58%</td>
</tr>
<tr>
<td>cationic amphiphilic cyclodextrin(^{20})</td>
<td>Human Cervical Adenocarcinoma (HeLa)</td>
<td>1 µM</td>
<td>30 min Visible Light</td>
<td>40%</td>
</tr>
<tr>
<td>Hyaluronate decorated chitosan hydrogel(^{21})</td>
<td>Murine Macrophages (RAW 264.7)</td>
<td>2 mM</td>
<td>12.5 min 652 nm</td>
<td>25%</td>
</tr>
<tr>
<td>PMMA (polymethyl methacrylate) nanoparticle(^{22})</td>
<td>Human mesenchymal stem cells</td>
<td>4.8 µM</td>
<td>5 min 405 nm laser</td>
<td>15%</td>
</tr>
<tr>
<td>cESG</td>
<td>Human Ovarian Carcinoma</td>
<td>0.6 µM</td>
<td>10 min 630 nm LED</td>
<td>15%</td>
</tr>
<tr>
<td>PMMA nanoparticle(^{23})</td>
<td>Murine Melanoma (B78H1)</td>
<td>~9 µM</td>
<td>5 min White Light</td>
<td>12%</td>
</tr>
<tr>
<td>octaarginine-conjugated β-cyclodextrin(^{24})</td>
<td>Human Cervical Adenocarcinoma (HeLa)</td>
<td>5 µM</td>
<td>15 min 310-510 nm</td>
<td>10%</td>
</tr>
<tr>
<td>Nitroaniline chromophore decorated PMMA nanoparticle(^{23})</td>
<td>Murine Melanoma (B78H1)</td>
<td>~4.5 µM</td>
<td>5 min White Light</td>
<td>&lt;1%</td>
</tr>
</tbody>
</table>

\(^{a}\)For papers reporting multiple treatment conditions, those resulting in the lowest cell viability after treatment are described.

From the perspective of particle toxicity, the high tolerability of cESG in in vitro MTT viability assays is a promising first step. But if IV administration of the complex is to be investigated, the behavior of the complex in the presence of proteins must be thoroughly characterized in vivo. In vitro studies demonstrated that cESG-siRNA interacted with proteins in fetal bovine serum, inducing aggregates that were not seen in cESG alone, cESG-TPPS, or in serum free media. While transfection was still achieved despite these aggregates, in vivo administration may result in agglomerations that may decrease efficacy or induce toxicity.
Assessment of the hemolytic activity of cESG and any cESG-conjugate complex may also help determine if in vivo tolerability testing is ethically justifiable.\textsuperscript{25}

A major hurdle to therapeutic gene delivery via viral vectors is difficulty scaling up production.\textsuperscript{26} As food grade ESG is commercially available in bulk quantities at low cost and cESG modification straightforward, scaling up synthesis is feasible. Both ESG and cESG displayed low cytotoxicity in our in vitro assay. In conjunction with successful cESG-mediated delivery of siRNA and the improved delivery of TPPS by encapsulation, this establishes cESG as a promising candidate for further investigations. cESG may be explored as a delivery vehicle for not only other siRNAs and photosensitizers, but also traditional therapeutic drugs and small molecules.

### 7.3 References


