Title
Sustained and observable release of therapeutics using porous silicon based materials

Permalink
https://escholarship.org/uc/item/4mx8b6rn

Author
Wu, Elizabeth Chung Pui

Publication Date
2010

Peer reviewed|Thesis/dissertation
UNIVERSITY OF CALIFORNIA, SAN DIEGO

SUSTAINED AND OBSERVABLE RELEASE OF THERAPEUTICS USING POROUS SILICON BASED MATERIALS

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Bioengineering by Elizabeth Chung Pui Wu

Committee in charge:
Professor Michael J. Sailor, Chair
Professor Michael J. Heller, Co-chair
Professor Sadik C. Esener
Professor William H. Freeman
Professor Gabriel A. Silva

2010
Copyright

Elizabeth Chung Pui Wu, 2010

All rights reserved.
The dissertation of Elizabeth Chung Pui Wu is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-chair

Chair

University of California, San Diego

2010
DEDICATION

To my husband Andrew
# Table of Contents

Table of Contents ........................................................................................................ v

List of Figures ............................................................................................................. xi

List of Tables ............................................................................................................... xvi

Acknowledgements ...................................................................................................... xvii

Vita ................................................................................................................................. xx

Abstract of the Dissertation ......................................................................................... xxi


## Chapter One ............................................................................................................. 1

1 Introduction To Nanostructured Materials For Drug Delivery ............................ 2

1.1 Introduction and Motivation ............................................................................. 2

1.2 Design of Nano-scale Drug Delivery Systems ............................................... 3

1.2.1 Particle Size ................................................................................................. 3

1.2.2 Surface Chemistry ....................................................................................... 4

1.2.3 Shape ........................................................................................................... 5

1.3 Materials for Drug Delivery .............................................................................. 6

1.3.1 Polymeric particles ..................................................................................... 6

1.3.2 Liposomes .................................................................................................. 8

1.3.3 Micelles ...................................................................................................... 9

1.3.4 Dendrimers ................................................................................................. 9

1.3.5 Carbon-Based Nanoparticles ..................................................................... 10

1.3.6 Inorganic Materials ................................................................................... 10

1.4 Porous Silicon .................................................................................................. 12

1.4.1 Introduction ................................................................................................ 12

1.4.2 Preparation of Porous Si ........................................................................... 12

1.4.3 Optical Properties of Porous Si ................................................................. 22

1.4.4 Biocompatibility ........................................................................................ 25

1.4.5 Chemistry of Porous Si ............................................................................. 26

1.4.6 Drug Delivery Using Porous Si ................................................................. 26
1.5 Ocular Drug Delivery ................................................................. 28
  1.5.1 Ocular Membranes and Barriers ........................................ 28
  1.5.2 Ocular Drug Delivery Systems .......................................... 32
1.6 Conclusions ........................................................................... 33
1.7 Scope of the Dissertation ........................................................ 34

**CHAPTER TWO** ........................................................................... 37
2 Oxidation-Triggered Release of Fluorescent Molecules or Drugs from Mesoporous
  Silicon Microparticles .................................................................. 38
  2.1 Abstract .................................................................................. 38
  2.2 Introduction .............................................................................. 39
  2.3 Experimental Methods .............................................................. 41
    2.3.1 Synthesis of Porous Si Microparticles ................................. 41
    2.3.2 Hydrosilylation of Porous Si Microparticles ....................... 42
    2.3.3 Loading of Alexa Fluor 488 and Doxorubicin .................... 42
    2.3.4 Physical Characterization of Porous Si Microparticles .......... 43
    2.3.5 Microscopy .......................................................................... 43
    2.3.6 Determination of Drug Loading ........................................... 44
    2.3.7 In Vitro Release Studies ....................................................... 44
    2.3.8 Cell Toxicity Assay .............................................................. 45
  2.4 Results and Discussion ............................................................. 46
    2.4.1 Preparation of Porous Si Microparticles .............................. 46
    2.4.2 Loading of Alexa Fluor 488 and Daunorubicin into Porous Si
      Microparticles ........................................................................... 50
    2.4.3 Oxidation-Triggered Fluorescence Increase of Dye Loaded Particles ... 52
    2.4.4 Oxidation-Triggered Release of Alexa Fluor 488 ................. 57
    2.4.5 Loading and Release of Doxorubicin From Porous Si Microparticles ... 60
    2.4.6 Functionality of Released Doxorubicin ............................... 63
  2.5 Conclusions ............................................................................. 67
CHAPTER THREE .......................................................... 69
3 Real-time Monitoring of Sustained Drug Release Using the Optical Properties of
Porous Silicon Photonic Crystal Particles ........................................... 70
3.1 Abstract .................................................................................. 70
3.2 Introduction ............................................................................. 70
3.3 Experimental Methods ............................................................. 73
  3.3.1 Formation of Porous Si Microparticles .............................. 73
  3.3.2 Characterization of Porous Si Microparticles .................... 75
  3.3.3 Loading of Daunorubicin into Porous Si Microparticles ....... 75
  3.3.4 In Vitro Porous Si Particle Degradation and Drug Release ... 76
  3.3.5 Determining Average Reflectivity Spectra of Porous Si Particles ...... 76
  3.3.6 Acquisition and Analysis of the Reflectivity of Individual Porous Si
      Particles .................................................................................... 77
  3.3.7 In Vitro Cellular Proliferation and Toxicity Assays .......... 77
3.4 Results and Discussion ............................................................ 78
  3.4.1 Fabrication of Daunorubicin-Loaded Porous Si Microparticles ... 78
  3.4.2 Characterization of Porous Si Particles ................................. 80
  3.4.3 In Vitro Release of Daunorubicin Covalently Attached to Porous Si
      Microparticles ........................................................................... 82
  3.4.4 Correlation of Spectral Shift with Drug Release ................. 85
  3.4.5 Distribution of the Reflectivity Spectra of Individual Particles ...... 90
  3.4.6 Effect of Released Daunorubicin on ARPE-19 Cells .......... 92
3.5 Conclusions ............................................................................ 98

CHAPTER FOUR ........................................................................ 100
4 Surface Modification of Porous Silicon for Long-term Delivery of Redox Active
  Therapeutics ................................................................................. 101
  4.1 Abstract ................................................................................. 101
  4.2 Introduction ............................................................................ 102
  4.3 Experimental Methods ........................................................... 104
4.3.1 Synthesis of Porous Si Microparticles ........................................ 104
4.3.2 Surface Modification of Porous Si Microparticles ......................... 104
4.3.3 Preparation of Mesoporous Silica Nanoparticles .......................... 105
4.3.4 Loading of Daunorubicin ....................................................... 106
4.3.5 Characterization of Released Daunorubicin ............................... 107
4.3.6 Daunorubicin Release from Porous Si Microparticles .................... 107
4.3.7 Cell Toxicity of Daunorubicin ............................................... 108

4.4 Results and Discussion ................................................................ 108
4.4.1 Modification of Porous Si by Thermal Hydrosilylation and Oxidation at 150°C ............................................................................. 108
4.4.2 Modification of Porous Si by Oxidation at 800°C ......................... 109
4.4.3 Loading of Daunorubicin to Porous Si and SiO₂ Particles .............. 111
4.4.4 Characterization of Released Daunorubicin .............................. 114
4.4.5 Cell Toxicity of Released Drug .............................................. 119
4.4.6 In Vitro Release of Daunorubicin ......................................... 122

4.5 Conclusions ............................................................................... 125

CHAPTER FIVE ......................................................................................... 127
5 Degradation and Biocompatibility of Porous Si and SiO₂ Microparticles in the Rabbit Eye ................................................................. 128
5.1 Abstract ................................................................................... 128
5.2 Introduction ............................................................................... 128
5.3 Experimental Methods ............................................................... 131
5.3.1 Synthesis of Porous Si Microparticles .................................... 131
5.3.2 Surface Modification of Porous Si Microparticles .................... 131
5.3.3 Loading of Daunorubicin into Porous Si and SiO₂ Particles ......... 132
5.3.4 Biodistribution of Non-Drug Loaded Porous Si Microparticles in the Rabbit Eye ................................................................. 133
5.3.5 Toxicity of Non-Drug Loaded Porous Si Microparticles .......... 134
5.3.6 Toxicity of Drug Loaded Porous Si and SiO₂ Microparticles ....... 134
A.4.4 Loading and Release of Foscarnet from Amine Functionalized Porous Silica Microparticles ................................................................. 178
A.4.5 Loading and Release of Foscarnet from Amine Functionalized Mesoporous Silica Nanoparticles .......................................................... 182
A.5 Conclusions ............................................................................... 189

REFERENCES ......................................................................................... 190
# List of Figures

**Figure 1.1** Schematic of the electrochemical etch cell for the formation of porous Si. .......................... 14

**Figure 1.2** Mechanism for the electrochemical etching of silicon. ............................................... 15

**Figure 1.3** Cross-sectional scanning electron micrograph image of two porous Si films showing the effect of dopant on pore structure ................................................................. 18

**Figure 1.4** Plan-view atomic force microscope image of a porous Si film containing a lateral gradient of pore sizes, illustrating the rage of pore dimensions that can be accessed. Image reproduced with permission from Ref. [33], copyright 2002 Wiley-VCH .................................................................................. 19

**Figure 1.5** Preparation of micrometer and nanometer size porous Si particles by ultrasonic fracture. .............................................................................................................................. 21

**Figure 1.6** The fabrication of a porous Si rugate film: application of a sinusoidal current waveform during the electrochemical etch can be used to fabricate a porous structure in which porosity varies periodically through depth ........................................ 23

**Figure 1.7** An example of a optical reflectance spectrum of a single porous Si rugate particle ................................................................................................................................. 24

**Figure 1.8** Illustration of the eye showing the biological barriers that prevent the delivery of many ocular therapeutics, reproduced with permission from Ref. [55], copyright 2008 Informa Plc. .................................................................................................................. 31

**Figure 2.1** Schematic of the loading of molecular payloads in porous Si microparticles by covalent attachment. ............................................................................................................. 48

**Figure 2.2** Scanning electron microscope (SEM) image of a representative sample of porous Si microparticles (a). A SEM view of one of the microparticles, revealing the mesoporous structure (b)........................................................................................................ 49

**Figure 2.3** Diffuse-reflectance Fourier-transform infrared spectra of porous microparticles .......................................................................................................................... 51

**Figure 2.4** Microscope images of porous Si microparticles with Alexa Fluor 488 attached, showing the increase in fluorescence intensity as the porous Si microparticles oxidize ........................................................................................................ 55
Figure 2.5 Comparison of the change in fluorescence intensity of an Alexa Fluor 488 dye grafted to porous Si microparticles as a function of time after addition of peroxynitrite (ONOO\(^-\)) .......................................................... 56

Figure 2.6 Oxidation of the porous Si matrix results in a fluorescence intensity increase of the dye and produces Si-O back bonds that are more susceptible to hydrolysis, allowing dye release ................................................................. 58

Figure 2.7 Release of covalently attached Alexa Fluor 488 from porous Si microparticles as a function of time in various solutions, demonstrating ROS-triggered release .................................................................................. 59

Figure 2.8 Release of doxorubicin from porous Si microparticles into PBS buffer .... 62

Figure 2.9 Viability of HeLa cells (MTT assay) after exposure to free doxorubicin, empty porous Si microparticles (empty-pSiMPs), and porous Si microparticles loaded with doxorubicin by covalent attachment (dox-pSiMPs) ......................... 64

Figure 2.10 Analysis of as received doxorubicin (top) and doxorubicin released from porous Si microparticles (bottom) by LC-UV with monitoring at 280 nm. .......... 66

Figure 3.1 Loading of daunorubicin into porous Si microparticles .................................. 79

Figure 3.2 (a) Scanning electron microscope (secondary electron) image of a representative sample of porous Si microparticles used in this study. (b) Representative white light reflectance spectrum from a single porous Si particle. 81

Figure 3.3 In vitro release of daunorubicin covalently attached to porous Si microparticles ................................................................................................................................. 84

Figure 3.4 Optical microscope images showing the color change of particles as they degrade in pH = 5, 7, or 9 buffer solutions .................................................................................................................................................. 88

Figure 3.5 Quantification of silicon dissolution (a), drug release (b) and spectral peak shift (c) as a function of time for drug-loaded porous Si photonic crystal microparticles in aqueous buffer solutions at pH 5, 7, and 9 ........................................... 89

Figure 3.6 Distribution of values of \(\lambda_{\text{max}}\) in an ensemble of particles, monitored during degradation in aqueous solution .......................................................................................................................... 91

Figure 3.7 Viability of ARPE-19 cells (MTT assay) after exposure to as-received daunorubicin, silicon released (as soluble orthosilicate) from porous Si
microparticles (containing the undecylenic acid linker chemistry but not containing any drug), and daunorubicin that had previously been attached to porous Si microparticles and then released by hydrolysis into aqueous solution.

**Figure 3.8** Fluorescence microscope images of ARPE-19 cells comparing the toxicity of as-received daunorubicin (“Free Drug”), daunorubicin that had previously been attached to porous Si microparticles (“Released Drug”), and the soluble by-products of porous Si dissolution from non drug loaded porous Si (“Released Si”).

**Figure 3.9** (top) Representative mass spectra of as-received daunorubicin generated by electron spray ionization (ESI) mass spectrometry.

**Figure 4.1** FTIR spectra of hydrosilylated porous Si particles (a), hydrosilylated particles treated with thermal oxidation at 150°C for 48 hours (b), and porous Si particles oxidized at 800°C for 1 hour (c).

**Figure 4.2** Schematic showing the reduction of daunorubicin by porous Si into semiquinone radical, then hydroquinone, and then further degradation products in which the amino sugar is eliminated.

**Figure 4.3** Schematic showing the loading of daunorubicin onto porous Si microparticles that were thermally oxidized and/or oxidized at 150°C (a) and loading of daunorubicin into porous SiO₂ microparticles prepared by thermal oxidation at 800°C (b).

**Figure 4.4** LC trace with UV detection at 480 nm of daunorubicin (a) and MS analysis of the peak detected in the LC trace of daunorubicin (b).

**Figure 4.5** LC traces with UV detection at 480 nm of compounds released from hydrosilylated particles (a), particles oxidized at 150°C for 48 hours (b), and particles oxidized at 800°C for 1 hour (c).

**Figure 4.6** High resolution MS of the sample (a) and simulation of the proposed compound (b) to confirm the presence of daunorubicin attached to Si through the succinic anhydride linker.

**Figure 4.7** Fluorescence microscope images of ARPE-19 cells comparing the toxicity of as-received daunorubicin to released drug from hydrosilylated particles, from
particles oxidized for 48 hours at 150°C after hydrosilylation, and from particles oxidized for 1 hour at 800°C .......................................................... 121

**Figure 4.8** Quantification of drug release (a) and Si dissolution (b) from porous Si and SiO₂ particles in PBS. Comparison rate of drug release and Si dissolution between silica particles prepared by thermal oxidation and the sol-gel route (c) ............... 124

**Figure 5.1** Fundus images of rugate structured porous Si particles hydrosilylated with undecylenic acid 4 days and 21 days after injection into the rabbit eye .......... 139

**Figure 5.2** Light microscopic images taken at 25x magnification (a) and 62.5x magnification (b), showing normal retina and choroid, 6 months after intravitreal injection .......................................................... 142

**Figure 5.3** Light microscopic images taken at 3 weeks and 3 months after intravitreal injection with hydrosilylated porous Si particles (OD) or BSS control (OS). Images show normal retinal morphology (taken at 62.5x magnification) .......... 144

**Figure 5.4** Intraocular pressures of eyes injected with daunorubicin loaded porous silica particles (OD) and control eyes injected with BSS (OS) ................. 147

**Figure 5.5** ERG b-wave amplitudes of eyes injected with daunorubicin loaded porous silica particles (OD) and control eyes (OS). Results indicate normal retinal function, with no statistical different between control and sample group .......... 148

**Figure 5.6** Light microscopic images taken at 3 weeks and 3 months after intravitreal injection with oxidized porous Si particles (OD) or BSS control (OS). Images show normal retinal morphology (taken at 62.5x magnification) ................. 149

**Figure 5.7** Fundus images showing the daunorubicin loaded oxidized porous Si particles in the vitreous on day 1 (a), 1 week (b), 1 month (c), and 2 months (d) after intravitreal injection .......................................................... 152

**Figure A.1** Schematic showing the reaction of the hydroxyl group on porous silica with the phosphate group of foscarnet ......................................................... 169

**Figure A.2** FT-IR spectra of foscarnet (a) and porous silica microparticles post reflux in a 24 mg/mL foscarnet solution (b) ......................................................... 170

**Figure A.3** TGA of foscarnet and porous silica microparticles after reflux with 24 mg/mL foscarnet solution ........................................................................ 171
**Figure A.4** Standard curve for $\lambda=625$ nm and $\lambda=445$ nm for the presence of foscarnet. Absorbance at 625 nm is due to the YbPV complex and the absorbance of 445 nm is due to the release of free PV ................................................................. 174

**Figure A.5** Scanning electron micrograph image of the porous Si particles prior to thermal oxidation (a) and the pore size of the particles prior to oxidation (b) .... 176

**Figure A.6** Release of Foscarnet from porous Si microparticles trapped by thermal oxidation. ................................................................. 177

**Figure A.7** Release of foscarnet from porous silica microparticles with pore sizes 10, 30 and 70 nm................................................................. 181

**Figure A.8** STEM image of mesoporous silican nanoparticles prepared without the addition of TEOS/APS in a dropwise manner (a-b). SEM image of mesoporous silica nanoparticles prepared with the addition of TEOS/APS in a dropwise manner (c). TEM image revealing the pore structure of the sol-gel derived porous silica nanoparticles (d)................................................................. 183

**Figure A.9** $\zeta$-potential titrations for mesoporous silica nanoparticles before and after functionalization with PEI ................................................................. 184

**Figure A.10** Comparison of the release of foscarnet from silica particles functionalized and not functionalized with PEI................................................................. 188
LIST OF TABLES

Table 5.1 Distribution of Si (displayed in µg) found in the various tissues of the eye.
Particles were injected in right eye (OD), and the fellow eye (OS) received an
injection of BSS. ................................................................. 138

Table 5.2 Clinical examination of the retina after injection with porous Si
microparticles hydrosilylated with undecylenic acid................................. 141

Table A.1 Energy-dispersive X-ray (EDX) spectroscopy mapping of porous silica
particles post reflux with 24 mg/mL foscarnet ........................................ 172

Table A.2 The amount of drug loading for various pore sizes.......................... 180

Table A.3 A summary of the amount of drug loading for PEI-functionalized and non
PEI-functionalized silica nanoparticles with and without EDC/Sulfo-NHS. .... 187
ACKNOWLEDGEMENTS

I would first like to thank my advisor, Prof. Michael Sailor, for allowing me to be part of the group, for showing me how to be a scientist, and for the guidance throughout these years. Thank you for the giving me the opportunities to go to conferences around the world, learning about other research areas and sharing our work. I truly value all that I have learned in your lab.

Thanks to my committee members who offered their time and advice throughout these years: Dr. William Freeman, Prof. Gabriel Silva, Prof. Sadik Esener, and Prof. Michael Heller.

To Prof. Jennifer Andrew, thank you so much for providing me with not only very helpful scientific advice, but also with your friendship. You somehow managed to be an awesome role model, coworker, and friend all at the same time. Thank you for keeping me motivated and helping me get through the frustrations of research. Thank you also for the trips to the cookie cart. Go team eyeball!

Alex Buyanin, I feel very lucky to have had the opportunity to work with you. Thank you for your constant enthusiasm and help throughout these years.

I have had the opportunity to work with many incredible post-docs in the lab, including Dr. Matt Kinsella, Dr. Timothy Kelly, and Prof. Ester Segal. Each of you has helped me so much and I have learned a lot from you.

Thank you to Dr. Su for helping me with all the mass spectrometry.

Thank you to all my collaborators that contributed to our projects presented in this dissertation: Dr. Lingyun Cheng, Lindsey Pearson, Laura Conner, Dr. Kathrin
Hartmann, and Dr. Jay Chhablani. Thank you also to our collaborators in France: Dr. Frédérique Cunin, Stephanie Pace, and Emilie Secret. I had a wonderful time in France and am very happy that I got to know each of you. Nous fabriquons de silicium poreux!!!

To Michelle Chen and Chiachen Wu (team stinky tofu): thank you for the good times in lab and outside of lab. Michelle, thanks for being an awesome travel buddy.

I have also had amazing group members that have provided constant support and motivation: Jen P., Adrian, Sara, Jen S., Luo, Ji-Ho, Maggie, Manny, Anne, Emily, Loren, Kyu-shik, Brian, Zhengtao, Joel, Wei, Ling, Shalini, Danny and all other current and former group members. Thank you for making the lab interesting and fun. Thank you Kim, Andrea and Ebony for keeping the lab running.

I would like to give special thanks to my family (mom, dad, and sister) for their love and patience. Thank you also to my in-laws for all your care in San Diego. Finally, to my husband, Andrew Lee: thank you for always listening to my practice presentations and complaints about the difficulty of research. Thanks for your understanding and support, especially all the times I worked odd or long hours. I know that without your constant encouragement I could not have done this.
Chapters one, two, three, and four are, in part or in full, reprints of the following publications:


The author of this dissertation was one of the primary authors or co-author on all publications.
VITA

EDUCATION

2010  PhD  Bioengineering  University of California, San Diego
2007  MS  Bioengineering  University of California, San Diego
2005  BS  Bioengineering  University of California, Berkeley

PUBLICATIONS


The sustained delivery of therapeutics with minimal systemic side effects would be a benefit in the treatment of many diseases. Numerous nanostructured materials in various shapes and sizes have been investigated to meet this challenge. This dissertation focuses in particular on the development of nanostructured porous silicon-based materials for observable and sustained drug delivery.
Previously, drugs loaded into porous silicon particles resulted in rapid drug release. In order to improve upon drug release kinetics, loading of drugs into porous silicon particles by physical adsorption and covalent attachment were compared, and the mechanism of drug release was studied. We found that drug loading by covalent attachment was able to eliminate burst release and provide extended drug release compared to previous results. One area that can benefit from such a sustained drug release system is ocular drug delivery. In this work, we investigated the use of porous silicon particles loaded with drug by covalent attachment for its potential in the treatment of the retina. Drug release was studied in a custom designed flow chamber designed to mimic the half-life of drugs in the human vitreous, and sustained drug release of up to one month was observed. In addition, the optical properties of porous silicon was used to monitor drug release.

An area of concern in using porous silicon as drug delivery carriers is the reactivity between porous silicon and redox active drug molecules such as anthracyclines. We demonstrate that reactive surface hydrides on porous silicon can reduce anthracyclines and create potential toxicity. However, we show that thermal oxidation can be used as a means to prevent this reduction. Finally, the degradation and toxicity of porous silicon and silica particles were studied in vivo using a rabbit eye model. We demonstrate that porous silicon and silica particles loaded with drugs are biocompatible. In addition, the color of the particles can be readily observed by a fundus camera, showing its potential as an indicator of drug release. These studies illustrate the possibility of using porous silicon based particles as a self reporting drug carrier for the long-term release of therapeutics.
Chapter One

INTRODUCTION TO NANOSTRUCTURED MATERIALS FOR DRUG DELIVERY
1 Introduction To Nanostructured Materials For Drug Delivery

1.1 Introduction and Motivation

Conventional forms of drug administration such as topical delivery, oral delivery or intravenous injection are unable to provide adequate treatment for many diseases.\(^1\) Current limitations include the inability to control pharmacokinetic profiles and drug biodistribution, poor drug stability, and potential immunogenicity.\(^2\) In addition, many of these therapeutics have a short plasma or vitreous half-life, thus requiring either a high dosage with potential toxicity or frequent dosages.\(^2, 3\) Nanostructured particulate medicine can offer many advantages over these traditional methods of drug administration. They can be tuned to provide specific release kinetics in order to release drugs in a controlled fashion, protect the drug from premature degradation, prevent drugs from interacting with healthy tissue, and enhance absorption of the drug into a specific tissue.\(^2, 4\) In addition, by being able to provide a high local concentration of the therapeutic at the target site while reducing the systemic concentration, potential toxic side effects can be minimized. Therefore, drug delivery systems with these capabilities would be able to enhance the therapeutic index of a given drug.\(^5\)

Various nano-scale particulate drug delivery systems have already been commercialized or are in late-stage clinical trials. For example, doxorubicin liposomes (Doxil) have been used for the treatment of refractory Kaposi’s Sarcoma, albumin-bound paclitaxel nanoparticles (Abraxane) have been approved for the treatment of
metastatic breast cancer, and polymer-protein conjugates such as PEG-L-asparaginase (Oncaspar) have been used for acute lymphoblastic leukemia. In each of these cases, the drug delivery system was found to be more effective than drug alone. For example, doxorubicin encapsulated in liposomes have an increased half-life and enhanced deposition in tumors, and Doxil has significantly reduced cardiotoxicity when compared with free doxorubicin. Despite these advances however, challenges still remain as these first generation delivery systems have low targeting efficiencies and lack precise control over drug pharmacokinetics. Studies on improving the targeting and control of drug release profiles are needed in order to provide for even more effective therapies.

1.2 Design of Nano-scale Drug Delivery Systems

The design of nano-scale materials for drug delivery requires an interdisciplinary field combining chemistry, biology, engineering, and medicine. Novel nanostructured materials being developed have shown promise in providing precise targeting and the controlled release of traditional therapeutics as well as proteins, vaccines, and nucleic acids. Some of the key characteristics in determining the properties and efficacy of these nanomaterials in vivo include size, surface chemistry, and shape.

1.2.1 Particle Size

Numerous methods have been used to fabricate micro- and nanoparticles of various sizes. In vivo properties such as circulation times, extravasation, targeting, immunogenicity, internalization, degradation, clearance and uptake mechanisms are all
affected by particle size. Micron sized particles (> 1 µm) are generally not considered suitable for delivery through intravenous injections due to difficulty in circulating through the bloodstream. They can be captured by Kupffer cells in the liver and can be physically trapped in capillary beds. Moreover, particles with diameters between 1-10 µm are most susceptible to phagocytosis. However, micron sized particles may be useful for other applications such as pulmonary delivery, where they can penetrate deep into the lungs after inhalation, or ocular delivery, where they may sink due to gravity and not impair the clarity of the ocular media.

For particles on the nanoscale, particles with a diameter greater than 5 nm will be filtered through the kidney after vascular delivery. Extravasation from blood to target tumors through the leaky vasculature requires particles with diameters less than 100 nm, whereas extravasation through normal vasculature requires sizes less than 1-2 nm. For nanoparticles up to 500 nm, endocytosis may occur by phagocytic or non-phagocytic cells. In drug delivery, internalization of the particles by the targeted non-phagocytic cells is desirable, whereas uptake by macrophages will result in faster particle clearance. Therefore, careful size selection is required to maximize particle targeting.

1.2.2 Surface Chemistry

Surface chemistry plays an important role in the inflammatory response and success of biomaterials. It has been found for example that hydrophilic or anionic implants elicit less foreign body reaction compared with cationic or hydrophobic surfaces. Therefore, the surface properties of a drug delivery carrier also greatly affect
their behavior *in vivo*. For example, the process of opsonization and clearance of the particles by the reticuloendothelial system (RES) is heavily influenced by surface chemistry. Hydrophilic and neutral surfaces are able to decrease protein adhesion and clearance by the RES.\(^5\) A major breakthrough in this area was the discovery that the addition of polyethylene glycol (PEG) polymers to the surface of nanoparticles can reduce serum protein adsorption and prolong the particle’s systemic circulation.\(^7\) However, the use of PEG only inhibits protein adsorption transiently, and opsonization will still ultimately occur.\(^5\)

The particle surface can also be functionalized with targeting ligands to achieve cellular targeting. For example, certain moieties can be used to bind with specific cancer cell-surface receptor proteins such as transferrin and folate receptors. By using these targeting ligands, receptor-mediated endocytosis can occur and increase particle internalization by the desired cell.\(^7\) Furthermore, the use of ligands to target specific organelles is also being explored.

### 1.2.3 Shape

Shape is an essential property that affects the phagocytosis, endocytosis, and circulation time of micro- and nanoparticles *in vivo*. For phagocytosis, the local geometry of the particle at which the macrophage makes contact determines whether macrophages will initiate internalization. It was found that elliptical disc shaped particles were fully internalized when the first point of contact with the macrophage was at the pointed end of the particle. On the other hand, when the first contact of the particle with the macrophage was with the flat region of the disc, the particles were not
internalized even after 12 hours. This occurred for particles of various sizes, with the extent of internalization being the only difference observed.\textsuperscript{8}

It had been previously thought that particles larger than ~150 nm are not endocytosed by non-phagocytic cells. However, it was shown that cubic particles with a side length of up to 3 µm were internalized by HeLa cells \textit{in vitro}.\textsuperscript{12} It was also found that aspect ratio greatly influenced internalization. Particles that had an aspect ratio of 3 were internalized four times more quickly than more spherical particles.

As mentioned above, the circulation time has also been shown to be influenced by particle shape. Filamentous micelles (filomicelles) as long as 18 µm have been shown to exhibit a significantly prolonged circulation time compared to micron sized spheroid particles. They were able to achieve a circulation half-life of ~5 days, and showed negligible uptake by macrophages compared with spheres of the same volume.\textsuperscript{13} These results indicate that particle shape plays an important role in the design of efficient drug delivery carriers.

\section*{1.3 Materials for Drug Delivery}

Many materials have been studied for use as nanostructured drug delivery carriers. Some of the most widely studied biomaterials for particle-based drug delivery systems will be discussed.

\subsection*{1.3.1 Polymeric particles}

Polymers are among the most common materials explored for synthesizing nano-scale drug carriers. Currently there are at least 12 polymer-drug systems that are
in Phase I or II clinical trials. Some of the most common polymers used are polylactide-polyglycolide copolymers (PLGA), polyacrylates, polycaprolactones, and natural polymers such as albumin, gelatin, collagen, and chitosan. Loading of therapeutics into polymers can be achieved by encapsulation within a polymer shell or matrix without chemical modification, or by covalent attachment to the polymer backbone. Release of therapeutics occurs through either diffusion out from the polymer matrix or shell, or by surface or bulk erosion of the polymer. Advantages of using polymers include their versatility in chemical composition, dimensions, and topology to allow for the controlled release of therapeutics in various drug delivery applications. For example, controlled release of macromolecules has been demonstrated by tailoring the composition of polymeric microspheres, resulting in release periods ranging from days to a month.

Hydrogels, which are hydrophilic, 3-dimensional cross-linked polymer networks that swell in the presence of water, have been widely studied for the controlled release of therapeutics. Release of therapeutics from hydrogels can be due to drug diffusion, hydrogel matrix swelling, and chemical reactivity of the drug and matrix. The advantages of using hydrogels include their biocompatibility, tunable properties, and ability to be designed to provide triggered release of therapeutics by responding to various stimuli such as ionic strength, pH, and temperature. In addition, therapeutics can be loaded after hydrogel synthesis, eliminating the risk of drug inactivation.

Currently, some concerns in using polymers as drug delivery systems include the inherent heterogeneity of polymer systems, with mixtures of chains of varying lengths. In addition, biocompatibility of polymer systems must also be addressed.
Polycations have often been shown to be cytotoxic, hemolytic, and likely to induce an inflammatory response. Polyanions are less cytotoxic but can induce an immune response such as cytokine release. Despite these drawbacks, polymer materials have shown much promise for in vivo biomedical applications, and have continued to be explored as drug delivery carriers.

1.3.2 Liposomes

Liposomes are spherical, self-assembled structures composed of lipid bilayers, and have been widely studied as drug delivery carriers. Hydrophilic molecules can be entrapped inside the internal aqueous phase and hydrophobic molecules can be incorporated within the external membrane layer. Liposomes have been attractive for use in biological applications because of its biocompatibility, biodegradability, and its ability to protect the loaded drug from the external environment. Furthermore, the size, charge, and surface properties of liposomes can be easily tuned. A variety of liposomal drugs have been approved for clinical application or are undergoing clinical trials, including DaunoXome (daunorubicin) for the treatment of Kaposi’s sarcoma and Platar (platinum compounds) for the treatment of solid tumors. These successes have fuelled increased interest in the research of liposomes for drug delivery. The current limitations of liposomes as drug delivery carriers include a quick burst release of drugs from the liposomes, fast clearance from the blood by the RES, high production cost, and poor storage stability. Studies are being done to address these drawbacks.
1.3.3 **Micelles**

Micelles are colloidal dispersions composed of amphiphilic molecules and have a hydrophilic core and a hydrophobic shell. They self-assemble into closed lipid monolayers under certain concentrations and temperatures, and are usually around 5-100 nm in size. There are several benefits of using micelles as a drug delivery system. First, micelles can be used to deliver water insoluble drugs, improving the bioavailability of many hydrophobic compounds and reducing their toxicity. Certain amphiphilic molecules can also improve the half-life of the micelle in blood circulation. Finally, their small size makes it possible for the micelles to penetrate across physiological barriers. For example, they have been found to be capable of passively targeting tumors through the enhanced permeation and retention (EPR) effect. Of particular interest has been micelles composed of diblock copolymers, which are often more stable than micelles composed of traditional surfactants. Further research on the controlled and triggered degradation of these micelles may lead to a successful micelle drug delivery carrier.

1.3.4 **Dendrimers**

Dendrimers are synthetic, highly branched, and symmetric polymers. They are composed of branched monomers, or dendrons, that emanate radially from a central core. Their chemical composition and molecular weight can be precisely controlled to determine its solubility and biodegradability. Polyamidoamine (PMAM) dendrimers in particular have attracted much interest for biological applications because they are biocompatible and water soluble, can be easily conjugated with drugs, and their small
size (<5 nm) allows them to be cleared from the blood through the kidneys. An early example of the use of PAMAM dendrimers is the delivery of methotrexate for the treatment of tumors. It was found that tumor growth was significantly reduced in mice after 15 weeks of biweekly injections of the methotrexate loaded dendrimers compared to control. However, some of the drawbacks of dendrimers include the high cost and difficulty of synthesis compared to other materials.

1.3.5 Carbon-Based Nanoparticles

Carbon-based materials have also generated interest as drug delivery carriers because they are chemically inert and the surface can be functionalized with various biomolecules. Carbon nanotubes (CNT), for example, are ultra light-weight and have excellent thermal and chemical stability. Single-walled carbon nanotubes (SWCNT) are constructed from a single sheet of graphite and have diameters on the order of 1 nm. Multi-walled carbon nanotubes (MWCNT) consist of multiple sheets of graphite and range from 2-100 nm diameter. However, there have been studies that suggest these materials are toxic, which could limit their use in vivo. For example, CNTs have been found to inhibit cell proliferation and induce cell death in vitro, induce an inflammatory response, and possess toxicity in vivo.

1.3.6 Inorganic Materials

Inorganic materials for nanostructured drug carriers comprise mostly of metal-based materials, magnetic materials, and silicon-based materials. Gold nanoparticles, for example, may be used for hyperthermia-based therapeutics, where they are able to
absorb radiation, then heat and destroy the surrounding tumor tissue. An example is a
cancer therapy that was developed based on the absorption of near-infrared light by
gold nanoshells. Tumor tissue implanted in mice were rapidly heated above the thermal
threshold and destroyed, whereas control tissues remained undamaged. However,
although metal nanoparticles are biologically inert, they are not biodegradable or small
enough to get cleared easily. Long-term toxicity could result due to accumulation of
these particles in the body. Magnetic particles such as iron oxide have also been studied
for controlled transport of therapeutics and hyperthermia treatment. Some current
challenges include providing a high drug loading capacity and accurate ligand mediated
targeting of tumor tissue.

Porous silicon-based particles can be fabricated by electrochemical etching and sol-gel chemistry. The advantages of using such systems include chemical
stability, large surface areas for high drug loading capacity, tunable pore sizes and
volumes, and biocompatibility. Silica particles formed from the sol-gel process have
been made in various sizes, ranging from nanometers to up to 2 µm in diameter,
providing use in various applications. Mesoporous silica nanoparticles (100-200 nm),
for example, formed by surfactant templated polycondensation chemistry, have been
loaded with superparamagnetic iron oxide and chemotherapeutic drugs. They were
found to be able to target the folate receptor on cancer cells and deliver non water
soluble drugs inside those cells. Chemical, photochemical, and redox based gating
systems have also been employed for the controlled release of therapeutics from
mesoporous silica particles. Porous silicon-based structures therefore offer great
promise as a drug delivery carrier. In the following section, porous silicon and silica
particles formed by electrochemical etching and its use as a drug delivery carrier will be discussed.

1.4 Porous Silicon

1.4.1 Introduction

Porous silicon (Si) was accidentally discovered by Uhlir at Bell Laboratories in the mid 1950s. He was trying to develop a means to electrochemically machine silicon wafers for use in microelectronic circuits. He found that under the appropriate conditions of applied current and solution composition, the silicon did not dissolve uniformly but instead fine holes were produced, which propagated primarily in the <100> direction in the wafer.\textsuperscript{26} Since its discovery, porous Si has been researched use in numerous applications due to its large surface area within a small volume, controllable pores sizes, convenient surface chemistry, and compatibility with conventional silicon microfabrication technologies. The use of porous Si for biomedical applications was first promoted by Leigh Canham in the 1990s, when he demonstrated that porous Si is biocompatible and biodegradable.\textsuperscript{27-29} Since then, porous Si or SiO\textsubscript{2} matrixes have been widely studied for drug delivery applications.\textsuperscript{30}

1.4.2 Preparation of Porous Si

Porous Si is generated by etching crystalline Si wafers in aqueous hydrofluoric acid (HF) solutions contained in a teflon etch cell (Figure 1.1). It is typically carried out in a two-electrode configuration under galvanostatic conditions. The Si wafer acts as the anode, and in the presence of fluoride ions, oxidized Si is removed from the
surface as either SiF$_4$ or SiF$_6^{2-}$ (Figure 1.2). The process occurs in two different regimes, a simple four-electron process represented by Equation 1.1(1), and a combination of two-electron processes in which the overall reaction involves both electrochemical and chemical oxidation, Equation 1.1(2)$^{26,31,32}$.

**Equation 1.1:**

$$\text{Si} + 4h^+ + 6F^- \rightarrow \text{SiF}_6^{2-} \quad (1)$$

$$\text{Si} + 2h^+ + 2H^+ + 6F^- \rightarrow \text{SiF}_6^{2-} + H_2 \quad (2)$$

The two-electron process predominates at lower applied potentials and is the main porous silicon forming reaction. The four-electron process is dominant when F ions are unable to get to the surface fast enough to remove the Si, resulting in electropolishing. This occurs when the concentration of HF is too low or when the current density is large. The oxidizing equivalents in these reactions are valence band holes, represented as “h$^+$” in the equations, and are driven to the surface by the applied electric field and diffusion. As the etch progresses, silicon atoms remaining on the surface become terminated with hydrogen atoms. These hydrogen atoms impart a strongly hydrophobic nature to the porous Si surface, and a surfactant such as ethanol must be included in the electrolyte to allow the pores to propagate and the etch to continue.
Figure 1.1 Schematic of the electrochemical etch cell for the formation of porous Si.
Figure 1.2 Mechanism for the electrochemical etching of silicon (a). The effect of fluoride concentration: the upper branch depicts oxidation of silicon when excess HF is present and the lower branch represents when water attacks the surface resulting in electropolishing (b).
With the appropriate choice of dopant type, dopant concentrations, and preparation conditions, a wide range of pore sizes are accessible in the porous Si electrochemical system. Illustrative cross-sectional electron micrograph images of two porous Si samples are shown in Figure 1.3. In general, the pore diameters of porous materials are categorized into three different size regimes: micro (<2 nm), meso (2-50 nm), and macro (>50 nm). All three regimes are available with porous Si. The electrochemical process is sufficiently flexible to provide control of pore dimensions in the x-y plane and in the z-direction on a Si wafer. The dimensions of the pores can be controlled within a single sample in the z-direction by temporal modulation of the current density, and in the x-y plane by localized placement of a small counter-electrode. A plan-view atomic force microscope image of a sample prepared by applying a current density gradient across the wafer is presented in Figure 1.4. The image illustrates the control over pore size that can be exerted just by controlling the etching current density.

Whereas the size of the pores is fixed by solution conditions, current density, and sample resistivity, the thickness of a layer is separately determined by the duration of the etch; the longer one etches, the deeper the pores propagate into the Si wafer. A uniform pore can be achieved because porous Si is highly resistive. Charge transport in the fine Si filaments of porous Si is highly restricted; thus they no longer participate in the electrocorrosion reaction once they are formed. Corrosion current is therefore concentrated at the interface between the bulk, highly conductive silicon substrate and the already-formed porous layer. In addition, certain crystallographic faces generated in the etch are more stable than others, resulting in an overall pore propagation (in most
circumstances) that favors the <100> direction. This tunability of the pore dimensions, porosity, and surface area is extremely useful for drug delivery applications, as it gives control over the loading and release of therapeutics.
Figure 1.3 Cross-sectional scanning electron micrograph image of two porous Si films showing the effect of dopant on pore structure. The sample on the left is prepared from an n-type sample and the sample on the right is prepared from a highly doped p++ type sample. They were prepared under the same conditions of current density and electrolyte composition. Images courtesy of Luo Gu, UCSD.
Figure 1.4 Plan-view atomic force microscope image of a porous Si film containing a lateral gradient of pore sizes, illustrating the rage of pore dimensions that can be accessed. Image reproduced with permission from Ref. [33], copyright 2002 Wiley-VCH.
For *in vivo* drug delivery applications, it is oftentimes desirable to prepare micrometer-scale and smaller particles. Micro- to nano-particles of porous Si can be prepared by ultrasonication of porous Si films. The general procedure is outlined in Figure 1.5. First, the porous Si layer is etched into a single-crystal silicon substrate in ethanolic HF solution. The aqueous HF/ethanol electrolyte is replaced with one that contains a relatively low concentration of HF (typically 1 part 49% aqueous HF to 10 parts ethanol). The entire porous nanostructure can then be undercut from the Si substrate by application of a current pulse. The freestanding hydrogen-terminated porous Si film is then placed in a liquid (pure ethanol is often used, but many liquids including water are suitable) and a vial of the mixture is subjected to ultrasound radiation in a common ultrasonic cleaner. The process fractures the porous Si film into small particles, whose average size can range from a few microns to a few nanometers, depending on the liquid used and the duration of the treatment. The particles can then be filtered if a specific size cut is needed.
Figure 1.5  Preparation of micrometer and nanometer size porous Si particles by ultrasonic fracture.
1.4.3 Optical Properties of Porous Si

A unique property of porous Si that makes it advantageous for drug delivery is its ability to be engineered as a 1-D photonic crystal. Photonic crystals are periodic nanostructured materials that diffract visible light.\(^{39}\) In the case of porous Si, a photonic structure can be produced by modulating current density during the electrochemical etching process. This is possible because the amount of Si dissolved is directly related to the current density passed at that instant.\(^{22}\) By applying a sinusoidal current waveform, a porosity that varies periodically throughout the depth of the porous film is produced, resulting in a rugate film,\(^{40}\) as shown in Figure 1.6. The variation in the porosity represents a variation in the refractive index of the material. Light is reflected from the interface at each refractive index change, and for particular wavelengths of light, there is a constructive interference, resulting in an intense reflected spectral peak (Figure 1.7). Essentially, the optical spectrum that is obtained from the rugate film represents the Fourier transform of the porosity modulation in the film. The reflection peak in the optical spectrum is dependent on the refractive index of the material, which changes as the matrix is degraded or as drugs are released, providing a simple method to monitor the extent of drug release.
Figure 1.6 The fabrication of a porous Si rugate film: application of a sinusoidal current waveform during the electrochemical etch can be used to fabricate a porous structure in which porosity varies periodically through depth.
Figure 1.7 An example of an optical reflectance spectrum of a single porous Si rugate particle.
1.4.4 Biocompatibility

A main concern for all drug delivery carriers is the biocompatibility and biodegradability of the material. Fortunately, porous Si is bioresorbable material\textsuperscript{27, 41} in which the primary degradation product is orthosilicic acid (Si(OH)\textsubscript{4}). Si is an essential trace element that is required for proper bone and connective tissue growth,\textsuperscript{42} and Si(OH)\textsubscript{4} is the natural form of Si found in the body and can be removed by the kidneys.\textsuperscript{41} Anderson and colleagues further showed that dissolution of porous Si under physiological conditions can be controlled by the porosity and pore size.\textsuperscript{41} Many \textit{in vitro} studies have been done regarding the biocompatibility of porous Si. It has been found, for example, that porous Si can support the culture of mammalian tissues without toxicity.\textsuperscript{43, 44} Fewer \textit{in vivo} studies have been conducted, but some studies have shown that porous Si implants can elicit a foreign body response, promoting calcification.\textsuperscript{45} Recently however, the biocompatibility of porous Si in the tissues of the eye has been demonstrated.\textsuperscript{46, 47} In addition, porous Si nanoparticles have also been injected intravenously into mice and have been shown to accumulate in tumors and then slowly degrade with no detectable side effects.\textsuperscript{48}

It is important to note that although porous Si is bioresorbable, as porous Si degrades, high concentrations of the released silicic acid compounds could potentially be a source of toxicity.\textsuperscript{49} However, because silicic acid can be eliminated through urine excretion, the rate of degradation of the porous Si drug carrier must be considered. The chemistry that allows for the control of the stability of porous Si in aqueous conditions is therefore crucial in the design of a drug delivery device.
1.4.5 Chemistry of Porous Si

Immediately after the electrochemical etch, porous Si has reactive surface hydride groups that are unstable in aqueous media. Fast dissolution times may not be amenable for long-term drug delivery applications. In addition, the hydrophobicity that the hydrogen terminated surface imparts is not suitable for in vivo applications. There are various routes that can be used to create a more stable and hydrophilic porous Si surface. The formation of a Si-C bond through hydrosilylation is one method to yield a stable surface.\textsuperscript{50} Hydrosilylation on porous Si involves the reaction of an alkene or an alkyne with a Si-H group, and was first demonstrated by Buriak through a photochemical or Lewis acid catalyzed reaction.\textsuperscript{50} Boukherroub later demonstrated the use of thermal hydrosilylation, and also developed a simple method of microwave hydrosilylation for creating hydrophilic surfaces on porous Si.\textsuperscript{51, 52} Due to the simplicity of the reaction, microwave hydrosilylation was used in this research as the hydrosilylation method for placing functional groups on the porous Si surface.

Another means to improve the stability of porous Si is through oxidation. Oxidation of porous Si can be achieved by thermal oxidation in air, by ozone oxidation, or by treatment with milder chemical oxidants such as dimethyl sulfoxide (DMSO).\textsuperscript{47} Oxidation imparts a hydrophilic surface to porous Si, making it more suitable for in vivo applications and allowing it to load hydrophilic drugs into the pores.

1.4.6 Drug Delivery Using Porous Si

One of the first uses of porous Si particles as a drug delivery carrier was demonstrated by Foraker et al. in delivering insulin across Caco-2 cell monolayers.
They showed that drug permeation rates across the cell layer can be significantly enhanced (10-fold) by using porous Si particles compared to liquid formulations.\textsuperscript{53} Another example is the study of porous Si for oral drug delivery by Salonen et al. The advantage of using porous Si particles for oral drug delivery is that porous Si can be fabricated to withstand the harsh environments of the gastrointestinal (GI) tract and stomach. Salonen and coworkers found that by encapsulating drugs into the porous Si microparticles, a delay in the release of drugs with otherwise high dissolution rates could be achieved.\textsuperscript{30} Anglin et al. have also demonstrated the possibility of using porous Si to monitor the extent of drug release. They were able to show that the release of dexamethasone from a porous Si film could be monitored by measuring the optical thickness changes using a CCD spectrometer.\textsuperscript{54} Complete release of dexamethasone occurred within several hours.

These few examples show that porous Si is a promising material as a drug delivery carrier. However, several challenges still remain. Currently, long-term release of therapeutics (days to months) has not been demonstrated. In the treatment of chronic diseases, it would be advantageous to have a drug delivery system that can provide sustained drug release for prolonged time periods. Another challenge is to demonstrate the ability to monitor drug release from porous Si microparticles by color change for \textit{in vivo} applications. Finally, the \textit{in vivo} biocompatibility and degradation of porous Si particles loaded with therapeutics needs to be evaluated. The next few chapters of this thesis will address these issues, in particular the use of porous Si particles in the treatment of ocular diseases.
1.5 Ocular Drug Delivery

The eye consists of 2 main segments: the anterior segment and the posterior segment. The anterior segment consists of the cornea, iris, pupil, ciliary body, and aqueous humor. The posterior segment consists of the retina, choroid, macula, and optic nerve. In this research, the focus is on the delivery of drugs to the posterior segment of the eye for the treatment of diseases affecting the retina, the light-sensitive part of the eye.

1.5.1 Ocular Membranes and Barriers

Ocular drug delivery, particular to the posterior segment, is a challenging field because the structure of the eye restricts entry of many therapeutics. This is due to the existence of tight cellular membranes and barriers which strictly control fluid and solute transport. An illustration showing the eye and its biological barriers that prevent the targeting and delivery of ocular therapeutics is shown in Figure 1.8. For the delivery of therapeutics by topical administration, the tear film and lacrimal fluid must first be passed. The tear film is composed of various nutrients, electrolytes, proteins, lipids and mucin, and is highly anti-adhesive. It therefore can protect the cell membrane by inhibiting cell-cell and cell-protein interactions. In addition, the turnover rate of the tear film is on the order of 2-3 minutes, resulting in the clearance of eye drops within the first 15-30 seconds of application.

After passing the tear film, another barrier that is reached is the cornea. The cornea consists of several layers, including the corneal epithelium, stroma, and corneal endothelium. The corneal epithelium inhibits the passage of mainly hydrophilic
compounds, the stroma poses a barrier for highly lipophilic drugs, and the endothelium has selective receptor-mediated transporters in order to protect the anterior chamber. Generally speaking, drugs applied through topical administration may be able to diffuse into the aqueous humor after crossing the tear film and corneal barriers, but will not reach the retina and vitreous at sufficient concentrations for the treatment of retinal diseases. One reason is that the blood-aqueous barrier restricts the passage of molecules between the posterior and anterior segments. In addition, the continuous turnover of the aqueous (2-3 mL/min) results in inefficient transfer of therapeutics between the anterior and posterior segments of the eye.\textsuperscript{55}

Therapeutics that are able to bypass the cornea can reach the conjunctival epithelium. Although some of the drugs will then be able to cross the sclera and reach the posterior section of the eye, the majority will be carried away by the systemic circulation through this route. Therefore, delivery through the conjunctiva and the sclera is considered to be inefficient for many ophthalmic drugs, with the exception of various peptides and oligonucleotides.\textsuperscript{55}

Finally, the blood-retinal barrier (BRB) is located in the posterior section of the eye and is composed of retinal capillary endothelial (RCE) and retinal pigment epithelial (RPE) cells. The RPE allows only selective nutrients to be exchanged between the retina and the choroid and the RCE cells inhibit the penetration of proteins and small hydrophilic compounds. Therefore, the BRB effectively inhibits many ophthalmic drugs from reaching the posterior segment of the eye when administered systemically, either through oral or intravenous administration. Because such a small
fraction of drug administered through these routes reach the posterior segment, a high dosage is often required, posing problems of systemic toxicity.

Injection of therapeutics directly into the posterior segment to bypass these barriers allows for high concentrations of the drug in the vitreous and retina and low systemic concentrations. However, due the short vitreous half-life of many of ophthalmic drugs, frequent intravitreal injections are oftentimes required. Frequent intravitreal injections risk endophthalmitis, vitreous hemorrhaging, retinal detachment, and cataract formation. Therefore, a drug delivery device capable of providing long term controlled release of therapeutics would be highly beneficial.
Figure 1.8 Illustration of the eye showing the biological barriers that prevent the delivery of many ocular therapeutics, reproduced with permission from Ref. [55], copyright 2008 Informa Plc.
1.5.2 Ocular Drug Delivery Systems

An ideal ocular drug delivery system would capable of providing long-term and controlled release of therapeutics without toxicity and interference with the clarity of the ocular media. For many applications, zero order kinetics, eliminating the burst release in which a significant amount of drug is released initially, would be preferred. Various non-biodegradable and biodegradable implantable systems have been developed that are capable of sustained release. For example, Vitransert® (Bausch & Lomb) is a non-biodegradable polymer that is commercially available for the controlled release of ganciclovir in the vitreous to treat cytomegalovirus retinitis (CMV). The device is coated with polyvinyl alcohol (PVA) and ethylene vinyl acetate (EVA). By combining PVA, a permeable polymer, with EVA, an impermeable polymer, zero-ordered sustained release of ganciclovir is obtained for up to 6 months. Retisert® (Bausch & Lomb) was developed using similar methods to provide up to 2.5 years of release of fluocinolone acetonide for the treatment of severe uveitis.\(^3\,55\)

Biodegradable implants have also been studied to provide sustained release of ocular therapeutics. For example, rod-shaped poly(d,l-lactic) implants encapsulating dexamethasone showed release over a period of 28 days in normal eyes.\(^3\) In addition, biodegradable implants are also being evaluated for commercialization. Oculex Pharmaceuticals, for example, has developed a biodegradable dexamethasone implant composed of PLA and PGA for the treatment of macular edema. The phase II trials showed a significant improvement in visual acuity compared to no drug therapy. The implant is currently undergoing Phase III trials. Despite the excellent drug release
kinetics achieved by implantable devices, complications exist. These devices require surgical implantation and if necessary, surgery to remove and implant another. Such invasive methods has risks such as vitreous hemorrhage, retinal detachment, endophthalmitis, and macular edema.³

Injectable colloids capable of providing long term release of therapeutics would reduce the risks associated with intravitreal injections and would be less invasive than implants. In has been found that particles 200 nm – 2 µm resulted in dense vitreous opacities, whereas particles less than 200 nm and microparticles greater than 2 µm resulted in minor vitreous opacities.³ However, smaller particles have a lower drug loading capacity along with faster release and clearance. Therefore, microparticles larger than 2 µm may be suitable for ocular drug delivery. Kompella et. al have demonstrated the release of budesonide, and anti-VEGF treatment for age-related macular degeneration, from PLA nano- and micro-particles. They found that the nanoparticles provided sustained release for approximately 2 weeks after an initial burst release. The microparticles did not exhibit a burst release and maintained sustained release over a period of 6 weeks. In addition, they found that both types of particles were able to inhibit VEGF expression.⁵⁷ Therefore the use of nano- or micro-particles for ocular drug delivery is a promising platform and warrants further research.

1.6 Conclusions

Traditional forms of drug administration do not meet the treatment needs of a variety of diseases. Biocompatible and biodegradable porous micro- or nano-particles provide a promising platform for the targeted, sustained release of therapeutics. One
area in particular that is in need of a controlled drug delivery device is the treatment of retinal diseases. Although implantable devices have shown excellent drug release kinetics, injectable micro- and nano-particles provide for a less invasive means to provide long-term therapy. In addition, because the eye is optically transparent and can be visualized non-invasively, a self-reporting drug carrier capable of providing real-time drug release information is desirable.

1.7 Scope of the Dissertation

The objective of this dissertation was to develop and investigate the use of porous Si-based particles as observable and long-term drug delivery carriers, in particular for application in the field of ocular drug delivery.

Chapter two focuses on long-term drug release using porous silicon. Loading by physical adsorption and covalent attachment are compared, and the mechanism of drug release is studied. This chapter demonstrates that drug loading by covalent attachment to the porous Si matrix provides extended drug release compared to loading by physical adsorption.

In chapter three, the use of porous silicon particles loaded with drug by covalent attachment is investigated for its potential in ocular drug delivery. Drug release is studied in a custom designed flow chamber designed to mimic the half-life of drugs in the human vitreous. In addition, the use of color change as a means to monitor drug release in vivo is discussed.

Chapter four focuses on the reactivity between porous silicon and redox active drug molecules such as anthracyclines. This chapter shows that reactive surface
hydrides on porous Si can reduce anthracyclines and create potential toxicity. The use of thermal oxidiation as a means to prevent this reduction is presented.

Chapter five describes the degradation and toxicity of porous silicon and silica particles \textit{in vivo} using a rabbit eye model. This chapter demonstrates that porous silicon and silica particles loaded with drugs are biocompatible.

Appendix A describes various methods and chemical modifications for the loading of an anti-viral drug into mesoporous silica nanoparticles.
Chapter one, in part, is a reprint (with co-author permission) of the material as it appears in the following publication: M.J. Sailor and E.C. Wu, Photoluminescence-based sensing with porous silicon films, microparticles, and nanoparticles. *Adv Funct Mater* 2009, 19, 3195-3208. The author of this dissertation is co-author of this manuscript.
Chapter Two

**Oxidation-Triggered Release of Fluorescent Molecules or Drugs from Mesoporous Silicon Microparticles**
2 Oxidation-Triggered Release of Fluorescent Molecules or Drugs from Mesoporous Silicon Microparticles

2.1 Abstract

The fluorescent dye Alexa Fluor 488 or the anticancer drug doxorubicin is attached to the surface and inner pore walls of mesoporous Si particles by covalent attachment, and the oxidation-induced release of each molecule is studied. The molecules are bound to the Si matrix using a 10-undecenoic acid linker, which is attached by thermal hydrosilylation. Loading capacity of the microparticles using this method is \(~0.5\) and \(45\) mg/g of porous Si microparticle for Alexa Fluor 488 and doxorubicin, respectively. The Si-C-bound assembly is initially stable in aqueous solution, although oxidation of the underlying Si matrix results in conversion to silicon oxide and slow release of the linker-molecule complex by hydrolysis of the Si-O attachment points. When the attached molecule is a fluorophore (Alexa Fluor 488 or doxorubicin), its fluorescence is effectively quenched by the semiconducting silicon matrix. As the particle oxidizes in water, the fluorescence intensity of the attached dye increases due to growth of the insulating silicon oxide layer and, ultimately, dye release from the surface. The recovery of fluorescence in the microparticle and the release of the molecule into solution are monitored in realtime by fluorescence microscopy. Both processes are accelerated by introduction of the oxidizing species peroxynitrite to the aqueous solution. The oxidation-triggered release of the anticancer drug doxorubicin to HeLa cells is demonstrated.
2.2 Introduction

Traditional methods of drug administration such as oral delivery or injection usually result in rapid release and clearance of the drug. A high initial drug dose is therefore necessary in order to maintain a therapeutic concentration over a period of time, which may produce toxic side-effects. Controlled drug release technologies can improve the efficacy of traditional drug therapies by reducing the systemic concentration of free drug. Various drug delivery vehicles have been employed, such as liposomes, micelles, gelatin nanoparticles, solid lipid nanoparticles, silica-based nanoparticles, and porous Si. Porous Si possesses several properties that make it advantageous as a drug delivery system, including low toxicity, a high surface area and tunable pore sizes and volumes. Chemical modification of the porous Si surface can also be achieved, providing many means to adjust the chemical stability of the material as well as to load a particular drug of interest. As with all biodegradable drug delivery carriers, the toxicity of the degradation products is an important factor. Si is an essential trace element that is required for proper bone and collagen growth. The bioavailable form of silicon consists of various oxo anions of orthosilicic acid (Si(OH)$_4$), which are rapidly removed by the kidneys in the human body. This is also the primary degradation product of porous Si in aqueous media, and the rate of production of orthosilicic acid is dependent on the porosity of the porous Si sample.

A variety of drugs such as ibuprofen, dexamethasone, and doxorubicin have been placed in porous Si matrices. In most reports, drug loading is accomplished
by adsorption of the drug to the inner pore walls of a suitably modified porous Si sample. These preparations typically release the drug rapidly. For long-term drug delivery applications it is desirable to minimize rates of desorption and leaching of drug from the fixture; one means to accomplish this is to attach the drug to the porous Si matrix by covalent attachment. This approach offers the added possibility of triggering release by incorporating a physiologically responsive linker; for example, an enzyme cleavable peptide sequence. The method described here uses covalent attachment to load Alexa Fluor 488 or doxorubicin into porous Si microparticles. Alexa Fluor 488 is a stable fluorescent molecule that is used in this study to model drug release. Doxorubicin is an anti-cancer agent that also possesses intrinsic fluorescence properties. The model drugs are attached to a linker and grafted to the porous Si surface by means of Si-C bonds. The Si-C surface bond is stable in aqueous media, however the Si-Si back bonds of the assembly can be oxidized, resulting in insertion of oxygen to form Si-O-Si linkages. Unlike the Si-C bond, the Si-O bond is susceptible to hydrolysis in aqueous media. Thus, oxidation of the underlying Si matrix can result in oxidation-triggered release of the linker-molecule complex.

A second unique aspect of the porous Si system is the ability of the material to efficiently quench photoluminescence from a fluorophore by energy transfer. We find that oxidation of porous Si eliminates the quenching pathway, providing a means to monitor the progress of oxidatively triggered drug or molecule delivery. We show that the process is accelerated in the presence of biologically relevant reactive oxygen species (ROS).
In this study, doxorubicin was used as a test drug. Doxorubicin is a highly toxic drug that is used for chemotherapy and has a narrow therapeutic concentration range due to undesirable side-effects such as cardiotoxicity and myelosuppression. The covalent method described here allows for drug release only when the covalent bonds are broken or when the porous Si matrix is oxidized and degraded. We observe a significant, extended release of doxorubicin that is covalently attached to the porous Si matrix compared to doxorubicin that is physically adsorbed onto the porous Si matrix. We conclude that porous Si microparticles loaded with a drug through covalent attachment is a viable candidate for long-term drug release applications.

2.3 Experimental Methods

2.3.1 Synthesis of Porous Si Microparticles

Porous Si microparticles were prepared from the electrochemical etching of highly doped, (100)-oriented, p-type Si wafers (Boron-doped, ~1 mΩ resistivity) in a 3:1 solution of 48% aqueous hydrofluoric acid (HF):ethanol (HF from Fisher, Inc. CAUTION: HF is highly toxic and contact with skin should be avoided). A Si wafer with an exposed area of 3.2 cm² was contacted on the back side with a strip of aluminum foil and mounted in a Teflon etching cell with a platinum counter-electrode. The wafer was etched at a constant current density of 248 mA/cm² for 2 min. The resulting porous layer was then lifted off by electropolishing in a 3.33% HF in ethanol solution for 2 min at a current density of 6.2 mA/cm². The etching and electropolishing procedure was repeated 4 times per wafer and the resulting porous layers were ultrasonicated in ethanol for 20 min.
2.3.2 Hydrosilylation of Porous Si Microparticles

Approximately 25 mg of the porous Si microparticles prepared in the manner described above were placed in a 10 mL Pyrex beaker and immersed in 1 mL of undecylenic acid (95%, purchased from Sigma-Aldrich). The microparticles in undecylenic acid were then heated in a commercial consumer microwave oven (Sears Kenmore 700 W) for 4 min at 280 Watts. The particles were then rinsed with hexane and ethanol to remove excess undecylenic acid.

2.3.3 Loading of Alexa Fluor 488 and Doxorubicin into Porous Si Microparticles

For loading of Alexa Fluor 488 (Invitrogen), approximately 4 mg of porous Si microparticles were suspended in 500 μL of ethanol. Afterwards, 5 μL of a 2 mg/mL solution of Alexa Fluor 488 in water and 25 μL of a 10 mg/ml solution of N-(3-Dimethylaminopropyl)-N2-ethylcarbodiimide hydrochloride, or EDAC (commercial grade, Sigma Aldrich Chemicals) were added. The microparticles were then agitated for 2 h at room temperature and rinsed thoroughly with PBS. Doxorubicin hydrochloride (Sigma-Aldrich Chemicals) was loaded into porous Si microparticles in a similar manner. Approximately 4 mg of porous Si microparticles were suspended in 500 μL of 10% dimethyl sulfoxide (DMSO) in Dulbecco’s phosphate buffered saline (PBS) solution. Afterwards, 200 μL of a 1 mg/mL solution of doxorubicin in water and 150 μL of a 10 mg/mL solution of EDAC were added. The particles were then agitated for 2 hours at room temperature and rinsed with methanol 5 times.
2.3.4 Physical Characterization of Porous Si Microparticles

SEM images were obtained using a Phillips XL30 ESEM Field Emission Gun (FEG) electron microscope operating at an accelerating voltage of 20 kV. Diffuse reflectance mode Fourier-transform infrared (FTIR) spectra were obtained with a Nicolet-Magna 550 spectrometer. Nitrogen adsorption-desorption isotherms of porous Si microparticles were recorded at 77 K using a Micromeritics ASAP 2010 volumetric apparatus. Prior to the adsorption experiment, approximately 20 mg of porous Si samples were outgassed overnight in-situ at 313 K. The surface area of the sample was measured by the BET (Brunauer-Emmett-Teller) method, which yields the amount of adsorbate corresponding to a molecular monolayer. The pore dimensions were determined using the BdB (Broekhof-de Boer) method from the nitrogen adsorption curve.\textsuperscript{32-34}

2.3.5 Microscopy

Fluorescent images were obtained using the Nikon Eclipse LV150 microscope with a Photometrics HQ\textsuperscript{2} camera. For all acquired images, a 10x objective with a numerical aperture of 30 was used. Fluorescence images were acquired with either 50 ms or 100 ms exposure times. Measurement of fluorescence intensity of Alexa Fluor 488 was achieved using an excitation filter wavelength of 540-580 nm and an emission filter wavelength of 600-660 nm. The computer program MetaMorph from Molecular Devices Operation was used for image acquisition, processing, and analysis.
2.3.6 Determination of Drug Loading

Porous Si microparticles were completely dissolved in a solution of 1M KOH for 10 min. The solution was then neutralized with an equal volume of 1M HCl to recover the fluorescence spectrum of free dye or drug. A Perkin Elmer LS50B fluorescence spectrometer was used for fluorescence measurements. For measurement of the amount of dye loaded, the solution was excited at 495 nm and the emission from 500-600 nm was measured. The peak was found to be at 520 nm and the concentration was calculated based on a calibration curve. For doxorubicin, the solution was excited at 470 nm and the intensity of the emission spectrum was measured between 500-650 nm. A calibration curve for the emission at 580 nm was used to calculate the amount of doxorubicin loaded.

2.3.7 In Vitro Release Studies

Porous Si microparticles were rinsed thoroughly with PBS prior to drug release studies. 3-Morpholinosydnomine (SIN-1) purchased from Invitrogen, which generates superoxide and NO, was used to produce a flux of peroxynitrite. Approximately 600 _g of porous Si microparticles loaded with Alexa Fluor 488 was placed in a 35mm Petri dish and suspended with either PBS, cell media (DMEM), or cell media with 1mM concentration of SIN-1. They were incubated at 37 °C and agitated at 50 rpm, and 1 mL of the release medium was removed at various time intervals to determine the amount of dye released. Fresh PBS, cell media, or cell media containing SIN-1 (depending on the relevant experiment) was added back to the release solution to maintain a constant volume. Concentrations of the samples were determined by
measuring the fluorescence intensity of the released dye with an excitation of 495 nm. The peak intensity of Alexa Fluor 488 was found to be at 516 nm, and a calibration curve for the dye in PBS or in cell media was used to determine the amount of dye released in their respective medium. For doxorubicin release studies, approximately 2 mg of porous Si microparticles was placed in 15 mL of PBS solution at 37 °C and agitated at 100 rpm. At predetermined time intervals, 2 mL of the release medium was removed and was replaced with fresh medium. Drug concentration in the release samples was determined as in the drug loading determinations.

2.3.8 Cell Toxicity Assay

The HeLa cell line was utilized for cell toxicity experiments. Cells were routinely maintained at 37 °C in 5% CO$_2$ in DMEM culture medium. In preparation for the MTT assay, the cells were plated in a 96-well plate. Fifty microliters of porous Si particle dispersions or free doxorubicin solution of the requisite concentrations was introduced to each well with 100 µL of media. To perform the MTT assay, the media was removed after a 48 h incubation period and the wells were rinsed with 100 µL of PBS three times. MTT solution was added to each well and allowed to react for 4 h at 37 °C. MTT formazan crystals were then dissolved in the MTT solvent. Values were determined by subtracting the absorbance value measured at 690 nm from the value measured at 570 nm. A larger MTT absorbance minus-background value indicates a larger relative viability.
2.4 Results and Discussion

2.4.1 Preparation of Porous Si Microparticles

Porous Si films were synthesized by electrochemical etching of highly doped p-type Si wafers in an aqueous electrolyte containing HF and ethanol. The resulting porous film was lifted off and fractured into microparticles using ultrasound. Freshly etched porous Si is unstable in aqueous media due to oxidation of the reactive surface hydrides and therefore surface modification is necessary in order to improve stability. Step 1 in Scheme 1 illustrates functionalization of the freshly etched microparticles using microwave-assisted thermal hydrosilylation with undecylenic acid, in which a Si-C linkage is created. The molecules Alexa Fluor 488 or doxorubicin were then grafted to the carboxy terminus of the modified surface. 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) was used to link the hydrazide group of Alexa Fluor 488 to the carboxylic acid group on the porous Si surface. Doxorubicin was attached to the porous Si microparticles in a similar manner, via the primary amine of doxorubicin. In both attachment chemistries an amide bond is formed (Figure 2.1).

Scanning electron microscope images reveal that after ultrasonic fracture and chemical modification, the porous Si sample consists of microparticles with sizes in the range of 30-50 μm (Figure 2.2a). Each individual microparticle is permeated with mesopores of 20-30 nm diameter (Figure 2.2b). The specific surface area, the porous volume, and the pore size of the microparticles were determined using nitrogen adsorption measurements with the application of the BET (Brunauer-Emmett-Teller)
and the BdB (Broekhof-de Boer) methods. The hysteresis loop observed is associated with cylindrically shaped pores of approximately constant cross section. The average pore diameter was calculated to be $20 \pm 3 \text{ nm}$ for the chemically modified microparticles, consistent with the SEM images. The surface area ($S_{BET}$) was $\sim 260 \text{ m}^2 \text{(STP)/g}$ and the porous volume was $\sim 780 \text{ cm}^3 \text{(STP)/g}$. 
Figure 2.1 Schematic of the loading of molecular payloads in porous Si microparticles by covalent attachment. The process involves functionalization of the porous Si surface by thermal hydrosilylation of undecylenic acid (a), followed by EDC-medicated coupling of either the fluorescent molecule Alexa Fluor 488 (b) or the anticancer drug doxorubicin (c).
Figure 2.2 Scanning electron microscope (SEM) image of a representative sample of porous Si microparticles (a). A SEM view of one of the microparticles, revealing the mesoporous structure (b).
2.4.2 Loading of Alexa Fluor 488 and Daunorubicin into Porous Si Microparticles

Covalent attachment of the dye and drug were confirmed by Fourier Transform Infrared (FTIR) spectroscopy. The FTIR spectrum of freshly etched porous Si microparticles displays bands characteristic of surface hydride species (Figure 2.3a). A band at 2110 cm\(^{-1}\) associated with the \(\nu_{\text{Si-H}}\) stretching vibrations, a band corresponding to the \(\delta_{\text{Si-H}_2}\) scissors mode at 910 cm\(^{-1}\), and a band corresponding to a \(\nu_{\text{Si-H}}\) deformation mode (663 cm\(^{-1}\)) are apparent in the spectrum. After hydrosilylation with undecylenic acid, bands at 2928 cm\(^{-1}\) and 2857 cm\(^{-1}\) associated with \(\nu_{\text{C-H}}\) stretching modes and a band at 1715 cm\(^{-1}\) associated with the \(\nu_{\text{C=O}}\) stretching mode are observed (Figure 2.3b). Conjugation of the drug or dye molecules generate bands in the FTIR spectrum characteristic of amide I (1655 cm\(^{-1}\)) and amide II (1588 cm\(^{-1}\)), indicative of the formation of the linking amide bond (Figure 2.3c).\(^{77}\)
Figure 2.3 Diffuse-reflectance Fourier-transform infrared spectra of porous microparticles. (a) Freshly etched porous Si before chemical modification. (b) Porous Si microparticles after hydrosilylation with undecylenic acid. (c) Particles after attachment of Alexa Fluor 488. The y-axis is presented in relative Kubelka-Munk units.
2.4.3 Oxidation-Triggered Fluorescence Increase of Dye Loaded Particles

The photophysical interaction of the fluorophore Alexa Fluor 488 with the porous Si matrix was measured by fluorescence microscopy. The degree of fluorescence quenching relates to the extent of oxidation of the porous Si microparticles. In the as-prepared material, very low levels of fluorescence are observed from the dye grafted onto porous Si (Figure 2.4a). The weak fluorescence intensity is attributed to the high density of electronic states in the semiconductor, which efficiently quenches the excited state of the attached fluorophore. The system can be thought of as a solid-state analog of Fluorescence Resonance Energy Transfer (FRET), where the attached dye is the energy donor and the porous Si matrix is the acceptor. The exchange of energy between quantum dot-molecular dye conjugates has been observed previously, and it appears to be a general feature of systems where the donor-acceptor separation distance is small. In the specific case of porous Si, the quenching of fluorescence from dye molecules has been well-studied by Vial and others.

As the porous Si microparticles oxidize in aqueous solution (pH 10), the growing oxide layer increases the separation between the dye donor and the semiconductor acceptor, resulting in a steady increase in photoluminescence intensity associated with the particles. A ten-fold increase in the intensity of dye fluorescence is measured (MetaMorph image analysis software, average of 10-20 particles) after 24 h and a thirty-fold increase is observed after a few days (Figure 2.4b). Oxidation of the porous Si matrix was verified ex situ by the appearance of a broad band centered at
1000 cm\(^{-1}\) in the FTIR spectrum, associated with silicon-oxide stretching modes. Independent measurements verified that the intensity of fluorescence of the Alexa Fluor 488 dye itself is independent of pH in the range 7-10.

Covalent attachment allows the grafted molecule to be released only after the silicon host matrix dissolves. Degradation of porous silicon in aqueous solutions in the physiological pH range involves the oxidation of silicon into silicon dioxide (eq. 1), followed by hydrolysis with water (Equation 2.1) to release orthosilicic acid.\(^{41}\)

Equation 2.1

\[
\begin{align*}
\text{Si} + \text{O}_2 & \rightarrow \text{SiO}_2 \\
\text{SiO}_2 + 2 \text{H}_2\text{O} & \rightarrow \text{Si(OH)}_4 
\end{align*}
\]

The first step in this process, the oxidation of Si, occurs in aqueous solutions such as phosphate-buffered saline (PBS) or in cell media, although the reaction is slow. Oxidation can be accelerated by the addition of an oxidizing agent such as reactive oxygen species (ROS), in particular peroxynitrite (ONOO\(^{-}\)). Peroxynitrite, a biologically significant ROS, is a strong oxidant that is synthesized \textit{in vivo} when superoxide and nitric oxide (NO) are generated in close proximity.\(^{85}\) Peroxynitrite is thought to produce oxidative damage in tissues by oxidizing or nitrating biological molecules such as tyrosine.\(^{86}\) In this study, 1-2 mM of 3-morpholinosydnonimine (SIN-1) was used to generate physiologically relevant micromolar levels of ONOO\(^{-}\) in situ, in a pH 7 buffer solution (PBS).\(^{87, 88}\) As observed with the base-induced oxidation, oxidation by ONOO\(^{-}\) increases the fluorescence intensity of the microparticles (Figure 2.5), but only by a factor of ~2. However, the reaction is faster than base-induced
oxidation, and fluorescence intensity increases significantly within the first 15 min of exposure. Control experiments with PBS buffer show a much smaller rate of fluorescence increase. A control experiment involving incubation of free Alexa Fluor 488 with ONOO· does not produce a measurable change in fluorescence intensity on this timescale. As with base-induced oxidation, the recovery of fluorescence intensity is attributed to a reduction in the efficiency of excited state energy transfer between the attached dye and the porous Si matrix. The present interpretation is consistent with the previous work in the porous Si and porous SiO$_2$ systems.$^{80-82, 89, 90}$ This increase in fluorescence intensity measured from the dye-modified porous Si microparticles provides a convenient means to monitor the extent of oxidation of the porous Si constituent, the first step in the aqueous dissolution of the fixture.
Figure 2.4 Microscope images of porous Si microparticles with Alexa Fluor 488 attached, showing the increase in fluorescence intensity as the porous Si microparticles oxidize. (a) Bright-field (left) and fluorescence (right) images of the microparticles before oxidation. (b) Bright-field (left) and fluorescence (right) images after treatment with pH 10 buffer for 5 days, showing the significant increase in fluorescence intensity from the attached dye when the porous Si matrix oxidizes. Fluorescence images were obtained using 480 nm excitation and 535 ± 25 nm observation channels.
Figure 2.5 Comparison of the change in fluorescence intensity of an Alexa Fluor 488 dye grafted to porous Si microparticles as a function of time after addition of peroxynitrite (ONOO⁻). Controls with pure buffer (PBS) and with free dye (not attached to porous Si) are shown for comparison. The data are quantified by fluorescence microscopic measurement of the microparticles, and presented as the ratio of fluorescence intensity relative to the fluorescence intensity measured at time t = 0. The 535 ± 25 nm emission channel is monitored, using 480 nm excitation.
2.4.4 Oxidation-Triggered Release of Alexa Fluor 488

The dye molecules tethered to the porous Si matrix release into the solution as a result of the Si surface oxidation. It is well-established that the silicon-carbon bonds produced by hydrosilylation of porous Si are quite stable in aqueous media.\(^{50, 73}\) Oxidation of the porous Si matrix produces Si-O back bonds that are more susceptible to hydrolysis. Therefore, oxidation of the porous Si matrix as indicated by the fluorescence increase of the dye tethered to the particle is followed by the release of the dye (Figure 2.6). In this work, the hydrolysis-induced release of the attached fluorophore was studied in pH 10 solution, PBS, cell media, and cell media containing added ONOO\(^-\). Appearance of fluorophore in solution was quantified by absorbance and fluorescence spectrometry.

Addition of an oxidant can be used to trigger the release of molecules covalently attached to porous Si microparticles (Figure 2.7). In a 4 h period, the amount of fluorophore released from porous Si microparticles immersed in cell media is 2 times the amount released from microparticles immersed in aqueous PBS buffer. When ONOO\(^-\) is added to microparticles immersed in cell media, the amount of Alexa Fluor 488 released is 4 times the amount released when in PBS. The increased amount of fluorophore released in cell media compared to PBS is attributed to a greater rate of oxidation due to the presence of amines and surfactants in the cell media.\(^{91-94}\) The presence of ONOO\(^-\) in the cell media further accelerates the rate of oxidation. After 24 h, there is > 2-fold increase in the concentration of fluorophore in a solution medium containing ONOO\(^-\) compared to media alone, and > 10-fold increase compared to PBS.
Figure 2.6 Oxidation of the porous Si matrix results in a fluorescence intensity increase of the dye and produces Si-O back bonds that are more susceptible to hydrolysis, allowing dye release.
Figure 2.7 Release of covalently attached Alexa Fluor 488 from porous Si microparticles as a function of time in various solutions, demonstrating ROS-triggered release. The release rate is smallest in PBS buffer, intermediate in cell media, and greatest in cell media containing 1 mM SIN-1, which generates the strong oxidant peroxynitrite in situ. The data are quantified by fluorescence spectrometry of the supernatant solution, using a calibration curve for the emission of Alexa Fluor 488 at 516 nm in either PBS or in cell media with an excitation of 495 nm.
2.4.5 Loading and Release of Doxorubicin From Porous Si Microparticles

The oxidation-triggered hydrolysis reaction that releases the dye into solution is potentially useful for drug delivery applications. Previous work using porous Si as a drug delivery material have focused on using physical adsorption as a means to load drug, often resulting in rapid, burst-type release profiles.\textsuperscript{30, 47} Covalent attachment offers a means to prolong the period of release and to harness physiological stimuli to modify the rate of release.

In order to test the relevance of the present approach to realistic therapeutic conditions, the anti-cancer drug doxorubicin was loaded into porous Si microparticles by covalent attachment and release into PBS solutions held at 37 °C was quantified. Two particle preparations were used. The first preparation was rinsed with methanol after covalent attachment of the drug, and the second was rinsed with water. It was found that doxorubicin is not sufficiently soluble in water to allow efficient removal of the free drug with a water rinse. Thus particles with covalently attached doxorubicin that were rinsed with water contained both covalently bound and physically adsorbed doxorubicin. The temporal drug release profiles of the two covalent preparations were quantified and compared with profiles from samples in which doxorubicin was loaded solely by physical adsorption (Figure 2.8). Approximately 75 μg of doxorubicin was loaded per mg of particles in the covalent attachment protocols, with 45 μg/mg remaining after the methanol rinse; the physical adsorption protocol resulted in a doxorubicin loading of approximately 55 μg/mg.
For particles loaded with doxorubicin through physical adsorption, a burst of drug release is observed within the first 2 hours and all detectable drug is released within 24 h. Particles containing a combination of covalently attached and physisorbed doxorubicin (the water-rinsed samples) display a smaller initial burst followed by a gradual release over a period of ~ 24 hours. Particles loaded with doxorubicin through covalent attachment and rinsed with methanol to remove all physisorbed drug display a continuous, slower release of doxorubicin that lasts for > 5 days. For all three sample types, the measured quantity of doxorubicin released into solution is less than the amount that is initially loaded into the microparticles. The discrepancy is attributed to chemical degradation of doxorubicin during the course of the experiments, as doxorubicin is known to be moderately unstable in aqueous media at pH 7.\textsuperscript{95}
Figure 2.8 Release of doxorubicin from porous Si microparticles into PBS buffer. Complete drug release occurs within 1 d for doxorubicin loaded through physical adsorption, while the covalently attached drug is continuously released for > 5 d. The data are quantified by fluorescence spectrometry of the supernatant solution, monitoring the excitation spectrum between 500-650 nm and calculating the concentration based on a calibration curve for the emission at 580 nm using an excitation of 470 nm.
2.4.6 Functionality of Released Doxorubicin

Cellular assays were performed to determine if the released doxorubicin retains its functional toxicity. Doxorubicin is somewhat unstable in aqueous solutions at pH 7, and in addition the chemical modification used to attach the molecule to the porous Si host microparticles may interfere with doxorubicin’s mode of action. An MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide) toxicity assay was used to test cell viability. Cells were exposed to free doxorubicin (dox), empty porous Si microparticles (PSiMPs), and porous Si microparticles in which doxorubicin was loaded by covalent attachment (dox-pSiMPs). The samples of doxorubicin and porous Si microparticles were added to HeLa cells and assessed for cytotoxicity 48 h after exposure (Figure 2.9). The viability of cells exposed to doxorubicin-loaded porous Si microparticles was significantly lower than cells exposed to empty porous Si microparticles, and somewhat higher than cells exposed to the same quantity of free doxorubicin. Porous Si microparticle samples in which doxorubicin is loaded by physisorption display toxicity similar to the covalently attached material. It should be noted that porous Si is able to reduce MTT in the absence of cells, giving rise to an apparent lack of toxicity with this particular mitochondrial activity assay.44,96 Thus the significant toxicity observed for doxorubicin-loaded porous Si microparticles is likely to be an underestimate of the actual cellular toxicity. The data indicate that doxorubicin released from porous Si microparticles retains significant functional toxicity.
Viability of HeLa cells (MTT assay) after exposure to free doxorubicin, empty porous Si microparticles (empty-pSiMPs), and porous Si microparticles loaded with doxorubicin by covalent attachment (dox-pSiMPs). The x-axis displays the doxorubicin concentration in terms of the total amount of doxorubicin added per mL of media. For the empty microparticles, there is no doxorubicin loaded, but a mass of particles equal to the mass of doxorubicin-loaded microparticles is used (1 µg/mL on the x-axis corresponds to 46 µg of particles). The mass loading of the doxorubicin-loaded microparticles is 22 ng of doxorubicin per microgram of particles. Toxicity assessed 48 h after sample introduction.

Figure 2.9
As mentioned above, doxorubicin is unstable in aqueous media, and it is possible that the drug may react chemically with porous Si microparticles either before or after release. In addition, the chemical attachment method employed modifies the drug from its normal form. Indeed, HPLC-MS analysis of the supernatant from doxorubicin-loaded porous Si microparticles indicates the presence of degradation products (Figure 2.10). Compared with physical adsorption, the covalent attachment method allows prolonged release of drug in a controllable fashion, although the attachment chemistry requires modification of the molecular structure of the drug. Thus the type of drugs that can be used with this approach may be somewhat limited. The reaction of the porous Si surface with drug compounds will be discussed further in chapter 4.
Figure 2.10 Analysis of as received doxorubicin (top) and doxorubicin released from porous Si microparticles (bottom) by LC-UV with monitoring at 280 nm. The mass of the parent ion doxorubicin + H, [M+H]⁺, is 543.89, and was found in the LC trace peak of as received doxorubicin. MS analysis of the LC trace peaks from doxorubicin released from porous Si microparticles revealed degradation products and drug attached to the linker molecule.
2.5 Conclusions

Loading of small molecules into mesoporous Si microparticles can be accomplished by covalent attachment via Si-C bonding. The molecules are released into aqueous solutions in a two-step mechanism involving oxidation and subsequent dissolution of the porous Si matrix. When a fluorescent dye is used, energy transfer quenching provides a sensitive probe of the oxidation process; fluorescence intensity of attached dye increases as the porous nanostructure oxidizes from semiconducting Si to insulating SiO$_2$, and the intensity of fluorescence from the solution increases as the porous matrix dissolves and dye is released. The covalent attachment method was tested with the anti-cancer drug doxorubicin, and release of the active drug was verified by the MTT cellular viability assay. Exposure of molecule-loaded porous Si particles to a reactive oxygen species (ROS) such as peroxynitrite results in triggered release of the attached molecule. This method allows the grafted molecule to be released only when the covalent bonds are broken or when the matrix is degraded. Because elevated levels of ROS are often generated in the vicinity of diseased or tumor tissues, the triggered release mechanism presented in this work is relevant to medical therapeutic applications.
Chapter two, in part, is a reprint (with co-author permission) of the material as it appears in the following publication: E.C. Wu, J.H. Park, J.S. Park, E. Segal, F. Cunin, M.J. Sailor, Oxidation-triggered release of fluorescent molecules or drugs from mesoporous Si particles. *ACS Nano* 2008, 2401-2409. The author of this dissertation is the primary author of this manuscript.
Chapter Three

**REAL-TIME MONITORING OF SUSTAINED DRUG RELEASE USING THE OPTICAL PROPERTIES OF POROUS SILICON PHOTONIC CRYSTAL PARTICLES**
3 Real-time Monitoring of Sustained Drug Release Using the Optical Properties of Porous Silicon Photonic Crystal Particles

3.1 Abstract

A controlled and observable drug delivery system that enables long-term local drug administration is reported. Biodegradable and biocompatible drug-loaded porous Si microparticles are prepared from silicon wafers, resulting in a porous 1-dimensional photonic crystal (rugate filter) around 12 micrometers thick and 35 micrometers across. An organic linker, 1-undecylenic acid, is attached to the Si-H terminated inner surface of the particles by hydrosilylation and the anthracycline drug daunorubicin is bound to the carboxy terminus of the linker. Degradation of the porous Si matrix in vitro is found to release the drug in a linear and sustained fashion for 30 days. The bioactivity of the released daunorubicin was verified on retinal pigment epithelial (RPE) cells. The degradation/drug delivery process is monitored in situ by digital imaging or spectroscopic measurement of the photonic resonance reflected from the nanostructured particles, and a simple linear correlation between observed wavelength and drug release is observed. Changes in the optical reflectance spectrum are sufficiently large to be visible as a distinctive red to green color change.

3.2 Introduction

The need for drug delivery systems capable of providing safe, sustained release to the target tissues is evident in many diseases, including various ocular diseases.97
The existence of barriers such as the blood-retinal barrier and the tight junctions of the retinal pigment epithelium pose great difficulty in delivering drugs systemically to the vitreous, retina, or choroid.\textsuperscript{55} Often, the standard treatment for an ocular disease involves frequent intravitreal injections, risking complications such as infection and vitreous hemorrhaging.\textsuperscript{98-101} Proliferative vitreoretinopathy (PVR) is an example of an ocular disease that would benefit from a controlled delivery system. PVR occurs when there is uncontrolled proliferation of non-neoplastic cells that form contractile membranes during the wound healing process after retinal detachment.\textsuperscript{102, 103} Efficient drug administration to treat PVR is hindered by the short half-life of the therapeutics in the vitreous coupled with the retinal toxicity caused by a high bolus intravitreal injection.\textsuperscript{102} Although an implantable system may provide for more controlled release kinetics, drawbacks include the need for surgery in the implantation and (sometimes) removal of these devices.\textsuperscript{99} A biodegradable and injectable system capable of providing long-term sustained release of a therapeutic to the vitreous for the treatment of PVR would therefore be more advantageous.

Porous Si has many advantages as a drug delivery system,\textsuperscript{30, 47} including the controllability of pore size and volume,\textsuperscript{31} the ease of chemical modification to load various drugs,\textsuperscript{50} a high surface area,\textsuperscript{31} biocompatibility\textsuperscript{43, 53} and resorbability.\textsuperscript{27, 41, 48} Si is required for proper bone and collagen growth, and the degradation product of porous Si \textit{in vivo} is orthosilicic acid (Si(OH)\textsubscript{4}), which is the natural form of Si found in the body and is readily excreted by the kidneys.\textsuperscript{104, 105} Additionally, previous studies have shown no toxicity from porous Si particles injected into the rabbit or rat eye.\textsuperscript{46, 98}
The \textit{in vitro} degradation pathway for porous Si involves two steps: oxidation of the Si matrix to SiO$_2$, followed by hydrolysis to the soluble orthosilicic acid species. The oxidant in the first step can be water or various bioavailable reactive oxygen species (ROS), and this oxidation reaction is significantly slowed if the porous Si surface is modified with a Si-C bonded organic species. Furthermore, the Si-C chemistry provides a convenient means to attach drugs intended for slow release—the low reactivity of Si-C bonds in aqueous media provides an effective means to limit the release of a tethered drug. Si-C chemistry is significantly more stable than the silanol (Si-O) chemistry typically employed to attach molecules to SiO$_2$ surfaces. For example, release of doxorubicin covalently attached to porous Si via Si-C links occurs over several days rather than hours. The rate of release can be increased by addition of ROS and it can be slowed by using more hydrophobic linkers or a more extensive carbonization chemistry. Since water is a competent but kinetically slow oxidant for Si-C modified porous Si matrices, the approach holds potential for long-term \textit{in vivo} drug release applications.

An interesting aspect of the materials science of porous Si is that it can be prepared in the form of multilayers of varying optical density, allowing the fabrication of photonic crystals. In this work we generate a type of photonic crystal known as a rugate filter, in which the porosity varies smoothly and periodically as a function of depth. This gives the porous Si particles an intense reflectivity peak at a predetermined wavelength. Shifts in this wavelength have been used to monitor the loading and release of various biomolecules. Moreover, the reflectivity spectrum
can be prepared in the near infrared region, allowing observation through human tissues\cite{110} or other biological media.

In this study, daunorubicin is loaded into particles of porous Si photonic crystals by covalent attachment. Daunorubicin is an anthracycline antibiotic that has been shown to be modestly efficacious as an intraocular anti-proliferative agent for the treatment of experimental PVR\cite{111}. Unfortunately, current use includes an infusion of 7 \( \mu \)g/mL of drug into the eye at the end of surgery, and the eye is exposed to the drug for only several minutes. Even with this application, probably due to the binding of daunorubicin to DNA, there is a weak but real clinical benefit as shown by a reduction in the extent of retinal re-detachment\cite{112}. In order to control PVR, the drug must be able remain in the vitreous long enough to inhibit cell proliferation effectively without exhibiting toxicity to the retina or other tissues\cite{112}. However, daunorubicin has a short half-life in the vitreous and a narrow therapeutic concentration range\cite{102,113,114}. Therefore, daunorubicin is not currently used for the treatment of PVR due to the toxicity that can occur with a high initial dose\cite{113}. We are investigating if sustained release of daunorubicin \textit{in vitro} can be achieved by sequestering the drug in porous Si particles. In addition, we want to develop a method to non-invasively monitor drug release \textit{in vivo} by the color change of the particles.

3.3 Experimental Methods

3.3.1 Formation of Porous Si Microparticles

Porous Si microparticles were prepared by electrochemical etch of highly doped, (100)-oriented, p-type Si wafers (Boron-doped, 0.98 m\( \Omega \)•cm resistivity) in a 3:1
solution of 48% aqueous hydrofluoric acid (HF):ethanol (CAUTION: HF is highly toxic and contact with skin should be avoided). A Si wafer with an exposed area of 8.04 cm\(^2\) was contacted on the backside with a strip of aluminum foil and mounted in a Teflon etching cell with a platinum counter-electrode. The wafer was etched using the current density waveform:

\[
J = A_0 + A \cos(kt + \alpha)
\]

where \(J\) is applied current density, \(A_0\) is current density offset (in mA/cm\(^2\)), \(A\) is current density amplitude (mA/cm\(^2\)), \(k\) is frequency (s\(^{-1}\)), \(t\) is time (s), and \(\alpha\) is phase shift (s\(^{-1}\)).

The values used for \(A_0\), \(A\), and \(\alpha\) were 90.2 mA/cm\(^2\), 12.4 mA/cm\(^2\), and 0, respectively. Thus the maximum current density value of the waveform was 102.6 mA/cm\(^2\) and the minimum was 77.8 mA/cm\(^2\). The value of \(k\) was adjusted to yield a reflectance peak whose maximum occurred at \(~600\) nm; a typical value was 2.15. The waveform was etched for a total of 300 s (corresponding to \(~80\) repeats), producing a layer 12 \(\mu\)m thick. The porous layer was then removed from the substrate by electropolishing in a 3.33% HF in ethanol solution for 2 min at a current density of 6.2 mA/cm\(^2\). The etching and electropolishing procedure was repeated 8 times per wafer and the resulting porous layers were placed in absolute ethanol and broken up into microparticles by ultrasound for 30 min. To perform the hydrosilylation chemistry, approx. 40 mg of porous Si microparticles were placed in a 10 mL Pyrex beaker and immersed in 2 mL of undecylenic acid. The mixture was then heated in a commercial consumer microwave oven for 4 min (280 Watts). The particles were rinsed with hexane and ethanol to remove excess undecylenic acid.
3.3.2 Characterization of Porous Si Microparticles

Scanning electron microscope (SEM) images were obtained using a Phillips XL30 ESEM Field Emission Gun (FEG) electron microscope operating at an accelerating voltage of 3 kV. Nitrogen adsorption-desorption isotherms of porous Si microparticles were recorded at 77K using a Micrometrics TriStar volumetric apparatus. Prior to the adsorption experiment, approximately 30 mg of porous Si particles were purged overnight with nitrogen at 40 °C. The surface area of the sample was measured by the Brunauer-Emmett-Teller (BET) method, which yields the amount of adsorbate corresponding to a molecular monolayer. Pore size distribution was determined using the Barrett-Joyner-Halenda (BJH) method.

3.3.3 Loading of Daunorubicin into Porous Si Microparticles

Approximately 5 mg of porous Si microparticles were suspended in 650 μL of 10% dimethyl sulfoxide (DMSO) in Dulbecco’s phosphate buffered saline (PBS) solution containing 50mM EDAC and 5mM Sulfo-NHS. Afterwards, 200 μL of a 1 mg/mL solution of daunorubicin hydrochloride in water were added. The particles were then vortexed for 2 hours at room temperature and rinsed with ethanol 5 times. To determine the amount of daunorubicin loaded, porous Si microparticles were completely dissolved in a solution of 1M NaOH for 10 min. The solution was neutralized with 1M HCl to recover the fluorescence spectrum of the free drug. To quantify the amount of drug released, the solution was excited at 480 nm and the photoluminescence emission spectrum was measured in the range 500-650 nm. The
emission maximum at 590 nm was used to calculate the amount of daunorubicin released, based on a calibration curve.

### 3.3.4 In Vitro Porous Si Particle Degradation and Drug Release

Porous Si particle degradation and drug release were studied using a custom designed flow cell chamber with a volume of 4.5 mL and a flow rate set to mimic the half-life of daunorubicin in the human eye. A solution of phosphate buffered saline (PBS) was flowed through the chamber containing the drug-loaded particles at a rate of 450 µL/h. The eluted PBS solutions were collected and analyzed for silicon and daunorubicin. Silicon content was determined by inductively coupled plasma optical emission spectrometry (ICP-OES). Samples were filtered through a 0.1 µm filter and 1 mL of the sample was diluted with Millipore water (17.3 mΩ resistivity) to a total volume of 5 mL. The Si atomic emission peaks at 212.412, 251.611, and 288.158 nm were monitored. Daunorubicin concentration was determined using fluorescence spectrometry as described above. Aliquot volume was typically 150 µL.

### 3.3.5 Determining Average Reflectivity Spectra of Porous Si Particles

Reflectance spectra of porous silicon particles were recorded using an Ocean Optics USB 2000 spectrometer. A tungsten light source with a spot size of approximately 1 mm was focused onto the particles in solution through a bifurcated fiber optic cable. The optical axis was normal to the plane containing the porous Si particles. Results of reflectivity spectra acquired from at least 5 regions per sample were averaged.
3.3.6 Acquisition and Analysis of the Reflectivity of Individual Porous Si Particles

A Nikon LV-150 fluorescence microscope was modified to allow illumination of the sample stage with a narrow-band light generated by a 100 W tungsten filament lamp in combination with a SpectraPro 275 scanning monochromator. A CoolSnap HQ2 (Photometrics) 14 bit, monochromatic camera was used to acquire reflectivity images. Metamorph software (Molecular Devices) was used to control the camera and the stepper motor of the monochromator to allow the generation of spectral image stacks. Reflectivity images were acquired as the monochromator was scanned from 500-725 nm in 1 nm steps. All images were acquired using a 10x objective with a live binning of 1 and an exposure time of 20 s to produce 520 x 696 pixel images. The result was a spectral image stack with 226 images. The images were then cropped to 480 x 480 pixels for analysis. Image stacks were analyzed using a code developed in MATLAB, which yielded the wavelength maximum of the reflectivity spectrum for each particle. The value of wavelength maximum for each particle was determined from the average of 5 pixels.

3.3.7 In Vitro Cellular Proliferation and Toxicity Assays

Cell proliferation and toxicity experiments were performed on retinal pigment epithelial cells (ARPE-19), grown in DMEM F-12 with 10% FBS and 1% antibiotic-antimyotic (100x). The data were analyzed for 3 samples at various concentrations: (1) daunorubicin as-received, diluted to 0.45 or 0.5 µg/mL in PBS as the highest concentration, (2) daunorubicin released from drug-loaded porous Si particles, with
0.45 or 0.5 µg/mL as the highest concentration, and (3) silicic acid released from non-drug loaded porous Si particles, equivalent in weight to the drug-loaded particles used in (2). Various concentrations of the 3 samples (50 µL) were added to 60-70% confluent cells in a 96-well plate containing 100 µL media in each well. Cells were incubated with the particular sample for 48 h and analyzed. The MTT assay, a quantitative colorimetric assay that measures living, metabolically active cells, was used to measure cell proliferation. Cell viability was assessed using the live/dead cell assay with calcein AM and ethidium homodimer-1. Calcein AM is hydrolyzed into the green-flourescent calcein in live cells and ethidium homodimer-1 stains dead cells with compromised membranes. A final concentration of 0.5 µM Calcein AM and 1.5 µM ethidium homodimer-1 was used.

3.4 Results and Discussion

3.4.1 Fabrication of Daunorubicin-Loaded Porous Si Microparticles

Porous Si films possessing a periodic, smoothly varying porosity profile in the 
z-direction (perpendicular to the wafer surface) were prepared. The periodic gradient was imposed using a cosine etch current waveform. The resulting porous film approximates a rugate optical filter, displaying a single narrow band in the reflectivity spectrum at 600 ± 5 nm. In order to improve stability and provide an attachment point for the drug, the freshly etched particles were functionalized with undecylenic acid using microwave-assisted thermal hydrosilylation. Daunorubicin was subsequently grafted onto the carboxy terminus of the undecanoic acid-modified surface via EDC chemistry, as shown in Figure 3.1.
Figure 3.1 Loading of daunorubicin into porous Si microparticles. The process involves functionalization of the hydrogen-terminated porous Si surface (Si-H) by microwave-assisted hydrosilylation of undecylenic acid (a), followed by EDC-mediated coupling of daunorubicin via the pendant amino group (b).
3.4.2 Characterization of Porous Si Particles

The resulting microparticles are approximately 35 x 45 x 12 µm in size. The specific surface area, porous volume and average pore size were determined using nitrogen adsorption measurements with the application of the BET (Brunauer-Emmett-Teller) and the BJH (Barrett-Joyner-Halenda) methods.\textsuperscript{74, 75, 118} The BET surface area was determined to be ~260 m\textsuperscript{2} (STP)/g and the BJH adsorption cumulative pore volume was determined to be ~550 cm\textsuperscript{3} (STP)/g. The BJH adsorption average pore diameter was found to be 9.6 ± 0.04 nm. Approximately 20 µg of daunorubicin could be loaded per mg of porous Si particles. The amount of drug loaded was determined by quantifying the amount released upon immersion of the particles in 1M NaOH. In this highly caustic solution, the particles were observed to dissolve completely within 30 min. All 20 µg of daunorubicin are covalently bound, as we found previously that rinsing by ethanol/methanol is able to remove all unbound drug.\textsuperscript{106} A reflectivity peak at approximately 660 nm results after immersion of drug loaded particles into buffer (Figure 3.2b).
Figure 3.2 (a) Scanning electron microscope (secondary electron) image of a representative sample of porous Si microparticles used in this study. (b) Representative white light reflectance spectrum from a single porous Si particle.
3.4.3 In Vitro Release of Daunorubicin Covalently Attached to Porous Si Microparticles

It has been suggested that PVR can be prevented by inhibiting cell growth at the cell proliferation stage, which can last for ~6 weeks. Therefore, release of an anti-proliferative should be sustained for >1 month.\textsuperscript{99, 103} To mimic the half-life of therapeutics in the vitreous in order to determine if sustained release could be achieved on a timescale relevant to chronic ophthalmic diseases, we designed a flow cell chamber designed to hold approximately the volume of the human vitreous (4.5 mL).\textsuperscript{119} Pure PBS solution was flowed through the cell at a rate of 450 µl/hour. At this flow rate, dissolved materials will clear from the cell with a half-life of 5 hours. This mimics the half-life for clearance of free of daunorubicin in the vitreous.\textsuperscript{114} To test the accuracy of the system, 20 µg of as-received daunorubicin was spiked into the chamber and the concentration of drug in the effluent from the cell was monitored. Experimentally, 97% of the spiked daunorubicin was removed in 24 h (Figure 3.3a), which agrees well with the amount expected (96.4%) based on a steady-state dilution calculation that assumes the continuous flow rate and reservoir volume of the experiment.

The degradation of Si and release of covalently attached daunorubicin was therefore studied using this flow cell chamber. Particles contained in the flow chamber were continuously flushed with fresh PBS solution over a period of 30 days and the effluent was collected and assayed for soluble Si content by ICP-OES analysis and for daunorubicin by fluorescence spectroscopy daily (Figure 3.3b). The rate of dissolution of the porous Si carrier scales with the total exposed surface area of the microparticles.
The rate of Si degradation decreased gradually over the 30-day test period, suggesting that the exposed surface area decreased during this time period. Correspondingly, a slight decrease in drug concentration was observed as the total surface area of the particles decreased. There was a strong correlation between the amount of silicon removed and the amount of drug released into solution: over the 30 day period approximately 88% of the drug content and 80% of the silicon content of the microparticles were released.

When loaded into the particles, the residence time of daunorubicin was greatly extended compared to the free drug. Sustained release of daunorubicin was achieved for 30 days, at which time about 88% of the drug had been released. The amount of daunorubicin in solution remained well under the toxic intravitreal dose throughout the duration of the experiment (Figure 3.3c).\textsuperscript{112} If it had been administered as free drug, the initial concentration of the drug loaded in the particles would have exceeded the toxic dose (4 µg/mL were injected; the toxic dose is 3.15 µg/mL). Doses of 5 µg/mL per eye have been applied to the vitreous at the end of surgery and have shown to be efficacious in human PVR.\textsuperscript{120} Given that the known half-life of the drug is approximately 5 hours and the IC\textsubscript{50} on proliferation for daunorubicin is approximately 0.15 µg/mL, it is expected that the drug will fall to subtherapeutic levels in 30 hours. In the present system, therapeutic levels are maintained for 30 days\textsuperscript{121-123} (Figure 3.3c). The data show the drug concentration is maintained at well under the toxic limit for a period of time that is 24 times longer than what could be obtained with a single intravitreal injection of free drug,\textsuperscript{99, 103} suggesting that this approach may be applicable to PVR treatment.
Figure 3.3 In vitro release of daunorubicin covalently attached to porous Si microparticles. (a) Control experiment, showing the rapid clearance ($t_{1/2} = 5$ h) of an aliquot of free daunorubicin injected into the flow chamber with a continuous flow of 450 µL/h of pure PBS solution. The solid line shown is the calculated % cleared as a function of time in hours, based on the elution rate and chamber volume. (b) Percent of daunorubicin (solid circles, “drug”) and silicon (open circles, “Si”) released from porous Si microparticles into the effluent stream under the same flow conditions as in (a). (c) Steady-state concentration of daunorubicin in the chamber as a function of time for the experiment described in (b). A therapeutic level of drug is maintained for > 30 d.
3.4.4 Correlation of Spectral Shift with Drug Release

A non-invasive indicator of the amount of residual drug remaining in the fixture is a desirable feature for drug delivery systems, and the present system accomplishes this by harnessing the optical reflectivity properties of the porous Si photonic crystal. A structural color is imparted to the particle during its preparation by application of a sinusoidal current density waveform during the electrochemical etch. This produces a periodic porosity modulation that propagates in the direction of the electrochemical etch, which is the <100> crystallographic direction. In the present case, the stratified refractive index of the resulting particles gives rise to a sharp reflectance peak in the optical spectrum. As aqueous solution replaces the drug molecules inside the pores, or as the porous Si matrix degrades, the refractive index in the porous Si layers decreases, resulting in an observable blue shift of the color. As reported previously, a primary pathway for release of the attached drug from these hydrosilylated materials is the oxidation and subsequent hydrolysis of the Si-C and Si-Si bonds in these particles. Therefore, in principle, the degradation rate of the particles controls the release rate of daunorubicin.

Particles (2 mg) were immersed in 2 mL of buffer solutions in a Petri dish to allow time-resolved microscopic imaging and spectroscopy of the particles as they dissolved in the media. The strong reflectivity peak generated by the rugate optical filter structure gives the particles a distinctive color. The evolution of this color was monitored at three different pH values: pH = 5, 7, and 9, each over a period of 7 days. Although the relevant pH for the eye is 7-7.4, using various pH buffers provided a
means to adjust the degradation rate in order to get different drug release rates for different rates of color change. The optical microscope images are presented in Figure 3.4. For the pH = 9 buffer, the particles turn from red to orange, then yellow, and finally green during the 7 d exposure period. The blue shift is not as extensive in pH = 7 buffer; after 7 d the initially red particles have turned yellow or yellow-green. Particles soaked in pH = 5 buffer display the least apparent color change, turning from red to orange during the 7 day exposure period.

The spectral shifts observed in the images of Figure 3.4 were quantified using a CCD spectrometer and a tungsten illumination source fitted to an optical microprobe. For each dish, 3 regions were analyzed. The optical probe was focused to a spot of diameter ~1 mm, which collected data from ~150 particles in a scan. Thus spectra from a total of 450 particles were averaged per data point. The largest spectral change was observed for particles immersed in the pH 9 buffer and the smallest was observed for particles in pH 5. After a period of 7 days, a blue shift of ~70 nm was observed for particles in pH 9 buffer, at pH 7 the shift is 60 nm, and at pH 5 the shift is only 40 nm (Figure 3.5c).

The optical and spectral changes in the porous Si microparticles were correlated with the appearance of free daunorubicin and silicon in the buffer solutions, and the results are presented in Figure 3.5 as a function of time for the three pH values studied. Concurrent with the spectral measurements, 1 mL of the buffer solution was removed and assayed for daunorubicin using the fluorescence method described above. After removal of the assay aliquot, an equal volume of the relevant (fresh) buffer solution was replaced in the dish. The rate of appearance of Si (Figure 3.5a) and of daunorubicin
(Figure 3.5b) in the solution is dependent on the pH of the buffer. Over a period of 7 days, 60% of the drug was released from particles in pH 9 buffer, 50% of the drug was released from the particles in pH 7 buffer, and 40% of the drug was released from the particles in pH 5 buffer. Degradation of the microparticles releases daunorubicin and soluble silicon in the form of orthosilicate at a rate that increases with increasing pH. A linear relationship between the cumulative amount of daunorubicin released and the magnitude of the spectral blue shift is observed (Figure 3.5d) for all pH values tested, for percentages of released drug < 60% of the total amount of loaded drug.

The reflected spectral peak from the photonic microparticles provides a non-invasive means of monitoring the degree of degradation of the materials and a surrogate means of monitoring the amount of drug that has been released into solution. In the present experiments, the 1 mm diameter spot probed by the optics encompasses hundreds of particles, allowing the establishment of a statistically significant correlation between spectral peak shift and drug release. The correlation between spectral peak shift and amount of drug released is approximately linear in the range 0-60% of total drug released (Figure 3.5d), supporting the hypothesis that the photonic signature from the particles could be used to monitor drug release in vivo. However, the clinical utility of the approach would be improved if the spectral change could be monitored by visual inspection through a medical ophthalmoscope or a fundus camera. Thus we imaged the particles under a microscope, using white light illumination and a digital camera. The results clearly indicate that a detectable color change occurs as the particles degrade, and that this color change correlates with the quantity of drug released.
Figure 3.4 Optical microscope images showing the color change of particles as they degrade in pH = 5, 7, or 9 buffer solutions. The degree of color change correlates with the amount of drug released (see Figure 4). The scale bar is 100 μm.
Figure 3.5 Quantification of silicon dissolution (a), drug release (b) and spectral peak shift (c) as a function of time for drug-loaded porous Si photonic crystal microparticles in aqueous buffer solutions at pH 5, 7, and 9. The shift correlates with percent of daunorubicin cumulatively released from the particles in an approximately linear fashion over the range 0-60% drug released (d).
3.4.5 Distribution of the Reflectivity Spectra of Individual Particles

At any given point in time during the dissolution reactions described above, the values of $\lambda_{\text{max}}$ in the reflectivity spectra of the particles displayed a range of values. This distribution was quantified in a separate set of experiments in which several series of microscope images were acquired as a function of illumination wavelength. The same experimental conditions as described in the section above were applied, immersing the porous Si microparticles in pH 5, 7, and 9 buffers. At a given time point, the microscope stage was illuminated by light passed through a 0.275 m monochromator, which was scanned over the wavelength range 500-725 nm in 1 nm steps. Image stacks were then analyzed using a MATLAB code and 5 contiguous pixels were measured and averaged per particle. A total of 30 particles were measured in each set; results are displayed in Figure 3.6. The distribution of $\lambda_{\text{max}}$ values shifts to the blue end of the visible light spectrum as the particles degrade in the solution. Initially, the distribution approximates a Gaussian, but with increasing time in the buffer solution, the distribution becomes skewed, with the maximum of the distribution appearing closer to the short wavelength. The width of the wavelength distribution does not change significantly with dissolution time.
Figure 3.6 Distribution of values of $\lambda_{\text{max}}$ in an ensemble of particles, monitored during degradation in aqueous solution. Each bar represents a 4 nm wavelength range about the value indicated on the x-axis. Photographic color images of the representative ensembles are shown at the bottom. The scale bar is 100 μm.
3.4.6 Effect of Released Daunorubicin on ARPE-19 Cells

The contractile membranes formed in PVR are made up of cellular components, including retinal pigment epithelial (RPE) cells, glial cells, fibroblasts, and inflammatory cells. These cells are capable of synthesizing collagen, which imparts a whitish appearance to the membranes.\textsuperscript{99, 103} Preventing the proliferation of these cellular components may inhibit the development of PVR. Pure daunorubicin is known to effectively inhibit growth of RPE cells. However, in the present study this active molecule was attached to the porous Si surface using a linker molecule. It is possible that such a chemically modified drug will not display the same therapeutic efficacy as the parent compound. In addition, porous Si or its degradation byproducts may participate in side-reactions with the drug,\textsuperscript{96} for example, the chemically related therapeutic doxorubicin has been shown to be reduced by porous Si under certain conditions,\textsuperscript{106} and porous Si has been shown to be a competent reductant for benzoquinone,\textsuperscript{125} which is one of the structural units contained in both daunorubicin and doxorubicin. For these reasons it was important to validate the efficacy of the drug delivered from the microparticles.

In vitro cell toxicity and proliferation assays were performed using ARPE-19 cells. The assays were run on the water-soluble components extracted from the porous Si particles rather than on the particles themselves, in order to simplify interpretation of the results. The daunorubicin that is released from porous Si particles into PBS solution was tested for its ability to inhibit cellular proliferation and toxicity on RPE cells using an MTT assay (\textbf{Figure 3.7}). The soluble by-products of porous Si dissolution
(primarily orthosilicate species) and as-received daunorubicin were also tested. It was found that daunorubicin extracted from porous Si microparticles inhibits cellular proliferation of RPE cells at concentrations lower than as-received daunorubicin. The soluble by-products of porous Si dissolution (from non-drug loaded particles) do not inhibit cell growth. Fluorescence images resulting from live/dead cell staining experiments also reveal that daunorubicin released from porous Si microparticles exhibits toxicity towards RPE cells (Figure 3.8). As-received daunorubicin displays the decreased cellular growth and toxic effects expected from this drug, but it shows a lower degree of toxicity relative to the same quantity of daunorubicin released from microparticles. Control experiments established that the source of additional toxicity cannot be attributed to silicic acid released from the porous Si particles; experiments run with extracts from porous Si particles that contained only the linker chemistry (no attached drug) show no significant toxicity (Figure 3.7). The concentration of drug used in each assay was verified by fluorescence spectroscopy.

The redox chemistry of the quinone group with porous Si is thought to play a role in the enhanced cellular toxicity observed. As discussed above, porous Si can reduce quinone to a semiquinone radical. Mass spectra obtained on the water-soluble extracts of the daunorubicin-modified porous Si microparticles display a molecular ion consistent with reduction of the quinone group of daunorubicin to a semiquinone radical (Figure 3.9). Chemically modified drug (daunorubicin bound to undecylenic acid linker) was not detected (Figure 3.9). We propose that superoxide radicals, formed as the semiquinone free radical is oxidized back into the parent quinone, are responsible for the higher toxicity observed with released daunorubicin relative to the
as-received drug. These results suggest that a lower overall quantity of drug may be needed to inhibit RPE cell proliferation, if the drug is delivered via porous Si microparticles. However, the fact that this system shows enhanced toxicity to RPE cells opens the possibility that it may show unwanted toxicity to other cells of the eye, or that it may introduce other deleterious effects. Such a possibility suggests that a thorough \textit{in vivo} study is needed to assess the efficacy of the porous Si drug delivery system, and it underscores a limitation of the approach--covalent attachment of drugs to a potentially reactive porous Si matrix may not be appropriate for many \textit{in-vivo} applications.
Figure 3.7 Viability of ARPE-19 cells (MTT assay) after exposure to as-received daunorubicin, silicon released (as soluble orthosilicate) from porous Si microparticles (containing the undecylenic acid linker chemistry but not containing any drug), and daunorubicin that had previously been attached to porous Si microparticles and then released by hydrolysis into aqueous solution. The $x$-axis displays the daunorubicin concentration in terms of the total amount of daunorubicin added per milliliter of media. The “Released Si” trace represents the soluble fraction of material released from a mass of porous Si microparticles that is equal to the mass of porous Si microparticles used in the “Released Daunorubicin” trace.
Figure 3.8  Fluorescence microscope images of ARPE-19 cells comparing the toxicity of as-received daunorubicin (“Free Drug”), daunorubicin that had previously been attached to porous Si microparticles (“Released Drug”), and the soluble by-products of porous Si dissolution from non drug loaded porous Si (“Released Si”). A control experiment using PBS buffer only is shown for comparison (“Control-PBS”). Green and red in images corresponds to calcein AM (live cells) and ethidium homodimer-1 (dead or damaged cells), respectively. The scale bar is 100 μm.
Figure 3.9 (top) Representative mass spectra of as-received daunorubicin generated by electron spray ionization (ESI) mass spectrometry. A m/z corresponding to 527.86 represents $[\text{M}+\text{H}]^+$, where M is daunorubicin. (bottom) Representative mass spectra of the water-soluble extracts of the daunorubicin-modified porous Si microparticles. A m/z corresponding to 528.88 represents $[\text{M}+\text{H}]^+$, where M is daunorubicin semiquinone. A m/z corresponding to 550.95 assigned to $[\text{M}+\text{Na}]^+$. 
3.5 Conclusions

In summary, daunorubicin can be loaded into porous Si particles via covalent attachment and it is slowly released as the porous Si matrix degrades. Using porous Si microparticles, sustained release of daunorubicin at therapeutic concentrations (for PVR treatment) can be maintained for a period of 30 days, significantly longer than could be obtained with an injection of comparable quantities of free drug. The released drug is able to inhibit the growth of RPE cells at even lower concentrations than as-received daunorubicin, suggesting that lower doses of the drug could be effective with this formulation. The extent of degradation of the porous Si particles can be monitored by the observed color change of the particles, and the spectral shift directly correlates with the amount of drug released. The data presented represent an effective means of providing extended drug release from an intravitreally injected material that can be monitored in a non-invasive clinical setting.
Chapter three, in part or in full, is a reprint (with co-author permission) of the material as it appears in the following publication: E.C. Wu, J.S. Andrew, L.C. Cheng, W.R. Freeman, L. Pearson, M.J. Sailor, Real-time monitoring of sustained drug release using the optical properties of porous silicon photonic crystal particles, *Biomaterials* 2010. The author of this dissertation is the primary author of this manuscript.
Chapter Four

SURFACE MODIFICATION OF POROUS SILICON

FOR LONG-TERM DELIVERY OF REDOX

ACTIVE THERAPEUTICS
4 Surface Modification of Porous Silicon for Long-term Delivery of Redox Active Therapeutics

4.1 Abstract

Chemical modification of porous Si microparticles was used to improve its compatibility for providing long-term release of daunorubicin, a redox active anthracycline drug. Three different surface chemistries were prepared: thermal hydrosilylation with undecylenic acid, thermal oxidation at 150°C, and thermal oxidation at 800°C. It was found that following thermal hydrosilylation, remaining Si-H species reduced daunorubicin into various degradation products, and upon release from porous Si, no unmodified daunorubicin was present. Oxidation at 150°C reduced the amount of surface hydrides, resulting in the partial recovery of unmodified daunorubicin upon release from the particles. Complete oxidation of porous Si to SiO₂ eliminated all reactive hydrides species and no reduction of daunorubicin was detected. *In vitro* cell toxicity assays on retinal pigment epithelial (RPE) cells showed increased toxicity for drug released from hydrosilylated particles and particles oxidized at 150°C. However, released drug from particles oxidized at 800°C showed no increased toxicity compared with as-received daunorubicin. Furthermore, particles oxidized at 800°C provided the most prolonged rate of daunorubicin release. Therefore, thermal oxidation of porous Si microparticles was successfully demonstrated to provide a compatible system for the long-term release of daunorubicin.
4.2 Introduction

Controlled drug delivery systems are important in improving current therapies by decreasing systemic toxicity and increasing the amount of drug to the target site.\(^1\) Porous silicon (Si) is an attractive material for drug delivery due to its high surface area,\(^31\) tunable pore sizes and volumes,\(^31\) and biocompatibility.\(^43\) In addition, multilayers of varying optical densities can be created using porous Si, producing a photonic crystal.\(^71\) The wavelength shift in the reflectivity peak resulting from this multilayer structure can be used to monitor the loading and release of biomolecules,\(^108,109\) allowing porous Si to be used as a self-reporting drug carrier. For these reasons, porous Si has been widely studied for use in drug delivery.\(^30,47\)

Freshly prepared porous Si, however, has reactive surface hydrides with a reducing potential that could react with various drug molecules. Porous Si has already been found to be able to interfere with the MTT cell viability assay by directly reducing MTT into formazan, providing a signal that is equivalent to that given by the reductase enzymes present in live cells.\(^44,96\) It has also been shown that an unmodified porous Si surface is able to reduce benzoquinone to hydroquinone.\(^125\) Therefore, in the use of porous Si as a drug delivery carrier, the reaction between the drug of interest with surface hydrides must be taken into account, especially with the use of redox active molecules. For example, in the process of loading cisplatin into porous Si microparticles, a portion of cisplatin is reduced to Pt\(^0\). In this case, the metallic platinum that forms partially caps the
pores, effectively trapping active cisplatin inside the porous Si matrix to provide for sustained drug release.\textsuperscript{128} However, reduction of other drug compounds may be more problematic. Anthracyclines such as doxorubicin, epirubicin, and daunorubicin have been found to be effective against a wide range of human malignant neoplasms, but have dose-limiting cardiotoxicity.\textsuperscript{126, 127} This observed toxicity has been attributed to the \textit{in vivo} reduction of the quinone moiety to a semiquinone free radical.\textsuperscript{129} The semiquinone in daunorubicin, for example, can disproportionate to the hydroquinone or back to the starting quinone, and the hydroquinone eliminates the amino sugar, and further degradation of the compound occurs.\textsuperscript{130} In this process, it has be proposed that the production of reactive oxygen radicals such as superoxide or the hydroxyl radical can produce DNA damage.\textsuperscript{126, 129} Due to the reductive environment produced by porous Si, reduction of anthracycline drugs may occur. Therefore, increased toxicity may be observed with the use of porous Si as a drug delivery carrier for anthracyclines.

In this study, we evaluated the use of thermal oxidation as a means to eliminate surface hydrides in order to prevent reduction of the loaded drug, using daunorubicin as our model redox active compound. We found that only by complete conversion of Si to SiO\textsubscript{2} are surface hydrides eliminated, and modification of daunorubicin through reduction does not occur. In addition, extended release of daunorubicin from porous SiO\textsubscript{2} was achieved compared with release from hydrosilylated particles. Therefore, surface modification of porous Si can be used to protect the loaded redox active drug from reduction inside the porous matrix as well
as to improve drug release rates for long-term drug delivery applications.

4.3 Experimental Methods

4.3.1 Synthesis of Porous Si Microparticles

Porous Si microparticles were prepared from the electrochemical etching of highly doped, (100)-oriented, p-type Si wafers (Boron-doped, ~1 mΩ resistivity) in a 3:1 solution of 48% aqueous hydrofluoric acid (HF):ethanol. A Si wafer with an exposed area of 9 cm² was contacted on the back side with a strip of aluminum foil and mounted in a Teflon etching cell with a platinum counter-electrode. The wafer was etched at a constant current density of 90.2 mA/cm² for 5 minutes. The resulting porous layer was then lifted off by electropolishing in a 3.33% HF in ethanol solution for 2 minutes at a current density of 6.2 mA/cm². The electropolished layers were ultrasonicated in ethanol for 30 min.

4.3.2 Surface Modification of Porous Si Microparticles

Three different surface modification chemistries were explored for loading daunorubicin: 1) thermal hydrosilylation with undecylenic acid, 2) thermal hydrosilylation with undecylenic acid followed by thermal oxidation at 150°C for 48 hours, and 3) thermal oxidation at 800°C followed by carboxylic acid functionalization. Thermal hydrosilylation was achieved by immersion of porous Si microparticles in 1 mL of undecylenic acid (95%, purchased from Sigma-Aldrich) in a 10 mL Pryex beaker. The microparticles in undecylenic acid were then heated in a commercial consumer microwave oven (Sears Kenmore 700 W) for 4 min at 280 Watts. For
thermal oxidation at 150°C, hydrosilylated particles were placed in a ceramic spoon and heated in Thermo Scientific furnace chamber for 48 hours.

For complete oxidation of porous Si to porous SiO$_2$, particles were placed in a ceramic spoon and heated from room temperature to 800°C for 1 hour. The particles were allowed to cool and were rinsed thoroughly with ethanol. The resulting porous SiO$_2$ particles were then reacted with 2% concentrated HCl in water for 1 hour, rinsed 3 times with water and dried. The particles were then vortexed in a 1% 3-aminopropyltrimethoxy silane in ethanol solution for 1 hour for amine functionalization, rinsed with ethanol, and dried. Finally, amine-functionalized porous SiO$_2$ particles were reacted with 0.1 M succinic anhydride in DMF for 24 hours and rinsed with water to obtain a carboxylic acid terminated surface.

Surface functionalization was characterized by Fourier Transform Infrared (FTIR) spectroscopy. Attenuated total reflectance (ATR) measurements were made using a Nikolet 6700 FT-IR spectrometer.

4.3.3 Preparation of Mesoporous Silica Nanoparticles

For the synthesis of mesoporous silica nanoparticles, 400 mL of water and 500 mL of methanol were mixed in a large flask. Afterward, 3.53 g of hexadecyltrimethylammonium chloride (CTACl) and 2.258 mL of a 1M sodium hydroxide solution were added to the water/ethanol solution and stirred until a homogenous solution was present. 1.163 mL of tetramethylorthosilicate (TMOS) and 0.152 mL of 3-Aminopropyltrimethoxysilane (APS) were mixed under nitrogen and the TMOS/APTMS solution was added to the methanol/water solution. The solution was
stirred for 8 hours at 500 rpm and aged overnight. The solution was then filtered through a 0.2 μm filter and the collected particles were rinsed with deionized water. The particles were dried for 72 hours at 45°C. The surfactant (CTACl) was removed by heating the particles in a 1% concentrated HCl in ethanol solution at 60°C for 3 hours. The particles were filtered and rinsed 3 times with ethanol and 3 times with deionized water. The silica nanoparticles were reacted with 0.1 M succinic anhydride in DMF for 24 hours and rinsed with water to obtain a carboxylic acid terminated surface.

4.3.4 Loading of Daunorubicin

Approximately 5 mg of porous Si or SiO₂ microparticles were suspended in 650 μL of 10% dimethyl sulfoxide (DMSO) in Dulbecco’s phosphate buffered saline (PBS). For loading by covalent attachment, 50 mM EDC and 5 mM Sulfo-NHS were added to the 10% DMSO in PBS solution. For loading by physical adsorption, EDC/Sulfo-NHS was not used. Afterward, 200 μL of a 1 mg/mL solution of daunorubicin hydrochloride in water were added to the particles. The particles were vortexed for 2 hours at room temperature. To determine the amount of daunorubicin loaded in the particles, the drug loading solution before and after the loading reaction was compared. Additionally, the microparticles were also rinsed with water (physical adsorption) or ethanol (covalent attachment) 3 times and the amount of drug in the rinse solutions was taken into account for determining the amount of drug loading.
4.3.5 Characterization of Released Daunorubicin

Approximately 4 mg of particles loaded with daunorubicin particles were immersed in 2 mL of PBS in a glass vial. The particles were immersed for a period of 24 hours at room temperature. The solution was then centrifuged and the supernatant collected for liquid chromatography-mass spectrometry (LC-MS) analysis. A Shiseido C-18 column (2.0 mm ID x 5 mm length, 3 µm) and a gradient of 20% to 95% of pure methanol with 0.1% formic acid in 18 minutes were used. LC analysis with UV detection at 480 nm was performed prior to MS analysis. Electron spray ionization (ESI) mass spectrometry was used to detect daunorubicin and its degradation products. High resolution ESI-FT-MS (Orbit-Trap-MS) was used to identify the compound attributed to daunouubicin attached to the succinic anhydride linker.

4.3.6 Daunorubicin Release from Porous Si Microparticles

Daunorubicin was loaded into the microparticles by covalent attachment. To determine the release of daunorubicin in vitro, approximately 4 mg of particles were placed in a glass vial and immersed in 1.5 mL of PBS. Every 24 hours, 1.5 mL of PBS was collected from the vial and replaced with 1.5 mL of fresh PBS. Concentrations of the samples were determined by measuring the fluorescence intensity of the released drug with an excitation of 480 nm. The emission spectrum was recorded between 500-650 nm and the peak intensity of daunorubicin was found to be 590 nm. A calibration curve for daunorubicin in PBS was used to determine the amount of drug released. Si concentration was determined using inductively coupled plasma optical emission spectrometry (ICP-OES).
4.3.7 Cell Toxicity of Daunorubicin

Daunorubicin was loaded into the three particle types by physical adsorption. Approximately 4 mg of drug loaded particles or non-drug loaded particles were placed in glass vial containing 1.5 mL of PBS. After 24 hours, the solution was centrifuged and the supernatant collected. The amount of daunorubicin in the supernatant was determined by fluorescence spectrometry. Daunorubicin released from the particles were diluted to a highest concentration of 2.7 µg/mL, and followed by subsequent serial dilutions. Supernatant collected from non-drug loaded particles were diluted in the same manner as their respective drug loaded particles. Cell toxicity experiments were performed on retinal pigment epithelial cells (ARPE-19). 100 µL of various concentrations of the samples were added to 50-60% confluent cells in a 48-well plate containing 200 µL media in each well. Cells were incubated with the particular sample for 48 hours and analyzed for toxicity (calcein AM and ethidium homodimer-1 live/dead assays).

4.4 Results and Discussion

4.4.1 Modification of Porous Si by Thermal Hydrosilylation and Oxidation at 150°C

Due to the instability of as prepared porous Si microparticles, porous Si microparticles functionalized with undecylenic acid were prepared, as described in previous chapters. This procedure results in a porous Si surface terminated with carboxyl groups, allowing for further functionalization. However, FTIR reveals that microwave assisted thermal hydrosilylation did not eliminate all surface hydrides, as
shown by the presence of the ν Si-H band at 2098 cm\(^{-1}\) (**Figure 4.1a**). To eliminate the remaining reactive surface Si-H species, hydrosilylated particles were thermally oxidized at 150°C for 48 hours. FTIR analysis reveals that this mild oxidation procedure eliminated surface hydrides on porous Si (**Figure 4.1b**), but complete conversion of the entire Si matrix to SiO\(_2\) has not occurred. Therefore, as the porous Si matrix begins to degrade, surface hydrides will be exposed.

### 4.4.2 Modification of Porous Si by Oxidation at 800°C

For complete conversion of Si to SiO\(_2\), eliminating Si-H species, porous Si particles were thermally oxidized for 1 hour at 800°C. In order to obtain a surface terminated by carboxyl groups for drug loading, the particles were reacted in a solution containing 2% concentrated HCl in water. This was followed by silanization with 3-aminopropyltrimethoxy silane to obtain amine-functionalization. The amine-functionalized particles were then reacted with 0.1M succinic anhydride in DMF.\(^{131}\) The carboxylic acid group was obtained by the ring opening of succinic anhydride through reaction with the amine group on the surface of particles.\(^3\) FTIR shows successful conjugation with the appearance of the ν C=O band at 1719 cm\(^{-1}\), the amide I band at 1640 cm\(^{-1}\), and the amide II band at 1556 cm\(^{-1}\), as shown in **Figure 4.1c**.
Figure 4.1 FTIR spectra of hydrosilylated porous Si particles (a), hydrosilylated particles treated with thermal oxidation at 150°C for 48 hours (b), and porous Si particles oxidized at 800°C for 1 hour (c).
4.4.3 Loading of Daunorubicin to Porous Si and SiO₂ Particles

Daunorubicin, an anthracycline quinone drug, was used as a model redox active compound in this study. Modification of daunorubicin following loading into hydrosilylated porous Si microparticles has been previously observed due to the reductive environment of porous Si. We propose that this is due to the reduction of daunorubicin by the remaining Si-H species following hydrosilylation, and that this reduction can be minimized by thermal oxidation to reduce the presence of surface hydrides (Figure 4.2). Covalent attachment of daunorubicin to the particles via EDC/Sulfo-NHS chemistry was used to couple the carboxylic acid group on the porous Si surface to the amine group of daunorubicin for long-term drug release (Figure 4.3). Drug loading was determined by measuring the decrease in the amount of daunorubicin in the drug loading solution and the amount of drug in the rinse solutions. For hydrosilylated particles, approximately 22.7 ± 0.4 µg of drug was loaded per mg of particle. For particles oxidized at 150°C for 48 hours, only 11.2 ± 1.0 µg of drug was loaded per mg of particle. This is due to the reduction in the number of carboxylic acid groups during the oxidation. For particles oxidized at 800°C for 1 hour, 38.9 ± .03 µg of drug was loaded per mg of particle, representing the highest loading efficiency of the 3 particle types.
Figure 4.2 Schematic showing the reduction of daunorubicin by porous Si into semiquinone radical, then hydroquinone, and then further degradation products in which the amino sugar is eliminated.
Figure 4.3 Schematic showing the loading of daunorubicin onto porous Si microparticles that were thermally oxidized and/or oxidized at 150°C (a) and loading of daunorubicin into porous SiO₂ microparticles prepared by thermal oxidation at 800°C (b).
4.4.4 Characterization of Released Daunorubicin

The compounds released from the particles in PBS over a period of 24 hours were analyzed using LC-MS. LC with UV detection on as-received daunorubicin revealed a single peak at a retention time of 10.4 minutes (Figure 4.4a). In addition, MS analysis revealed a m/z of 528.07, attributed to [M+H]^+, and a m/z of 550.21, attributed to [M+Na]^+ (Figure 4.4b). LC with UV detection on compounds released from drug loaded hydrosilylated samples showed multiple peaks between a retention time of 10-17 minutes (Figure 4.5a). MS analysis on each of the LC trace peaks indicated that daunorubicin had been reduced and degraded. For example, MS analysis of the LC trace peak at 11.7 minutes showed a m/z of 406.17, suggesting the presence of 7-deoxydaunomycinone semiquinone. None of the peaks in the LC trace contained a m/z corresponding to unmodified daunorubicin.

The LC trace for released compounds from particles oxidized at 150°C revealed multiple peaks, but fewer peaks were obtained compared to release compounds from hydrosilylated particles. In addition, the LC trace peak with a retention time at 10.8 minutes was found to contain unmodified daunorubicin (Figure 4.5b). MS analysis on that peak showed a m/z at 528.08, corresponding to [M+H]^+, and a m/z at 550.20, corresponding to [M+Na]^+. Therefore, with a decrease in surface hydrides through oxidation at low temperatures, unmodified drug can be obtained, although degradation products were still present. This occurred because SiO₂ is susceptible to hydrolysis and at low temperatures, complete oxidation has not occurred,⁴⁷ and therefore surface hydrides will appear as the particles are degraded. The LC trace for compounds
released from particles oxidized at 800°C revealed a main peak with a retention time of 10.5 (Figure 4.5c). MS analysis of this peak showed a m/z of 528.12 and 550.26, corresponding to [M+H]^+ and [M+Na]^+, respectively, where M is unmodified daunorubicin. In addition, MS analysis of the LC trace peak at 14.2 minutes suggested a compound in which daunorubicin remains attached to Si through the succinic anhydride linker. In this case, daunorubicin was released as hydrolysis of the Si-O back bonds occurred\textsuperscript{106}, but hydrolysis of the amide bond linking daunorubicin to the linker molecule has not occurred. High resolution MS was performed to confirm this proposed structure (Figure 4.6). Simulation of the proposed molecule produced a mass of 769.2247. The measured mass of the sample was 769.2253, confirming the compound is daunorubicin attached to Si through the linker molecule, and not degradation products due to the reduction of daunorubicin.
Figure 4.4 LC trace with UV detection at 480 nm of daunorubicin (a) and MS analysis of the peak detected in the LC trace of daunorubicin (b).
Figure 4.5 LC traces with UV detection at 480 nm of compounds released from hydrosilylated particles (a), particles oxidized at 150°C for 48 hours (b), and particles oxidized at 800°C for 1 hour (c).
**Proposed Structure**

<table>
<thead>
<tr>
<th>Theoretical Mass</th>
<th>Mass Measured</th>
<th>Delta (ppm)</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>769.2247</td>
<td>769.2253</td>
<td>0.8</td>
<td>C$<em>{34}$ H$</em>{42}$ N$<em>2$ O$</em>{15}$ Si Na</td>
</tr>
</tbody>
</table>

**Figure 4.6** High resolution MS of the sample (a) and simulation of the proposed compound (b) to confirm the presence of daunorubicin attached to Si through the succinic anhydride linker.
4.4.5 Cell Toxicity of Released Drug

The activity of released drug from porous Si or SiO$_2$ particles was tested. Daunorubicin was loaded by physical adsorption in order to obtain higher concentrations in a relatively short period of time. Non drug loaded particles (“empty particles”) were used as a control. After immersion of particles in PBS for 24 hours, the supernatant was collected from the drug loaded samples and diluted to 2.7 µg/mL, as determined by fluorescence spectroscopy. The supernatant from the empty particles were diluted in the same manner as their drug loaded counterpart. The fluorescence emission from each sample was attributed to the various compounds observed in its respective LC trace. The concentration of released drug therefore includes all compounds emitting at the same wavelength as unmodified daunorubicin.

Each sample was incubated with retinal epithelial (RPE) cells for 48 hours. Toxicity was assessed using the Live/Dead assay with calcein AM and ethidium homodimer-1. Consistent with previous findings$^{132}$, released drug from hydrosilylated particles exhibited a higher toxicity than as-received daunorubicin (Figure 4.7). Supernatant collected from non drug loaded hydrosilylated particles exhibited no toxicity. Therefore, the increased toxicity observed is due to the production of radical species as daunorubicin degradation compounds are produced.

Released drug from particles oxidized at 150°C also exhibited a much higher toxicity compared to as-received daunorubicin despite the presence of unmodified daunorubicin. However, control experiments measuring the toxicity of the supernatant collected from non-drug loaded particles oxidized at 150°C also exhibited significant
toxicity. The toxicity from non drug loaded particles can therefore be attributed to the instability of the grafted undecylenic acid on the porous Si surface, which is consistent with the decreased drug loading capacity of these particles after oxidation at 150°C due to the decrease in carboxyl groups.

Released drug from particles oxidized at 800°C showed similar toxicity and ability to inhibit cell proliferation as as-received daunorubicin. In addition, the control using the supernatant from non-drug loaded particles oxidized at 800°C for 1 hour exhibited no toxicity. These results are consistent with the findings from LC-MS, indicating that released drug from particles oxidized at 800°C are mostly in the form of unmodified daunorubicin, with a portion of daunorubicin still attached to Si through the succinic anhydride linker. Therefore, it would be expected that released drug from the oxidized particles would behave in the same manner as as-received daunorubicin.
Figure 4.7 Fluorescence microscope images of ARPE-19 cells comparing the toxicity of as-received daunorubicin to released drug from hydrosilylated particles, from particles oxidized for 48 hours at 150°C after hydrosilylation, and from particles oxidized for 1 hour at 800°C. A control experiment using only PBS is shown. The scale bar is 100 µm.
4.4.6 In Vitro Release of Daunorubicin

An important aspect in drug delivery is the ability to provide sustained release of a therapeutic, and it has been shown that surface chemistry influences the rate of drug release. Long-term release of drug from porous Si microparticles hydrosilylated with undecylenic acid has previously been demonstrated. However, increased in vitro toxicity induced through the reduction of drug by surface hydrides may cause increased in vivo toxicity. Therefore, to determine if long-term drug release can be maintained by using less reactive surface chemistries, in vitro release of daunorubicin was evaluated. Mesoporous silica nanoparticles (500 nm) were also prepared by the sol-gel route for comparison to silica particles prepared by the thermal oxidation of porous Si. Characterization of these nanoparticles will be discussed in Appendix A.

Particles were placed in 1.5 mL of PBS and the PBS was collected each day and analyzed for drug release by fluorescence spectrometry and Si degradation by ICP-OES. It was found that the fastest rate of drug release occurred with particles oxidized at 150°C following hydrosilylation (Figure 4.8a). However, Si degradation was not significantly higher than for hydrosilylated particles (Figure 4.8b). Therefore, the increased drug release is due to the instability of the undecylenic acid linker when oxidized at 150°C. On the other hand, completely oxidized particles (800°C, 1h) resulted in the slowest release rate of daunorubicin, releasing approximately 2.5% of the loaded drug after 7 days. Similarly, degradation of these particles was also the slowest, releasing 185 µg of Si after 7 days. Additionally, daunorubicin released from oxidized particles at 800°C was slower compared to release from silica nanoparticles prepared by
the sol-gel route. Over the 7 day period, approximately 7.4% of the loaded daunorubicin and 350 µg of Si were released from mesoporous silica nanoparticles. Thus, silica nanoparticles released approximately 3 times the amount of drug compared to the silica microparticles, while only releasing approximately 2 times the amount of Si (Figure 4.8c). These results indicate that the degradation rate for silica nanoparticles prepared by the sol-get route may be slower than the drug release rate. Further studies are needed to establish the long-term drug release and degradation properties. However, current results suggest that porous silica microparticles prepared by thermal oxidation is able to provide for the most extended sustained drug release.

As mentioned previously, an advantage of using porous Si formed by electrochemical etching as a drug delivery system is the ability to utilize its unique optical properties to monitor the extent of drug release. Recently, it was shown that oxidation of porous Si up to 950°C can preserve the structures required for optical monitoring of biomolecules. Therefore, in addition to maintaining the ability for long-term drug release, oxidation of porous Si microparticles at 800°C also preserves the ability for being a self-reporting drug carrier. This is advantageous especially in the field of ocular drug delivery, as the eye is relatively transparent and allows for non-invasive monitoring.
Figure 4.8 Quantification of drug release (a) and Si dissolution (b) from porous Si and SiO$_2$ particles in PBS. Comparison rate of drug release and Si dissolution between silica particles prepared by thermal oxidation and the sol-gel route (c).
4.5 Conclusions

Despite the numerous advantages of using porous Si as a drug carrier, there is concern regarding the reactivity between loaded therapeutics and the reactive Si-H species. In this study, we showed that oxidation of porous Si at 800°C eliminated reactive hydride species and did not degrade daunorubicin, the model redox active drug. In addition, sustained release of daunorubicin was demonstrated by the covalent attachment of daunorubicin to oxidized porous Si particles. These findings indicate that through surface chemistry modification, a drug delivery system using porous Si can be achieved to maintain the integrity of the drug as well as to provide for long-term drug release.
Chapter four, in part, is a reprint (with co-author permission) of the material as it appears in the following publication: E.C. Wu, J.S. Andrew, A. Buyanin, J.M. Kinsella, M.J. Sailor, Surface modification of porous silicon for long-term delivery of redox active therapeutics, *Chem Comm*, manuscript in preparation. The author of this dissertation is the primary author of this manuscript.
Chapter Five

DEGRADATION AND BIOCOMPATIBILITY OF POROUS SI AND SiO₂ MICROPARTICLES IN THE RABBIT EYE
5 Degradation and Biocompatibility of Porous Si and SiO$_2$

**Microparticles in the Rabbit Eye**

5.1 Abstract

Porous Si-based microparticles have been demonstrated to be able to provide sustained release of therapeutics in an *in vitro* system designed to mimic the clearance of therapeutics in the human vitreous. In addition, previous studies have shown that thermally oxidized porous Si (SiO$_2$) particles are non toxic in the rabbit eye. In this study, we further investigated the potential of porous Si and SiO$_2$ microparticles for use in ocular drug delivery. Porous Si microparticles hydrosilylated with undecylenic acid as well as porous Si and SiO$_2$ microparticles loaded with daunorubicin were evaluated for biocompatibility and biodegradability using a rabbit eye model. It was found that porous Si particles hydrosilylated with undecylenic acid displayed no toxicity, and degraded in the vitreous over a period of 13 weeks. In addition, porous Si and SiO$_2$ particles loaded with daunorubicin were found to be biocompatible and did not induce any abnormalities in the retina. These results indicate that porous Si based particles may be a suitable carrier for the long-term delivery therapeutics.

5.2 Introduction

As discussed in chapters one and three, ocular drug delivery poses great challenges due to the difficulty of transporting therapeutics across barriers such as the blood-retinal-barrier (BRB) and the blood-aqueous barrier (BAB). In
addition, the half-life of many therapeutics to treat retinal diseases, especially those that have low molecular weights, are only on the order of several hours.\textsuperscript{134} Therefore, frequent intravitreal injections are often needed in order to maintain therapeutic concentrations in the eye, which causes patient discomfort and has serious risks such as retinal detachment and vitreous hemorrhage.\textsuperscript{99, 134} Thus, drug delivery carriers providing sustained release of ocular therapeutics are needed to improve upon current treatment.

In Chapter 3, it was shown that hydrosilylated porous silicon microparticles can potentially improve the therapeutic effect of daunorubicin for the treatment of proliferative vitreo-retinopathy (PVR), by providing sustained release of drug for up to one month.\textsuperscript{132} PVR is a retinal disease characterized by the formation of contractile cellular membranes, and is the major reason for surgical failure and blindness in the treatment of retinal detachment.\textsuperscript{135, 136} The pathogenesis of PVR involves cell migration, cell proliferation, membrane formation and membrane contraction.\textsuperscript{135} Many therapeutics designed to prevent PVR, such as daunorubicin, have targeted the cell proliferation state.\textsuperscript{111} With only a 5 hour half-life in the vitreous however,\textsuperscript{114} daunorubicin alone is only mildly effective in the prevention of PVR.\textsuperscript{112} The use of porous Si based particles as drug carriers has shown promise in improving the efficacy of treatment with daunorubicin. However, as with all biomaterials, the biocompatibility and biodegradability of the drug carrier must be investigated.

As discussed in Chapter 4, increased \textit{in vitro} toxicity of retinal pigment
epithelial (RPE) cells was observed as reactive surface Si-H species on hydrosilylated porous Si particles were able to reduce daunorubicin and produce radical species.\textsuperscript{137} The potential for these particles to cause \textit{in vivo} toxicity could therefore prevent the use of porous Si-based particles as an effective treatment for PVR. Current treatments of PVR, for example, includes vitrectomy, removal of the contractile membranes, and the use of a tamponade made of silicone oil to seal the retinal breaks.\textsuperscript{136, 138} However, silicone oils can cause inflammatory reactions and lead to damage of the retinal tissue by infiltration of inflammatory cells. Silicone oil vesicles have also been observed in cells in the posterior segment of the eye.\textsuperscript{138} In addition, because silicone oil does not degrade, it requires removal due to the complications that can occur. For these reasons, the use of silicone oil is not ideal despite it having other suitable properties such as stability and transparency,\textsuperscript{138} highlighting the importance of biocompatibility and biodegradability. Therefore, a successful drug delivery carrier would need to be both biocompatible and biodegradable.

Non drug loaded porous Si particles have already been investigated for its biocompatibility as a membrane to support ocular cells,\textsuperscript{46} as well as a drug carrier for the treatment of ocular diseases.\textsuperscript{98} In the study of porous Si as a potential drug carrier, porous Si microparticles that had been freshly etched, hydrosilylated with 1-dodecene, and thermally oxidized were injected in the rabbit eye and no toxicity was observed over a period of 4 months. The half-life of the particles depended on the surface chemistry, and it ranged from 1 week for freshly etched particles to 16
weeks for hydrosilylated particles. However, the biocompatibility and biodegradability of porous Si particles hydrosilylated with undecylenic acid or drug loaded porous Si particles has yet to be investigated. Therefore, in this study, we are investigating the biodegradability and biocompatibility of daunorubicin loaded porous Si and SiO\textsubscript{2} particles \textit{in vivo} using a rabbit eye model.

5.3 Experimental Methods

5.3.1 Synthesis of Porous Si Microparticles

Porous Si microparticles were prepared from the electrochemical etching of highly doped, (100)-oriented, p-type Si wafers (Boron-doped, \textasciitilde 1 m\Omega resistivity) in a 3:1 solution of 48% aqueous hydrofluoric acid (HF):ethanol. A Si wafer with an exposed area of 9 cm\textsuperscript{2} was contacted on the back side with a strip of aluminum foil and mounted in a Teflon etching cell with a platinum counter-electrode. The wafer was etched with a sinusoidal waveform current density as described in Chapter 3 (Section 3.3.1) for biodistribution studies, and at a constant current density of 90.2 mA/cm\textsuperscript{2} for 5 minutes for toxicity studies. The resulting porous layer was then lifted off by electropolishing in a 3.33% HF in ethanol solution for 120 seconds at a current density of 6.2 mA/cm\textsuperscript{2}. The electropolished layers were ultrasonicated in ethanol for 30 min.

5.3.2 Surface Modification of Porous Si Microparticles

Two different surface modification chemistries were explored for loading daunorubicin: thermal hydrosilylation with undecylenic acid and thermal oxidation at 800°C followed by carboxylic acid functionalization. Thermal hydrosilylation was
achieved by immersion of porous Si microparticles in 1 mL of undecylenic acid (95%, purchased from Sigma-Aldrich) in a 10 mL Pryex beaker. The microparticles in undecylenic acid were then heated in a commercial consumer microwave oven (Sears Kenmore 700 W) for 4 min at 280 Watts. The particles were then rinsed with hexane and ethanol to remove excess undecylenic acid.

For complete oxidation of porous Si to porous SiO$_2$, particles were placed in a ceramic spoon and heated from room temperature to 800°C for 1 hour. The resulting porous SiO$_2$ particles were then reacted with 2% concentrated HCl in water for 1 hour, and rinsed 3 times with water. The particles were then vortexed in a 1% 3-aminopropyltrimethoxy silane in ethanol solution for 1 hour for amine functionalization, and rinsed with ethanol. Finally, amine-functionalized porous SiO$_2$ particles were reacted with 0.1 M succinic anhydride in DMF for 24 hours and rinsed with water to obtain a carboxylic acid terminated surface.

5.3.3 Loading of Daunorubicin into Porous Si and SiO$_2$ Microparticles

Approximately 5 mg of porous Si or SiO$_2$ microparticles were suspended in 650 μL of 10% dimethyl sulfoxide (DMSO) in Dulbecco’s phosphate buffered saline (PBS). 50 mM EDC and 5 mM Sulfo-NHS were added to the 10% DMSO in PBS solution. Afterward, 200 μL of a 1 mg/mL solution of daunorubicin hydrochloride in water were added to the particles. The particles were then vortexed for 2 hours at room temperature. To determine the amount of daunorubicin loaded in the particles, the drug loading solution before and after the loading reaction was compared. Additionally, the
microparticles were also rinsed with or ethanol 3 times and the amount of drug in the rinse solutions were taken into account for determining the amount of drug loading.

5.3.4 Biodistribution of Non-Drug Loaded Porous Si Microparticles in the Rabbit Eye

12 New Zealand Red rabbits were used to study the biodistribution of hydrosilylated porous Si microparticles after injection into the rabbit eye. A sinusoidal waveform was used during the etching of the hydrosilylated porous Si microparticles to achieve a rugate structure. All of the animal handlings were carried out in accordance to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. One eye of each animal was injected with 100 µL of balanced salt solution (BSS) containing 3.2 mg of porous Si particles using a 27 gauge needle. However, due to the hydrophobic nature of the particles and the diameter of the syringe, a portion of the 3.2 mg of the particles was lost in the syringe during delivery. Therefore, 3.2 mg is an overestimate in the amount of porous Si injected. The fellow eye was injected with 100 µL of saline as a control.

After intravitreal injection, fundus photography was carried out to observe the color of the particles imparted by its rugate structure. At time points of: 1 week, 2 weeks, 3 weeks, 5 weeks, 9 weeks, and 13 weeks, 2 animals were sacrificed and the vitreous, choroid, retina, aqueous humor, and plasma were collected for analysis. Tissue samples were completely dissolved in concentrated nitric acid for a minimum of 1 week. The samples were centrifuged and any remaining porous Si particles were dissolved completely in 1M KOH. The dissolved samples were diluted in water and Si
content was measured by inductively coupled plasma optical emission spectroscopy (ICP-OES). A Si standard was made in 2% nitric acid in water.

5.3.5 Toxicity of Non Drug Loaded Porous Si Microparticles

4 New Zealand Red rabbits were used to study the toxicity of hydrosilylated porous Si microparticles after injection into the rabbit eye. All of the animal handlings were carried out in accordance to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. One eye of each animal was injected with 100 µL of balanced salt solution (BSS) containing 3.2 mg of porous Si particles using a 27 gauge needle. However, due to the hydrophobic nature of the particles and the diameter of the syringe, a portion of the 3.2 mg of the particles was lost in the syringe during delivery. Therefore, 3.2 mg is an overestimate in the amount of porous Si injected. The fellow eye was injected with 100 µL of BSS as a control. After intravitreal injection, the eyes were monitored with indirect ophthalmoscope, tonometer and biomicroscopic slit lamp on days 2, 7, 14, 28, 96, 125, 161, and 177 after injection. Animals were also sacrificed after 177 days and the eye globes were enucleated for histology evaluation, using hematoxylin and eosin (H&E) staining. During the study, two of the rabbits obtained an infection and thus only two rabbits were monitored for toxicity.

5.3.6 Toxicity of Drug Loaded Porous Si and SiO₂ Microparticles

12 New Zealand Red rabbits were used to study the toxicity of porous Si and SiO₂ microparticles after injection into the rabbit eye. 6 rabbits were used for injection with hydrosilylated porous Si particles and 6 rabbits were used for injection with
oxidized porous Si (SiO$_2$) particles. One eye of each animal was injected with 100 µL of balanced salt solution (BSS) containing 3 mg of particles using a 27 gauge needle. The fellow eye was injected with 100 µL of BSS as a control. After intravitreal injection, the eyes were monitored with indirect ophthalmoscope, tonometer and biomicroscopic slit lamp 3 days, 5 days, 14 days, and 3 months after injection. The electroretinogram (ERG) was also recorded at 14 days and 3 months after intravitreal injection. Animals were sacrificed after 3 weeks or 3 months and the eye globes were enucleated for histology evaluation with H&E staining.

5.3.7 Pharmacokinetics of Daunorubicin Loaded into Porous Silica Particles

5 New Zealand Red rabbits were used to study the pharmacokinetics of daunorubicin loaded into completely oxidized porous Si particles. One eye of each animal was injected with 100 µL of balanced salt solution (BSS) containing 3 mg of particles using a 27 gauge needle. The fellow eye was injected with 100 µL of BSS as a control. After intravitreal injection, approximately 50 µL of the aqueous humor was sampled at various time points for 2 months. Fundus imaging was also performed to observe the color and decrease in the amount of particles. Detection of daunorubicin in the aqueous was performed by liquid chromatography-mass spectrometry (LC-MS).
5.4 Results and Discussion

5.4.1 Preparation of Drug Loaded Porous Si and SiO\textsubscript{2} Particles

Porous Si microparticles functionalized with undecylenic acid and porous SiO\textsubscript{2} microparticles functionalized with succinic anhydride were prepared, as previously described.\textsuperscript{132, 137} Loading of daunorubicin into the particles was accomplished by covalent attachment via EDC/Sulfo-NHS chemistry. Approximately 22 µg of daunorubicin were loaded per mg of hydrosilylated porous Si particles and approximately 38 µg of daunorubicin were loaded per mg of porous SiO\textsubscript{2} particles.

5.4.2 Biodistribution of Hydrosilylated Porous Si Particles

Rugate structured porous Si particles hydrosilylated with undecylenic acid were injected into rabbit eyes. At various time points, the rabbits were sacrificed and the vitreous, retina, choroid, aqueous humor, and plasma were collected for Si analysis. Normal vitreous, retina and plasma from rabbits that had not received any injection of porous Si particles were used to determine baseline levels of Si. As shown in Table 5.1, the highest amount of Si at each time point was found in the vitreous, followed by the aqueous. A small amount of Si was detected in the retina and an even smaller amount of Si was detected in the choroid. No detectable Si amounts were found in the plasma, suggesting that degraded Si does not reach the systemic circulation and remain within the eye. The low levels of Si detected in weeks 1 and 2 may be attributed to the incomplete dissolution of porous Si particles after dissolution of the tissue. In addition, although only one eye received an injection of porous Si particles, Si was detected in both eyes, indicating the possibility of transport between the two eyes. This has
previously been observed, where injection of a therapeutic into one eye resulted in detection of the drug in the fellow eye.\textsuperscript{139}

Prior to sacrifice, fundus images were also obtained to determine if the color of the particles imparted by its rugate structure could be observed. 4 days after injection, fundus images reveals porous Si particles display a vivid red color, and the particles are suspended in the vitreous mainly near the injection site (Figure 5.1). After 3 weeks, the particles displayed a vivid green color, and a slight decrease in the amount of particles was observed (Figure 5.1). The particles still remained suspended in the vitreous near the injection site. After 8 weeks, only a small portion of the particles still displayed a green color. The majority of the particles displayed a brown color due to the shift of the photonic signature to the ultraviolet range as the particles degrade. A significant decrease in the amount of particles was also observed after 8 weeks, indicating that particles hydrosilylated with undecylenic are biodegradable in the eye.
Table 5.1 Distribution of Si (displayed in µg) found in the various tissues of the eye. Particles were injected in right eye (OD), and the fellow eye (OS) received an injection of BSS. Si measured by ICP-OES.

<table>
<thead>
<tr>
<th>Rabbit (week sacrificed)</th>
<th>Vitreous</th>
<th>Retina</th>
<th>Choroid</th>
<th>Aqueous</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OS</td>
<td>OD</td>
<td>OS</td>
<td>OD</td>
</tr>
<tr>
<td>54 (1)</td>
<td>28.20</td>
<td>40.90</td>
<td>0.26</td>
<td>0.15</td>
</tr>
<tr>
<td>55 (1)</td>
<td>20.90</td>
<td>45.50</td>
<td>0.17</td>
<td>0.14</td>
</tr>
<tr>
<td>56 (2)</td>
<td>26.24</td>
<td>25.41</td>
<td>0.13</td>
<td>0.12</td>
</tr>
<tr>
<td>57 (2)</td>
<td>29.39</td>
<td>61.94</td>
<td>0.16</td>
<td>0.15</td>
</tr>
<tr>
<td>58 (3)</td>
<td>31.35</td>
<td>57.15</td>
<td>0.13</td>
<td>0.11</td>
</tr>
<tr>
<td>59 (3)</td>
<td>27.85</td>
<td>29.04</td>
<td>0.12</td>
<td>0.10</td>
</tr>
<tr>
<td>60 (5)</td>
<td>18.53</td>
<td>19.19</td>
<td>0.14</td>
<td>0.15</td>
</tr>
<tr>
<td>61 (5)</td>
<td>29.35</td>
<td>40.63</td>
<td>0.14</td>
<td>0.13</td>
</tr>
<tr>
<td>62 (8)</td>
<td>27.26</td>
<td>31.33</td>
<td>0.10</td>
<td>0.13</td>
</tr>
<tr>
<td>63 (8)</td>
<td>27.40</td>
<td>43.31</td>
<td>0.13</td>
<td>0.10</td>
</tr>
<tr>
<td>64 (13)</td>
<td>37.51</td>
<td>16.10</td>
<td>0.10</td>
<td>0.14</td>
</tr>
<tr>
<td>65 (13)</td>
<td>26.83</td>
<td>21.43</td>
<td>0.11</td>
<td>0.11</td>
</tr>
</tbody>
</table>
Figure 5.1 Fundus images of rugate structured porous Si particles hydrosilyated with undecylenic acid 4 days and 21 days after injection into the rabbit eye.
5.4.3 Toxicity of Hydrosilylated Particles

Porous Si particles hydrosilylated with undecylenic acid were injected into the rabbit eye and evaluated for toxicity using an indirect ophthalmoscope and a biomicroscopic slit lamp. Clinical examination of the eye that received the injection of porous Si particles demonstrated clinical tolerance equal to that in the controls. The clinical examination results are summarized in Table 5.2. Conjunctival hyperemia was scored according to previously reported methods. A grade of 0, indicating normal vessels, was observed at all time points except at day 2, where a grade of 1 indicates injected vessels. Anterior chamber flare and cell was not observed and the lens and vitreous opacity were graded 0, indicating that the lens and vitreous remained clear. Retinal damage and optic nerve abnormalities were not observed. Additionally, intraocular pressure (IOP) measurements were also taken using a tonometer and 3 measurements from each eye at each time point were obtained and averaged. The IOP of eyes injected with particles was 19.0 ± 3.1 and the IOP of control eyes was 18.6 ± 2.9 (p=0.71) during the 6 month period, indicating no significant difference.

Pathological examination by light microscopy showed normal retinal and choroidal morphology (Figure 5.2). There were no obvious signs of inflammation and hemorrhage, and disarrangement of the photoreceptor layer, inner nuclear layer, and outer nuclear layer was not observed. In addition, vacuolization and retinal swelling or loss was not observed in the photoreceptor layer. These results indicate that porous Si microparticles hydrosilylated with undecylenic acid are biocompatible with ocular tissues.
Table 5.2 Clinical examination of the retina after injection with porous Si microparticles hydrosilylated with undecylenic acid.

<table>
<thead>
<tr>
<th>Exam Day</th>
<th>Animal ID</th>
<th>Eye</th>
<th>IOP (mmHg)</th>
<th>Conjunctiva hypermia</th>
<th>Lens opacity</th>
<th>Vitreal opacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>44</td>
<td>OD</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OS</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>45</td>
<td>OD</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>OS</td>
<td>19</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>44</td>
<td>OD</td>
<td>15</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>OS</td>
<td>17</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>45</td>
<td>OD</td>
<td>15</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>OS</td>
<td>12</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>44</td>
<td>OD</td>
<td>19</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>OS</td>
<td>22</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>45</td>
<td>OD</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>OS</td>
<td>19</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>44</td>
<td>OD</td>
<td>26</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>OS</td>
<td>19</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>45</td>
<td>OD</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>OS</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>28</td>
<td>44</td>
<td>OD</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>OS</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>45</td>
<td>OD</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>OS</td>
<td>19</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>96</td>
<td>44</td>
<td>OD</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>OS</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>45</td>
<td>OD</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>OS</td>
<td>22</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>125</td>
<td>44</td>
<td>OD</td>
<td>24</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>OS</td>
<td>22</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>45</td>
<td>OD</td>
<td>23</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>OS</td>
<td>23</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>161</td>
<td>44</td>
<td>OD</td>
<td>16</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>OS</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>45</td>
<td>OD</td>
<td>16</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>OS</td>
<td>17</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>177</td>
<td>44</td>
<td>OD</td>
<td>21</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>OS</td>
<td>21</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>45</td>
<td>OD</td>
<td>19</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>OS</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 5.2 Light microscopic images taken at 25x magnification (a) and 62.5x magnification (b), showing normal retina and choroid, 6 months after intravitreal injection.
5.4.4 Toxicity of Drug Loaded Hydrosilylated Particles

Pathological examination by light microscopy showed normal retinal and choroidal morphology compared to control eyes from three weeks to three months after intravitreal injection (Figure 5.3). Infiltration by inflammatory cells such as macrophages was not present and the images showed normal organization of the architecture of the retina. In addition, vacuolization and retinal swelling or loss was not observed. Although drug loaded hydrosilylated particles displayed toxicity in vitro, toxicity was not observed in vivo. This difference may be due to the inability of modified daunorubicin to diffuse away from the RPE cells in a cell culture dish in vitro, resulting in increased concentrations and toxicity. On the other hand, in vivo, small drugs and solutes have the capability to diffuse out of the vitreous. In addition, the use of RPE cells as an in vitro tissue culture model to evaluate toxicity may not accurately represent in vivo conditions, as most drugs are in contact with the retinal surface and not the RPE. These discrepancies may contribute to the conflicting toxicity results between the in vitro and in vivo model.
Figure 5.3 Light microscopic images taken at 3 weeks and 3 months after intravitreal injection with hydrosilylated porous Si particles (OD) or BSS control (OS). Images show normal retinal morphology (taken at 62.5x magnification).
5.4.5 Toxicity of Drug Loaded Oxidized Particle

During 3 months of observation, no clinical toxicity was observed. The IOP of eyes injected with drug loaded porous silica particles were 14.5, 21.7, 18.7, and 19.9 at days 3, 5, 14, and 90 respectively (Figure 5.4). IOP of control eyes were 15, 18.8, 19.6, and 20.1 at days 3, 5, 14, and 90 respectively. These results indicate no significant difference in IOP between control and sample groups. Retinal function was also assessed by ERG examination. There were no significant differences in the b-wave amplitudes of the dark-adapted, light-adapted, and flicker ERG between control eyes and eyes injected with porous silica particles (Figure 5.5). Similarly, normal ERG responses were observed in the a-wave amplitude, a-wave implicit, and b-wave implicit. Furthermore, pathological examination by light microscopy showed normal retinal and choroidal morphology compared to control eyes from three weeks to three months after intravitreal injection (Figure 5.6). There were no swelling of retinal cells and no infiltration by inflammatory cells. The photoreceptor, inner, and outer nuclear layers also maintained normal organization. Thus, drug loaded oxidized porous Si particles are non-toxic to the retina.

It was previously found that a dose of 27 nmol of daunorubicin to the rabbit eye resulted in toxicity to the retina.143 In this study, the total amount of silica microparticles injected was 3 mg, resulting in approximately 114 µg of daunorubicin (216 nmol dose) delivered to the vitreous. Due to the narrow therapeutic index of daunorubicin, the ability to deliver a high initial dose with free daunorubicin to maintain therapeutic concentrations for prolonged periods is currently not possible. However, by
sequestering daunorubicin into porous silica microparticles, a normally toxic dose can be delivered safely, providing a reservoir for the sustained release of daunorubicin to treat PVR.
**Figure 5.4** Intraocular pressures of eyes injected with daunorubicin loaded porous silica particles (OD) and control eyes injected with BSS (OS).
Figure 5.5 ERG b-wave amplitudes of eyes injected with daunorubicin loaded porous silica particles (OD) and control eyes (OS). Results indicate normal retinal function, with no statistical difference between control and sample group.
Figure 5.6 Light microscopic images taken at 3 weeks and 3 months after intravitreal injection with oxidized porous Si particles (OD) or BSS control (OS). Images show normal retinal morphology (taken at 62.5x magnification).
5.4.6 Drug Release from Oxidized Porous Si Particles

Oxidized porous Si microparticles loaded with daunorubicin were injected into one eye of each rabbit, with the fellow eye receiving an injection of BSS as control. After the intravitreal injection, aqueous humor taps from each eye were obtained at various time points and analyzed for the presence of daunorubicin as well as daunorubicin attached to Si through the succinic anhydride linker. Vitreous taps were not obtained due to the removal of SiO$_2$ particles during the acquisition of the vitreous. As drugs can be eliminated from the vitreous by the anterior route, we hypothesized that daunorubicin could diffuse into the aqueous humor. However, mass spectrometry did not detect daunorubicin or daunorubicin attached to Si in the aqueous humor. Therefore, a method to measure daunorubicin concentrations in the vitreous is needed in order to accurately assess the pharmacokinetics of daunorubicin released from porous SiO$_2$ particles.

Fundus photography was also carried out prior to each aqueous tap. Immediately after intravitreal injection, the particles displayed a vivid red color due to the presence of daunorubicin. The oxidized particles themselves are transparent. In addition, the particles had dispersed into the surrounding vitreous. After 12 days, a significant portion of the particles still remained dispersed in the vitreous and were red in color, indicating the presence of daunorubicin. After one month, a significant amount of the particles had been degraded and eliminated from the vitreous. However, the remaining particles still displayed a red color due to the presence of daunorubicin. After 2 months, the majority of the particles had been degraded and eliminated from the
vitreous. Remaining particles still displayed a red color indicating that daunorubicin had not been completely eliminated. These results suggest that daunorubicin loaded porous SiO$_2$ particles are able to provide release of daunorubicin for a period of 1 to 2 months. In addition, if drug release occurred linearly over a period of 2 months, the average drug release rate would be approximately 1.9 µg per day. This would result in a daily concentration of approximately 1.3 µg/mL, which is well above the therapeutic concentration but below the toxic concentration.$^{122, 143}$ Therefore, these results suggest that daunorubicin loaded porous silica particles can provide up to 2 months of therapeutic daunorubicin levels without reaching a toxic concentration. However, measurement of the concentration of daunorubicin in the vitreous and retina is needed to accurately assess the pharmacokinetics.
Figure 5.7 Fundus images showing the daunorubicin loaded oxidized porous Si particles in the vitreous on day 1 (a), 1 week (b), 1 month (c), and 2 months (d) after intravitreal injection.
5.5 Conclusions

Porous Si and SiO₂ microparticles have previously been demonstrated to be able to provide \textit{in vitro} long-term release of small molecule drugs, and we have now investigated its degradation and biocompatibility \textit{in vivo}. Over a period of 3 months, daunorubicin loaded porous Si and SiO₂ particles were found to be biocompatible and normal retinal function and morphology were observed. We have demonstrated that by loading daunorubicin into porous Si and SiO₂ particles, a normally toxic dose of daunorubicin can be delivered without any toxicity. This allows for the extended period of therapeutic concentration. In addition, the particles are cleared from the vitreous over a period between 2 to 3 months, indicating its potential for long-term \textit{in vivo} drug release.
Chapter Six

CONCLUSIONS
6 Conclusions

This dissertation describes the development of porous Si based materials for the long-term delivery of small molecule therapeutics. Sustained drug delivery carriers are being developed to improve the efficacy of therapeutics by providing effective drug concentrations for prolonged periods without high and potentially toxic initial drug doses. In particular, retinal diseases would greatly benefit from a controlled drug delivery system due to the difficulty of delivering therapeutics to the posterior section of the eye by systemic or topical application. Many materials have been studied for drug delivery, and porous Si has been demonstrated to be a biocompatible material with many advantages as a drug carrier. Therefore, we have focused on developing a porous Si based drug carrier for the treatment of retinal diseases. This chapter will summarize the work that has been accomplished as well as discuss future directions.

Our first step was to improve the drug release kinetics previously observed with porous Si. In order to accomplish this, we developed a method to load doxorubicin into porous Si particles by covalent attachment through a 10-undecenoic acid linker. Chapter 2 demonstrates that when doxorubicin is loaded into porous Si particles by covalent attachment, drug release occurs as the porous Si is oxidized and degraded. Therefore, an initial burst drug release due to diffusion is not observed. Through covalent attachment, extended drug release was achieved compared to release from particles loaded with doxorubicin by physical adsorption. In addition, it was demonstrated that the released drug was able to maintain functionality, inhibiting the growth of Hela cells in vitro.
The next step was to develop the system for use as an intravitreal drug delivery agent, in particular for the treatment of proliferative vitreoretinopathy (PVR). Daunorubicin was loaded into rugate structured porous Si particles using the same attachment chemistry as doxorubicin. In order to assess drug release kinetics for intravitreal drug delivery, a flow cell chamber was designed to mimic the half-life of therapeutics in the vitreous. Free daunorubicin was spiked into the chamber and its clearance rate from the chamber verified accuracy of the system. Chapter 3 shows that when daunorubicin is sequestered into porous Si particles, sustained release can be maintained for 30 days. When compared to a single dose injection, the porous Si particles can extend the period of therapeutic concentration by a factor of 24. Additionally, it was established that there is a linear correlation between drug release and the color change of the particles. Due to the transparency of the eye, the color of the particles allows porous Si to be a self-reporting drug carrier. However, it was found that released drug from the particles exhibited higher toxicity on retinal pigment epithelial cells in vitro compared to as-received daunorubicin. Thus, we investigated the possible reactions between porous Si and daunorubicin.

Chapter 4 describes the various surface chemistries that were investigated for the loading and release of daunorubicin. It was found that remaining reactive Si-H species on porous Si after hydrosilylation with undecylenic acid was able to reduce the quionone moiety of daunorubicin, resulting in degradation products. Upon release from the particles, only degradation products of daunorubicin were detected. Thermal oxidation at 150°C reduced the number of Si-H species and intact daunorubicin was
detected upon release. However, degradation products were still observed. Thermal oxidation at 800°C eliminated all Si-H species by conversion to SiO₂, and upon release from the particles, only intact daunorubicin and daunorubicin attached to the succinic anhydride linker was detected. A slower drug release rate from the particles oxidized at 800°C was also observed compared to hydorsilylated porous Si particles, indicating its potential as a long-term drug delivery carrier.

In addition, daunorubicin release from silica microparticles formed by the oxidation of electrochemically etched porous Si and from silica nanoparticles formed by the sol-gel route and were compared. It was observed that over a 7-day period, mesoporous silica nanoparticles released 3 times the amount of daunorubicin and degraded at 2 times the rate of the thermally oxidized silica microparticles. This suggests that the degradation rate of the silica nanoparticles may be slower than the release rate of daunorubicin, potentially leaving behind an empty porous silica matrix after complete drug release. However, due to the ease of fabrication of silica particles by the sol-gel route when compared to electrochemical etching, long-term release studies should be conducted. In particular, for application in ocular drug delivery, micron-sized mesoporous silica particles should be fabricated for comparison to micron sized silica particles formed by the thermal oxidation of porous Si. The difference in shape (spherical vs tabular) and pore morphology (uniform vs branched) may result in different long-term degradation properties and drug release kinetics. These studies should be conducted to establish the ideal route for the fabrication of silica microparticles for ocular drug delivery.
As with all materials designed for biomedical applications, the biocompatibility of the material must be addressed. In chapter 5, we showed that daunorubicin loaded porous Si and SiO₂ microparticles are non toxic and are biodegradable. Porous Si and SiO₂ microparticles were tested in an in vivo rabbit model. Clinical examination and histopathology over the 3 month period revealed normal retinal and choroidal morphology and no signs of inflammation were detected. In addition, the color of the particles were imaged using a fundus camera and its degradation was observed over a period of 2 months. The next step would be to establish a method to determine the pharmacokinetics of daunorubicin when released from the particles. Due to the difficulty of obtaining vitreous taps without disturbing the particles in the vitreous, the rabbit should be sacrificed at each time point in order to obtain the whole vitreous for drug analysis. In addition, daunorubicin loaded porous Si or SiO₂ particles should be evaluated by treatment of an animal disease model. Conjuctival fibroblasts with platelet rich plasma can be used to induce PVR in rabbits. Prior to disease induction, daunorubicin loaded particles can be intravitreally injected. The ability of these particles to inhibit the formation of PVR can be assessed to determine the efficacy of the drug delivery system described in this dissertation.
Appendix A

LOADING AND RELEASE OF FOSCARNET FROM
MESOPOROUS SILICA MICRO- AND NANO-
PARTICLES
A Loading and Release of Foscarnet From Mesoporous Silica Micro- and Nano-particles

A.1 Abstract

Mesoporous silica materials have been shown to be biocompatible and biodegradable materials with high drug loading capacity. For these reasons, the use of mesoporous silica particles as drug delivery carriers has been widely studied. The loading and release of foscarnet, an anti-viral, from silica micro- and nano-particles were investigated. Silica microparticles were synthesized by the electrochemical etching of Si followed by thermal oxidation. Silica nanoparticles were synthesized by the co-condensation of tetramethylothsilicate (TMOS) and 3-aminopropyltrimethoxysilane (APS), and were further functionalized with polyethylene imine (PEI). Loading of foscarnet was accomplished by trapping via oxidation, and also by attachment to the amine group on the silica particle surface. Trapping by oxidation resulted in a large burst release of the drug, releasing 100% of the drug within several hours. Loading by interaction of foscarnet with the amine groups on the particle surface prolonged the release period, and it was observed that increased porosity allowed for increased drug loading capacity in the silica microparticles. The highest amount of drug loading and the longest drug release period was observed for silica nanoparticles functionalized with PEI. Complete in vitro release of Foscarnet occurred after a period of 13 days, indicating a highly cationic surface can delay the release of foscarnet.
A.2 Introduction

As mentioned in previous chapters, ocular drug delivery poses a great challenge due to the existence of biological barriers that prevent therapeutics from reaching the posterior section of the eye when administered systemically or topically. Because the eye is an immune-privileged site, ocular controlled drug delivery systems can be utilized to provide sustained release of therapeutics. An example of an ocular disease in need of a controlled drug delivery system is cytomegalovirus (CMV) retinitis, a retinal disease that occurs in roughly one-third of patients with AIDS, and is the cause of over 90% of HIV-related blindness. With the increased availability of highly active antiretroviral therapy (HAART) for AIDS patients, CMV retinitis is currently not widespread in developed countries. However, it still poses a problem in developing countries, where it is largely left undiagnosed and untreated, and where HAART is difficult to obtain. In addition, CMV retinitis may still develop or relapse if HAART is not effective.

CMV retinitis is characterized by dense retinal whitening and retinal cell necrosis with adjacent intraretinal hemorrhage. Without treatment, CMV retinitis is a progressive infection that leads to vision loss and blindness. Current treatment includes several anti-CMV drugs, none of which eliminate the virus, and therefore long term treatment is required. Ganciclovir is the current standard of treatment for CMV retinitis. It can be administered as an intravenous infusion (daily or twice daily) or as an intravitreal injection. In addition, a ganciclovir implant that can be placed into the vitreous cavity surgically has also been developed, providing 6-8 months of release,
and was found to be more effective than intravenous ganciclovir. However, treatment with ganciclovir is not without drawbacks. Bone marrow suppression is the most common systemic side effect, and toxicity and drug resistance has been observed with the long-term use of ganciclovir. Therefore, long term release of other anti-CMV drugs needs to be developed.

Foscarnet (trisodium phosponoformate) is a pyrophosphate analog that binds reversibly near the pyrophosphate binding site of DNA polymerase without requiring further modification. It has also been shown to be effective against CMV retinitis, and can be delivered intravenously or intravitreally. However, because of the multiple negative charges at physiological pH, foscarnet has low oral bioavailability and poor penetration into cells. Although effective against CMV, the high concentrations (8-15 g/day) required for intravenous delivery of foscarnet may result in severe renal impairment. Intravitreal injections of foscarnet may help to reduce systemic toxicity, but the short half-life of foscarnet in the vitreous requires frequent intravitreal injections. A minimally invasive and sustained drug release device would therefore be beneficial in reducing the potential systemic toxicity as well as the risks associated with frequent intravitreal injections.

In this work, we are investigating the use of silica micro- and nano-particles for the intravitreal delivery of foscarnet. As described previously, mesoporous silica particles have been studied as drug delivery carriers due to its biodegradability, biocompatibility, and its high surface area and pore volume which allows for a high drug loading capacity. Various methods have been employed in order to provide
controlled release of therapeutics from silica particles.\textsuperscript{155} Controlled release of vancomycin from silica microspheres, for example, has been demonstrated by modification of the acid-base catalyzed sol-gel process followed by emulsification.\textsuperscript{153}

In this chapter, we investigate the use of both electrochemically etched and sol-gel derived mesoporous micro- and nano- particles for the loading and sustained release of foscarnet. These injectable materials would provide a less invasive method of delivering therapeutics to the vitreous compared with implantable devices and scleral plugs, which require surgery.

A.3 Experimental Methods

A.3.1 Direct Foscarnet Attachment to Porous Silica Microparticles

Porous silica microparticles were prepared by the electrochemical etch of p-type ($\rho = 1.03$ m$\Omega \cdot $cm) Si wafers with a 9 cm$^2$ exposed area in a HF solution (3:1 HF:Ethanol) using a Teflon etch cell. A 222 mA/cm$^2$ current density was applied for 2 minutes. The resulting porous film was lifted off with a current density of 2.1 mA/cm$^2$ for 3 minutes in a 3.33% HF in ethanol solution. The film was fractured into particles by ultrasound in ethanol for 20 minutes. The resulting Si particles were thermally oxidized at 800°C for 1 hour and then treated with 3:1 H$_2$SO$_4$:H$_2$O$_2$ solution for 5 minutes and rinsed three times with water. 30 mg of these hydroxylated silica particles were refluxed with 20 mL a of 24 mg/mL foscarnet (trisodium phosphonoformate) solution for two hours. The particles were rinsed three times with deionized water, and dried under vacuum until use.
Fourier Transform Infrared Spectroscopy (FT-IR) was performed to evaluate drug loading in porous silica microparticles. Attenuated total reflectance (ATR) measurements were made using a Nicolet 6700 FT-IR spectrometer. Drug loading was also determined by thermogravimetric analysis (TGA) and energy dispersive X-ray spectroscopy (EDX). For TGA, approximately 20 mg of foscarinet or foscarinet loaded porous silica particles were placed in a 90 µl alumina sample cup. Samples were heated at a constant rate of 10°C/minute up to 600°C using TA Instruments Q600 simultaneous TGA/DSC. EDX mapping was obtained using a Phillips XL30 ESEM Field Emission Gun (FEG) electron microscope.

A.3.2 Quantification of Foscarinet Release

The amount of foscarinet released was measured using metal complexation chemistry for the detection of phosphates. Briefly, a 10 mM pyrocatechol violet (PV) solution was made by dissolving PV in deionized water, and a 10 mM Yb\(^{3+}\) solution was made by dissolving YbCl\(_3\) in deionized water. The Yb\(^{3+}\) solution was added to 5 mL of pH 7.0 (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) buffer for a final concentration of 50 µM and was mixed for 10 seconds. The PV solution was then added to the Yb\(^{3+}\) solution, resulting in a final concentration of 50 µM PV and was mixed for 15 seconds. The YbPV solution was added to the foscarinet sample and reacted for twenty seconds. Solution absorbance was measured at wavelengths 445 nm and 625 nm. Absorbance difference between a water control and the foscarinet sample was compared to generate a standard curve.
A.3.3 Trapping of Foscarnet into Porous Silica Microparticles by Oxidation

Porous silica microparticles were prepared by the electrochemical etch of p-type ($\rho = 4.6$ $\Omega\cdot$cm) Si wafers with a 9 cm$^2$ exposed area in a HF solution (3:1 HF:Ethanol) using a Teflon etch cell. A 23 mA/cm$^2$ current density was applied for 8 minutes. The resulting porous film was lifted off with a current density of 6.7 mA/cm$^2$ for 30 seconds in 3.33% HF in ethanol solution. The film was fractured into particles by ultrasound in ethanol for 20 minutes. Particle size and pore size were determined by scanning electron microscopy (SEM). For loading of foscarnet, the resulting particles were dried and immersed in a 24 mg/mL solution of foscarnet for 5 hours at room temperature. The particles in solution were then placed in the furnace at 100°C for 3 days to oxidize and trap the drug.

A.3.4 Preparation of Amine-functionalized Porous Silica Microparticles

Porous Si was prepared by the electrochemical etching of p-type Si ($\rho = 1$-$1.4$ m$\Omega\cdot$cm) in a HF solution (3:1 HF:Ethanol) using a Teflon etch cell with 9 cm$^2$ exposed area. 3 different current densities and times were applied: 1.8 mA/cm$^2$ for 8 minutes, 8.9 mA/cm$^2$ for 6 minutes, and 72.2 mA/cm$^2$ for 2 minutes. The resulting porous film was lifted off with a current density of 2.1 mA/cm$^2$ for 3 minutes in a 3.33% HF in ethanol solution. The film was then fractured into particles by ultrasound in ethanol for 20 minutes. The Si particles were thermally oxidized at 800°C for 1 hour and then treated with 3:1 H$_2$SO$_4$:H$_2$O$_2$ solution for 5 minutes and rinsed three times with water. The particles were then reacted with 1% 3-Aminopropyltrimethoxysilane (APS) in methanol solution for 1 hour in a glass vial and rinsed three times with methanol.
Amine functionalization was confirmed using the ninhydrin test. 10% by weight of ninhydrin was added to ethanol and heated until completely dissolved.

**A.3.5 Preparation of Amine-functionalized Porous Silica Nanoparticles**

For the synthesis of mesoporous silica nanoparticles, 400 mL of water and 500 mL of methanol were mixed in a large flask. Afterward, 3.53 g of hexadecyltrimethylammonium chloride (CTACl) and 2.258 mL of a 1M sodium hydroxide solution were added to the water/ethanol solution and stirred until a homogenous solution was present. 1.163 mL of tetramethylorthosilicate (TMOS) and 0.152 mL of 3-Aminopropyltrimethoxysilane (APS) were mixed under nitrogen and the TMOS/APTMS solution was added to the methanol/water solution. The solution was stirred for 8 hours at 500 rpm and aged overnight. The solution was then filtered through a 0.2 μm filter and the collected particles were rinsed with deionized water. The particles were dried for 72 hours at 45°C. The surfactant (CTACl) was removed by heating the particles in a 1% concentrated HCl in ethanol solution at 60°C for 3 hours. The particles were filtered and rinsed 3 times with ethanol and 3 times with deionized water. To prepare polyethylene imine (PEI)-mesoporous silica particles (Mn: ~60,000, Mw: 750,000), 400 μL of PEI were added per 10 mg of silica particles. The mixture was vortexed for one hour at room temperature. The particles were rinsed three times with deionized water.
A.3.6 Loading and Release of Foscarnet from Amine-functionalized Silica Micro- and Nano-particles

5 mg of foscarnet and 10 mg of silica particles were immersed in 800 μL of 2-\(\text{(N-morpholino)}\)ethanesulfonic acid (MES) buffer in a glass vial. 100 μL of 1M EDC and 100 μL 0.05M Sulfo-NHS were then added to the glass vial to for a total volume of 1 mL. 1M 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 0.05M N-hydroxysulfosuccinimide (Sulfo-NHS) solutions were made by dissolving the compounds in 0.1 MES Buffer at pH 4.7. The mixture was reacted at room temperature for two hours. The particles were then rinsed three times with water and dried under vacuum for at least 24 hours. To evaluate the release of foscarnet, 10 mg of drug loaded silica micro- or nano-particles were placed in 2 mL of Millipore water in a 15 mL centrifuge tube. All 2 mL of the solution was replaced with fresh Millipore water at 24 hour intervals. The release was performed until no foscarnet was detected in the release solutions. The total amount of foscarnet released was determined as the total amount of foscarnet loaded.

A.4 Results and Discussion

A.4.1 Direct Foscarnet Attachment to Porous Silica Microparticles

Direct attachment of foscarnet to porous silica microparticles was based on the previously demonstrated direct reaction of phosphorus acids with a hydroxyl of a silanol or on a silica gel surface.\textsuperscript{156} Our approach was to use the hydroxyl group on the porous silica surface to react with the phosphate group of foscarnet in order to load foscarnet into the porous silica particles, as shown in Figure A.1. FT-IR was used to evaluate the
presence of foscarnet on the porous silica particles. Characteristic absorption bands corresponding to $\nu_3$ phosphate vibrations are between 950 and 1200 cm$^{-1}$. However, a strong peak at 1051 cm$^{-1}$, corresponding to $\nu_{\text{Si-O}}$ on the silica particles after reflux in a 24 mg/mL foscarnet solution was observed, overlapping with many of the main absorption bands of foscarnet (Figure A.2). Therefore, TGA and EDX were used to assess the presence of foscarnet on the porous silica particles. TGA of foscarnet reveals a decomposition temperature at around 500°C (Figure A.3). The initial decrease in weight at 100°C is attributed to the loss of water from the sample. No significant decrease in weight was observed at 500°C for porous silica microparticles post reflux with foscarnet (Figure A.3). Furthermore, EDX mapping reveals the presence of Si and O, with a trace amount of P, and the absence of Na, indicating foscarnet was not successfully loaded into porous silica microparticles using this method (Table A.1).
Figure A.1 Schematic showing the reaction of the hydroxyl group on porous silica with the phosphate group of foscarnet.
Figure A.2 FT-IR spectra of foscarnet (a) and porous silica microparticles post reflux in a 24 mg/mL foscarnet solution (b).
Figure A.3 TGA of foscarnet and porous silica microparticles after relux with 24 mg/mL foscarnet solution.
Table A.1 Energy-dispersive X-ray (EDX) spectroscopy mapping of porous silica particles post reflux with 24 mg/mL foscarnet.

<table>
<thead>
<tr>
<th>Element</th>
<th>App Conc.</th>
<th>Intensity Corrn.</th>
<th>Weight %</th>
<th>Atomic %</th>
</tr>
</thead>
<tbody>
<tr>
<td>O K</td>
<td>13.13</td>
<td>1.7677</td>
<td>54.96</td>
<td>68.20</td>
</tr>
<tr>
<td>Si K</td>
<td>6.63</td>
<td>1.0995</td>
<td>44.47</td>
<td>31.43</td>
</tr>
<tr>
<td>P K</td>
<td>0.10</td>
<td>1.2484</td>
<td>0.57</td>
<td>0.36</td>
</tr>
<tr>
<td>Totals</td>
<td></td>
<td></td>
<td>100.00</td>
<td></td>
</tr>
</tbody>
</table>
A.4.2 Determination of Foscarnet Release

A method based on the use of metal complexation chemistry to detect phosphates was adapted to quantify the amount of foscarnet released from porous silica micro- and nano-particles in water. A 1:1 YbPV complex was used as a colorimetric measurement of foscarnet. In the presence of phosphates, there is an ligand exchange, releasing the free PV dye (yellow). This reaction can be monitored using UV-visible spectroscopy: absorbance at 625 nm due to the YbPV complex or absorbance at 445 nm due to free PV. Therefore, with increasing concentrations of foscarnet, and therefore, phosphates, there should be a decrease in the absorbance at 625 nm and an increase in absorbance at 445 nm. A linear correlation between the absorbance at 625nm/445 nm and the concentration of foscarnet was observed (Figure A.4). We found that this colorimetric measurement of foscarnet was able to detect foscarnet with a sensitivity of 2 µM. This provides a more sensitive detection method compared with HPLC with UV detection, which allowed a quantification limit of 33 µM.
Figure A.4 Standard curve for $\lambda=625$ nm and $\lambda=445$ nm for the presence of foscarinet. Absorbance at 625 nm is due to the YbPV complex and the absorbance of 445 nm is due to the release of free PV.
A.4.3 Trapping of Foscarnet into Porous Silica Microparticles by Oxidation

As Si is oxidized into SiO$_2$, a volume expansion occurs in order to accommodate for additional oxygen atoms, resulting in a decrease in pore size. This method has been used to trap magnetite (Fe$_3$O$_4$) nanoparticles into a porous Si matrix. Due to the relatively high decomposition temperature of foscarnet (~500°C), thermal oxidation at lower temperatures may be used to oxidize the porous Si matrix while maintaining the integrity of the drug. A p-type Si wafer was chosen in order to generate smaller pore sizes to trap foscarnet, which has a molecular weight of 126 g/mol. Scanning electron micrograph images revealed pore sizes of 3.7 ± 0.4 nm (Figure A.5).

Release of foscarnet from the porous silica microparticles was assessed by placing 9 mg of particles in 2 mL of Millipore water. The total amount of foscarnet released was assumed to be the total amount of foscarnet loaded into the particles. Within 2 hours, 100% of the drug was released, indicating a quick release of the drug trapped into porous silica microparticles by thermal oxidation (Figure A.6). This large burst release can be attributed to the hydrophilicity of the drug and presence of multiple negative charges on foscarnet. In physiological pH, silica has a negatively charged surface, and therefore the negatively charged foscarnet does not have a strong affinity to the silica surface. It has been shown previously that the negatively charged silica surface can be used to efficiently load a positively charged antibody. Therefore, additional functionalization of the silica surface in order to obtain a positively charged surface may aid in drug loading and prolonged drug release.
Figure A.5 Scanning electron micrograph image of the porous Si particles prior to thermal oxidation (a) and the pore size of the particles prior to oxidation (b).
Figure A.6  Release of Foscarnet from porous Si microparticles trapped by thermal oxidation.
A.4.4 Loading and Release of Foscarnet from Amine Functionalized Porous Silica Microparticles

Amine-functionalization of silica microparticles to achieve a positively charged surface at physiological pH may help to facilitate the loading of foscarnet. In addition, covalent attachment of small molecule drugs to the surface of porous Si particles has been previously demonstrated to be able to provide long-term release drug release.\textsuperscript{106} Therefore, EDC/Sulfo-NHS chemistry was used to couple the amine group on the surface of the particle to the carboxylic acid group of foscarnet. Three current densities and times were applied to achieve particles with three different pore sizes to determine the effect of pore size on drug loading. A current density of 1.8 mA/cm\textsuperscript{2} for 8 minutes resulted in pore sizes of 9.7 ± 1.6 nm, a current density of 8.9 mA/cm\textsuperscript{2} for 6 minutes resulted in pore sizes of 30.8 ± 5 nm, and a current density of 72.2 mA/cm\textsuperscript{2} for 2 minutes resulted in pore sizes of 80 nm. Amine-functionalization of all three particle types was confirmed by a deep blue color that resulted after addition of the ninhydrin solution to the particles, indicating the presence of primary amines.\textsuperscript{162}

Loading of foscarnet into porous silica microparticles by EDC/Sulfo-NHS coupling chemistry resulted in different drug loading efficiencies for the 3 different particle types. The lowest amount of drug loading (8.1 µg/mg) was observed for particles with a pore size of 10 nm. The highest amount of drug loading (19.1 µg/mg) was achieved using particles with a pore size of 80 nm. Drug loading for the 3 different pore sizes is summarized in (Table A.2). An 8-fold increase in diameter resulted in an increase of drug loading by 2-fold. Release of foscarnet was also evaluated in water
over a period of 24 hours (Figure A.7). A significant burst release was observed for all 3 pore sizes. Within 2 hours, approximately 40% of the loaded drug was released. Complete release of the drug was observed within 24 hours. No significant difference in the release rates between the 3 particle types was observed, indicating that in the range of 10-80 nm, pore size does not have an effect on drug release rate. However, for pore sizes smaller than 5 nm, interaction between drug and particle surface could increase, resulting in delayed foscarinet release.
**Table A.2** The amount of drug loading for various pore sizes.

<table>
<thead>
<tr>
<th>Pore size (nm)</th>
<th>Porosity</th>
<th>Foscarnet Loaded per mg of Silica Particles (µg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>50%</td>
<td>8.1 ± 1.5 µg/mg</td>
</tr>
<tr>
<td>30</td>
<td>67%</td>
<td>12.8 ± 1.3 µg/mg</td>
</tr>
<tr>
<td>80</td>
<td>80%</td>
<td>19.1 ± 2.4 µg/mg</td>
</tr>
</tbody>
</table>
Figure A.7 Release of foscarinet from porous silica microparticles with pore sizes 10, 30 and 70 nm.
A.4.5 Loading and Release of Foscarnet from Amine Functionalized Mesoporous Silica Nanoparticles

In order to obtain particles with pore diameters smaller than 5 nm, silica nanoparticles were synthesized by the co-condensation of TEOS and APS, using CTACl as a template for the pores.\textsuperscript{163, 164} Scanning electron microscopy (SEM) and scanning transmission electron (STEM) was used to assess the morphology and size of the particles. It was found that when the TEOS/APS solution was not added drop-wise to the methanol/water solution, non-uniform particle sizes are obtained (Figure A.8). However, when TEOS/APS were added to the methanol/water solution in a drop-wise manner, uniform spherical particles of around 500 nm are produced (Figure A.8). Furthermore, transmission electron microscope (TEM) images revealed and ordered pore structure, with pore sizes of around 2.3 ± 0.3 nm. BET surface area was measured to be 655 m\textsuperscript{2}/g.

In addition, because foscarinet is a hydrophilic and negatively charged molecule, the silica nanoparticles were further functionalized with polyethylene imine (PEI), a highly branched cationic polymer. The ζ-potential was measured for the particles before and after functionalization with PEI. Prior to the addition of PEI, the silica nanoparticles had a ζ-potential of 10 mV at pH 7.5 with an isoelectric point at pH 7.8 (Figure A.9). After functionalization with PEI, the silica nanoparticles exhibited a ζ-potential of 30 mV at pH 7.5, with the isoelectric point increased to pH 9.8 (Figure A.9). The increase in isoelectric electric point of the particles could facilitate the attraction and reaction with the negatively-charged foscarinet.
Figure A.8 STEM image of mesoporous silican nanoparticles prepared without the addition of TEOS/APS in a dropwise manner (a-b). SEM image of mesoporous silica nanoparticles prepared with the addition of TEOS/APS in a dropwise manner (c). TEM image revealing the pore structure of the sol-gel derived porous silica nanoparticles (d).
Figure A.9 $\zeta$-potential titrations for mesoporous silica nanoparticles before and after functionalization with PEI.
The loading and release of foscarnet from PEI-functionalized and non PEI-functionalized particles were evaluated. Compared to non PEI-silica nanoparticles, PEI-silica nanoparticles had a significantly higher drug loading capacity, with a 6-fold increase in fosarnet loading. In addition, without the addition of EDC/Sulfo-NHS to facilitate the coupling between the carboxylic acid group of foscarnet with the amine groups on the particle surface, a significant amount of foscarnet can be loaded into the PEI porous silica nanoparticles. This was not observed for porous silica particles without PEI functionalization, with only 3.8 µg of foscarnet loaded per mg of silica particles in the absence of EDC/Sulfo-NHS. The amount of drug loading for each particle type is summarized in Table A.3. Although an increase in drug loading is observed with the use of EDC/Sulfo-NHS, a more significant increase in drug loading is observed with the increase in particle surface charge, indicating that a highly cationic particle greatly facilitates the loading of foscarnet into silica nanoparticles.

Release of foscarnet from the silica nanoparticles was also evaluated in water. For silica particles not functionalized with PEI, a significant burst release was observed within several hours, with 80% of the drug release after 24 hours. This showed a slight increase in the drug release period compared with the silica microparticles, where almost 100% of the drug was released after 24 hours. This may be due to the significant decrease in pore size for the silica nanoparticles compared with silica microparticles, allowing for increased drug-surface interaction. Release was not performed on silica particles where the drug was loaded without EDC/Sulfo-NHS due to the low amount of drug loading. When functionalized with PEI, the silica particles exhibited a significant
decrease in burst release. Whereas within 2 hours, 28% of the drug was release from the non PEI silica nanoparticles, only 9% of the drug was release from the PEI nanoparticles. Furthermore, the release period of foscarinet was significantly prolonged with the addition of PEI. Complete release of foscarinet was obtained after 6 days from the particles without PEI. Complete release of foscarinet was achieved after 13 days from the particles with PEI. These results indicate a promising platform for the sustained release of foscarinet from silica nanoparticles, as release in vivo may be prolonged compared to in vitro studies.\textsuperscript{147}

However, in vitro cell cultures have shown that 25 µg/mL of foscarinet is required to effectively inhibit the replication of CMV.\textsuperscript{165} Therefore, a higher drug loading capacity is required in order to provide therapeutic concentrations for the treatment of CMV retinitis. In addition, no significant difference was observed in the release rates between the particles in which drug was loaded by EDC/Sulfo-NHS versus drug loaded without EDC/Sulfo-NHS into the PEI functionalized nanoparticles. This suggests that the prolonged drug release is due to the increased surface charge of the particle and not by covalent attachment of the drug to the porous silica matrix. However, although PEI is able to facilitate drug loading and prolonged drug release, the toxicity that has been observed with PEI may not render it ideal for drug delivery.\textsuperscript{166} Additional studies on the use cationic polymers to functionalize the surface should be explored.
Table A.3 A summary of the amount of drug loading for PEI-functionalized and non-PEI-functionalized silica nanoparticles with and without EDC/Sulfo-NHS.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Foscarnet Loaded per mg of Si NP (μg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No EDC/Sulfo-NHS Non-PEI Silica Particles</td>
<td>3.8 ± 1.9 μg/mg</td>
</tr>
<tr>
<td>EDC/Sulfo-NHS Non-PEI Silica Particles</td>
<td>11.1 ± 1.1 μg/mg</td>
</tr>
<tr>
<td>No EDC/Sulfo-NHS PEI Silica Particles</td>
<td>45.1 ± 3.2 μg/mg</td>
</tr>
<tr>
<td>EDC/Sulfo-NHS PEI Silica Particles</td>
<td>62.5 ± 2.1 μg/mg</td>
</tr>
</tbody>
</table>
Figure A.10  Comparison of the release of foscarnet from silica particles functionalized and not functionalized with PEI.
A.5 Conclusions

Loading of foscarnet into mesoporous silica micro- and nano-particles can be accomplished via trapping by oxidation, attachment of the carboxylic acid group on Foscarnet to surface amine groups, and by attraction of foscarnet to a highly cationic surface. The highest amount of drug loading and the longest period of drug release were observed for PEI-functionalized silica nanoparticles. This indicates the importance of a positively charged surface in the loading and prolonged release of foscarnet. Although the use of PEI helped to facilitate foscarnet loading as well as to prolong drug release, other cationic polymers should be considered as alternatives, as PEI has been shown to exhibit toxicity. Furthermore, although 13 days of release indicate the possibility of using mesoporous silica nanoparticles for the sustained release of foscarnet, further development for increased drug loading is needed to provide therapeutic concentrations of foscarnet in the vitreous.
References


123. Garweg, J. G.; Wegmann-Burns, M.; Goldbum, D., Effects of daunorubicin, mitomycin c, azathioprine and cyclosporin a on human retinal pigmented epithelial,


