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UNIVERSITY OF CALIFORNIA, SAN DIEGO

**STUDIES ON THE TOXICITY AND CISPLATIN
LOADING OF POROUS SI MICROPARTICLES**

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

in

Bioengineering

by

Jennifer Shim Park

Committee in charge:

Professor Michael J. Sailor, Chair
Professor Karen L. Christman
Professor David A. Gough
Professor Michael J. Heller
Professor Stephen B. Howell

2010

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The dissertation of Jennifer Shim Park is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2010

Dedication

To my family and friends

Table of Contents

Signature Page.....	iii
Dedication.....	iv
Table of Contents.....	v
List of Figures.....	x
List of Schemes.....	xiii
List of Tables.....	xiv
Acknowledgements.....	xv
Vita.....	xviii
Publications.....	xix
Abstract of Dissertation.....	xx

Chapter One

Introduction

1.1	Introduction to the Research Problem	2
1.2	Statement of Purpose.....	3
1.3	List of Specific Research Questions.....	4
1.4	References	5

Chapter Two

Literature Review

2.1	Introduction to Porous Silicon.....	8
2.2	Fabrication of Porous Silicon	8
	2.2.1 Electrochemical-etching.....	9
	2.2.2 Stain-etching.....	14
2.3	General Particle-Based Cancer Drug Delivery.....	15

2.3.1	Polymeric Delivery Systems	18
2.3.2	Porous Inorganic Materials	19
2.3.2.1	Porous Alumina	19
2.3.2.2	Porous Titanium.....	20
2.4	Porous Silicon for Drug Delivery	20
2.4.1	Biocompatibility.....	21
2.4.2	Surface Chemistry	23
2.4.3	Tunable Pore Sizes and Surface Area	23
2.4.4	Optical Properties of Porous Si.....	24
2.4.5	Previous Delivery Systems	29
2.4.5.1	Chip-Based Delivery Modes.....	29
2.4.5.2	Particle-Based Systems	30
2.5	Ovarian Cancer Therapeutics	31
2.5.1	Ovarian Cancer.....	31
2.5.2	Historical Overview of Therapeutics	31
2.5.2.1	Platinum Compounds.....	32
2.5.2.2	Cisplatin Mode of Action	33
2.5.2.3	Limitations of Cisplatin Therapies	37
2.6	Studies with Porous Si and Cisplatin.....	38
2.6.1	Surface Reactivity of Porous Silicon	38
2.6.2	Discussion of Reductive Potential	39
2.6.3	Reductive Deposition of Platinum into Porous Si Particles.....	40
2.7	Summary	42
2.8	References	42

Chapter Three

Improving the Biocompatibility of Chemically-Modified Porous Si Microparticles With Ovarian Cancer Cells

3.1	Abstract.....	52
3.2	Introduction	52
3.3	Materials and Methods	55

3.3.1	Sample Preparation	55
3.3.2	Surface Area and Porosity Characterization	56
3.3.3	Post-Process Rinse and Fluorescent Dye Conjugation	57
3.3.4	Monitoring Degradation Rates of Microparticles in PBS.....	57
3.3.5	Determination of Toxicity by In-vitro Cell Viability Assay	58
3.3.6	In-Vivo Toxicity.....	59
3.4	Results and Discussion	59
3.4.1	Porous Si Etching and Characterization.....	59
3.4.2	Preparation of Fluorescent Dye Modified Porous Si Particles.....	63
3.4.3	Surface Chemistry Analysis by FTIR	68
3.4.4	Cellular Toxicity of Porous Si Microparticles with Ovarian Cancer Cells	71
3.4.5	Porous Si Microparticles Dissolution Rates.....	78
3.4.6	In-Vivo Toxicity Determination of Maximum Tolerated Dose	84
3.5	Conclusions	88
3.6	References	89

Chapter Four

Localized Delivery of Cisplatin, An Anti-Cancer Drug

4.1	Abstract.....	95
4.2	Introduction	96
4.3	Materials and Methods	100
4.3.1	Materials.....	100
4.3.2	Stain-Etched Microparticle Characterization.....	100
4.3.3	Surface Modification.....	101
4.3.4	Cisplatin and Hydrophobic Pt(IV) Pro-drug Loading.....	101
4.3.5	Platinum Loading Characterization.....	102
4.3.6	In-Vitro Drug Release	103

4.3.7	Cellular Toxicity with Cisplatin-loaded Microparticles.....	103
4.4	Results and Discussion	104
4.4.1	Preparation of Stain-etched Microparticles.....	104
4.4.2	Surface Functionalization for Drug Loading	105
4.4.3	Drug Loading	109
4.4.4	Discussion of Reactivity of Porous Silicon.....	112
4.4.5	Platinum Reduction Mechanism	113
4.4.6	Drug Release Characterization.....	116
4.4.7	Localized Delivery of Cisplatin-loaded Microparticles to Human Ovarian Cancer Cells.....	119
4.5	Conclusions	129
4.6	References	130

Chapter Five

Attachment of Lyp-1 Targeting Peptide

5.1	Abstract.....	136
5.2	Introduction	136
5.3	Results and Discussion.....	141
5.3.1	LyP-1 Conjugation to Platinum Surfaces.....	141
5.3.2	Cellular Toxicity of LyP-1 Conjugated, Drug-loaded Microparticles	149
5.4	Conclusions	151
5.5	References	152

Chapter Six

Future Works

6.1	Introduction.....	156
6.2	Materials and Methods.....	156
6.2.1	In-vitro Toxicity Studies of Cisplatin Loaded Porous Si Microparticles.....	156
6.2.2	In-vitro Binding of Lyp-1 Conjugated Cisplatin Loaded Porous Si Microparticles.....	157
6.2.3	In-vivo Toxicity Studies of Lyp-1 Conjugated Cisplatin loaded Porous Si Microparticles.....	158

Appendix A
Notes on the Study of
Porous Si-Based Polymer Replicas
Formed by Bead Patterning

A.1	Background.....	161
A.2	Materials and Methods	162
	A.2.1 Porous Si Preparation	162
	A.2.2 Bead Dispersion, Infusion, and Particle Collection	162
	A.2.3 Characterization.....	163
A.3	Results and Discussion	164
A.4	Conclusions	170
A.5	References	171

List of Figures

Chapter Two

Figure 2.1	Schematic of electrochemical setup.....	11
Figure 2.2	Mechanism of electrochemically-etched porous Si formation, adapted from reference ^[100]	12
Figure 2.3	Scanning electron micrograph of a single current density etched porous Si film observed from a cross-sectional angle	13
Figure 2.4	Reflectance spectrum of fabry-pérot film	26
Figure 2.5	Fast fourier transform of reflectance spectrum to generate plot of effective optical thickness.....	27
Figure 2.6	Example of a reflectance spectrum of a rugate (sine or cosine) etched film	28
Figure 2.7	Diagram of cisplatin-DNA adducts (from reference ^[74]).....	35

Chapter Three

Figure 3.1	Scanning electron microscopy of three types of porous Si microparticles for toxicity studies	61
Figure 3.2	Attachment of Alexafluor 594 hydrazide fluorescent dye coupling confirmed by light microscopy	64
Figure 3.3	Scheme of dye attachment chemistry	65
Figure 3.4	Discussion of particle degradation pathways and the involving chemistry	67
Figure 3.5	FTIR of surface modifications	70
Figure 3.6	Cellular toxicity assessed by sulforhodamine B viability assay with 12 particle experimental groups.....	73
Figure 3.7	Reaction of silicic acid generation from silane.....	74
Figure 3.8	Relative degradation rates of surface modified porous Si microparticles.....	80

Figure 3.9	Free silicic acid toxicity.....	83
Figure 3.10	Kidney and peritoneal tissue histology on toxicity-induced specimen	87

Chapter Four

Figure 4.1	Porous Si microparticles analyzed for elemental platinum by scanning electron microscopy and EDX.....	115
Figure 4.2	Drug release in PBS, hypertonic saline, and plasma; timed release fetal bovine serum for aqueated cisplatin particles.....	118
Figure 4.3	Cellular toxicity with cisplatin loaded porous Si microparticles demonstrating localized delivery to ovarian cancer cells.....	121
Figure 4.4	Cellular toxicity with hydrophobic Pt (IV) pro-drug in microparticles compared with free drug.....	123
Figure 4.5	High resolution XPS spectrum of Pt 4f region indicating the oxidation state of platinum on the microparticles.	124
Figure 4.6	Cellular toxicity of cisplatin loaded porous Si microparticles at a higher dose range.	125
Figure 4.7	Cellular toxicity of solid platinum particles on human ovarian cancer cells	126
Figure 4.8	High performance liquid chromatography – mass spectrometric analysis of hydrophobic Pt (IV) pro-drug.	127

Chapter Five

Figure 5.1	Structure of receptor to LyP-1, p32, based on the crystal structure (from reference [8]).	115
Figure 5.2	Structure of LyP-1 circular peptide with amino acid sequences.....	121
Figure 5.3	Schematic representation of LyP-1 conjugation onto drug loaded microparticle.	123

Figure 5.4	Fluorescence microscopy image verifying LyP-1 conjugation	124
Figure 5.5	Increased peptide doses and non-specific physical adsorption of peptide.....	125
Figure 5.6	Cellular toxicity of LyP-1 conjugated microparticles.....	125

Appendix A

Figure A.1	Scheme employed to produce uniform-sized porous Si-based polymer replicas	159
Figure A.2	Polymer bead infiltration into a porous Si photonic crystal and reflectivity spectrum.....	127
Figure A.3	Scanning electron micrographs of free-standing polymer replicas of porous silicon.....	127

List of Schemes

Scheme 4.1	Cisplatin loading into porous Si microparticles by reductive platinum plating.....	108
------------	--	-----

List of Tables

Chapter Three

Table 3.1	Scheme employed to produce uniform-sized porous Si-based polymer replicas	77
Table 3.2	Maximum tolerated dose (MTD) of porous Si microparticles injected intraperitoneally into balb/c mice	86

Chapter Four

Table 4.1	Drug loading of aqueated cisplatin and hydrophobic platinum (IV) prodrug in surface modified particles.....	127
-----------	---	-----

Chapter Five

Table 5.1	LyP-1 conjugation on platinum coated porous Si microparticles	140
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Park, J.S., Meade S.O., Segal, E., Sailor, M.J. Porous silicon-based polymer replicas formed by bead patterning. *Physica Status Solidi A*, 2007, 204, (5), 1383-1387.

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

Vita

May 2004

B.S., Chemical Engineering,
Department of Chemical Engineering,
University of Colorado, Boulder

June 2007

M.S., Bioengineering, Department of
Bioengineering, University of
California, San Diego

June 2010

Ph.D., Bioengineering, Department of
Bioengineering, University of
California, San Diego

Publications

Park, J.S., Lianglin Zhang, Howell, S.B., Sailor M.J. Attachment of LyP-1 targeting peptide to cisplatin loaded porous silicon microparticles. *Manuscript in preparation.*

Park, J.S., Jandial, D.D., Howell, S.B., Sailor, M.J. Porous silicon microparticles for localized delivery of cisplatin, an anticancer drug. *Manuscript in preparation.*

Park, J.S., Jandial D.D., Segal E., Wu, E.C., Howell, S.B., Sailor M.J. Enhanced biocompatibility of surface-modified porous silicon microparticles with ovarian cancer cells. *Manuscript in preparation.*

Park, J.S., Cao, C., and Sailor, M.J., A basic glucose sensor exercise for teaching optical sensors. *Manuscript in preparation.*

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Wu, E.C., Park, J.H., **Park, J.S.**, Segal, E., Cunin F., Sailor M.J. Oxidation-Triggered Release of Fluorescent Molecules or Drugs from Mesoporous Si Microparticles. *ACS Nano*, 2008, 2, (11), 2401-2409.

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Abstract of the Dissertation

STUDIES ON THE TOXICITY AND DRUG LOADING OF POROUS SI MICROPARTICLES BY UTILIZING ITS INTRINSIC REACTIVITY

by

Jennifer Shim Park

Doctor of Philosophy in Bioengineering

University of California, San Diego, 2010

Professor Michael J. Sailor, Chair

This thesis describes the fabrication, chemical modification, toxicity studies, and drug loading of nanostructured porous silicon for the purposes of developing a drug delivery device applied to epithelial ovarian carcinoma.

The first chapter is an introductory chapter, presenting the premise of the overall research problem, the statement of purpose, and a list of questions to be understood. The second chapter focuses on a review of the relevant literature and

describes the chemical and physical properties of porous silicon, the concepts and issues of current drug delivery devices and materials, applications in ovarian cancer therapeutics, and how porous silicon can address the issues regarding localized and controlled drug therapies.

The third chapter explores the use of porous silicon as a material that can be delivered intraperitoneally and addresses the limitations in toxicity that can arise. Due to the toxicity, the surface of the porous silicon nanostructure can be chemically modified and rinsed to control the rate of degradation into orthosilicic acid. This chapter defines the dose range of these particles that can be delivered IP as well as discusses the effect of modifying surface chemistry to reduce toxicity.

In chapter four, the concept of loading porous silicon microparticles with a therapeutic is demonstrated. The particles are loaded with cisplatin, an anti-cancer drug through a reaction instigated by the intrinsic properties of the material, trapping the drug into the pores by coating the surface with a platinum metal. Release and high localized delivery towards human ovarian cancer cells compared to free drug is discussed.

The last chapter of the thesis presents a new method for attaching LyP-1 targeting peptide to porous Si microparticles for delivery towards ovarian cancer.

Still much work needs to be completed in this area with regards to the targeting of LyP-1 in ovarian tumors. However, in this chapter we have demonstrated proof-of-concept that the peptide can be attached via thiol – platinum chemistry on the cisplatin loaded microparticles.

The appendices mainly focus on the use of porous Si / polymer composites formed by templating to utilize the unique properties of both materials. The optical reflectivity pattern of porous Si is imprinted into mono-disperse polymer beads in the first appendix, and the second addresses the use of a malleable polymer to attempt to demonstrate a change in optical properties of the composite structure due to mechanical deformation.

Chapter One

INTRODUCTION

1.1 Introduction to the Research Problem

A handful of studies have demonstrated the use of porous Si as biomaterial interfaced with mammalian cells ^[1-4], implantable in-vivo devices ^[5-7], and more recently use as a potential delivery material for therapeutic molecules ^[8-13]. One cannot deny that it does possess some meritable attributes for integration into biological systems. These include degradability into resorbable products like orthosilicic acid, Si(OH)_4 , that can be processed through the kidneys. As Si is the seventh most abundant element, various compositions of it exist as *silicone* (in lubricants, polishing agents, electrical insulators, and medical implants), *sodium silicate* - Na_2SiO_3 (in soaps, adhesives, preservatives), and the most abundant form - SiO_2 (present in sand and manufacture of glass and bricks). It is no doubt that small amounts will enter into our bodies if by accident into our diet, drinking sea-water while surfing, or by other means. It is even known that a deficiency in Si in our diets can cause abnormal growth in the development of bone and cartilaginous structures as found in an early study that deprived chicks of Si in their diet, ultimately finding that it was indeed an essential element. ^[14-17]

Thereby in this thesis we have sought to explore additional applications of porous Si for drug delivery. More ubiquitously researched materials include polymers, iron oxide, and various composite materials. The disease model we sought to target is intraperitoneal ovarian cancer. There is a need for better drug delivery for this particular disease as it is often known to undergo silent metastasis often being diagnosed well into the late stages. In the late stages, the chance of

five year survival drops to less than 25%. The current drugs that are used to treat this model have been a combination of those in the platinum alkylating neoplastic agent genre, like cisplatin and carboplatin, and additionally taxanes such as paclitaxel. There is a need for alternative delivery modalities to treat this type of cancer that spreads by shedding metastatic cells from the ovarian epithelium. Epithelial ovarian cancer is the most common of the three subclasses of this type of cancer. There is a need to provide improved delivery by reducing the systemic toxicity, develop more effective delivery that can potentially circumvent deactivation by plasma proteins and -thiol containing moieties, and possibly reduce the effect of induced cellular resistance.

1.2 Statement of Purpose

We propose to first discuss the design of constructing a feasible microparticle. Then, go into the limitations in toxicity and biocompatibility found by this material in terms of applying it towards an intraperitoneal model. We will discuss the reactivity of porous Si with certain classes of chemicals and how in this case the concept was used to trap a drug into the particle structure to obtain a high level of localized chemotherapeutic delivery compared to free drug on cells. Lastly, the appendix will touch upon some applications to create polymer-porous Si composite materials that utilize the unique optical properties of the porous Si in combination with the robust chemical and mechanical features of certain polymers.

1.3 List of Specific Research Questions

Chapter 3

- Can the biocompatibility of porous Si microparticles be improved in an in-vivo peritoneal model by modifying the surface chemistry with a group that slows down the degradation rate of the particle?
- Is the toxicity caused by a high flux in the Si being processed by the kidneys?
- Can the surface modified porous Si microparticles still be used as a therapeutic delivery vehicle in-vivo?

Chapter 4

- What is the best method to load porous Si microparticles with cisplatin, anti-cancer drug?
- What is the mode of drug loading?
- How much drug is loaded?
- What is the drug release behavior in varying physiological solutions?
- How toxic is the drug-loaded microparticle in comparison to free drug on human ovarian cancer cells?

Chapter 5

- Does Lyp-1 targeting peptide bind to ovarian cancer cells?
- How can Lyp-1 targeting peptide be attached to drug-loaded porous Si microparticles.
- How effective is the toxicity of the targeting peptide in comparison to non-peptide loaded microparticles.

Appendix A

- Can a mono-disperse polymer-porous Si replica be formed using a bead templating procedure?

Appendix B

- Is it possible to create a malleable polymer replica of porous Si?
- Can the optical properties be transferred by templating?
- Can the polymer replica change color (alter reflectivity spectrum) as we deform the structure?

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Chapter Two

LITERATURE REVIEW

2.1 Introduction to Porous Silicon

Porous silicon was first discovered unintentionally by Arthur Uhlir and Dennis Turner of Bell Laboratories and published in the Bell Labs Technical Reports in the 1950's.^[1, 2] They were attempting to develop a method to electrochemically polish semiconductor materials for use in microelectronic circuits. The efforts instead serendipitously resulted in a thin, brown film resulting from the formation of fine pores propagating in the <100> direction of the silicon wafer. Interest in the discovery of this porous material waned until findings in the early 1990's that islands of silicon filaments remaining after pore formation could induce quantum confinement effects resulting in light-emitting photo-luminescent properties.^[3, 4] Although there was initial excitement in the potential for applications in lasers, displays, and switches, eventually it diminished due to limitations in the material's chemical and mechanical stability. However, porous silicon has since been identified for its unique properties of high surface area, control of pore morphology, and ease of surface functionalization for broader applications in micro- and opto-electronics^[5], chemical^[6-12] and biological^[13-16] sensors, and more recently, drug delivery and biomedical devices^[17-21].

2.2 Fabrication of Porous Silicon

The material primarily used throughout this thesis is a film or particle form of mesoporous silicon, the resulting product synthesized by either electrochemical

etching or stain etching of a silicon wafer or particle. Both electrochemical etching and stain etching methods can result in the generation of nanometer-sized pores.

2.2.1 Electrochemical-etching

Porous Si formed by electrochemical-etching involves the anodization of a single-crystalline Si wafer with hydrofluoric acid (HF) electrolytic solutions. The material possesses rich morphological features and likewise a complex formation mechanism dependent on parameters like electrolyte concentration, dopant class and quantity, and orientation of the crystal lattice. Specific to the electrochemically-etched variants, the formation is also correlated to current density. Morphological characteristics of a material (morphology being defined as a pattern of matter distribution in space) tend to be one of the more qualitative features which are challenging to systematize for porous silicon. It can be roughly organized into categorical divisions such as size, pore orientation, branching, fill of macropores, and depth variation of the porous layer. ^[22] In the research presented in this thesis, the electrochemically prepared material is etched from highly doped (P-type) Si using 48% aqueous HF in ethanol at a ratio of either 1:1 or 3:1 by volume (**Figure 2.1**). The pore sizes ranged from 2-50 nanometers, in the mesoporous range. With altered processing conditions, smaller pores can be generated for example in the microporous range (< 1.5 nm) or the macroporous range (> 50 nm).

Various theories have been proposed for the mechanism of porous silicon formation that highlights aspects of morphological formation and electrochemical behavior at the silicon-electrolyte interface. However, due to the many variations in morphological characteristics, there has not yet been a global theory established that describes pore formation over all size regimes. A general mechanism for porous Si formation by electrochemical-etching has been proposed (**Figure 2.2**). A typical cross-sectional view of a porous silicon film imaged by scanning electron microscopy is shown in (**Figure 2.3**).

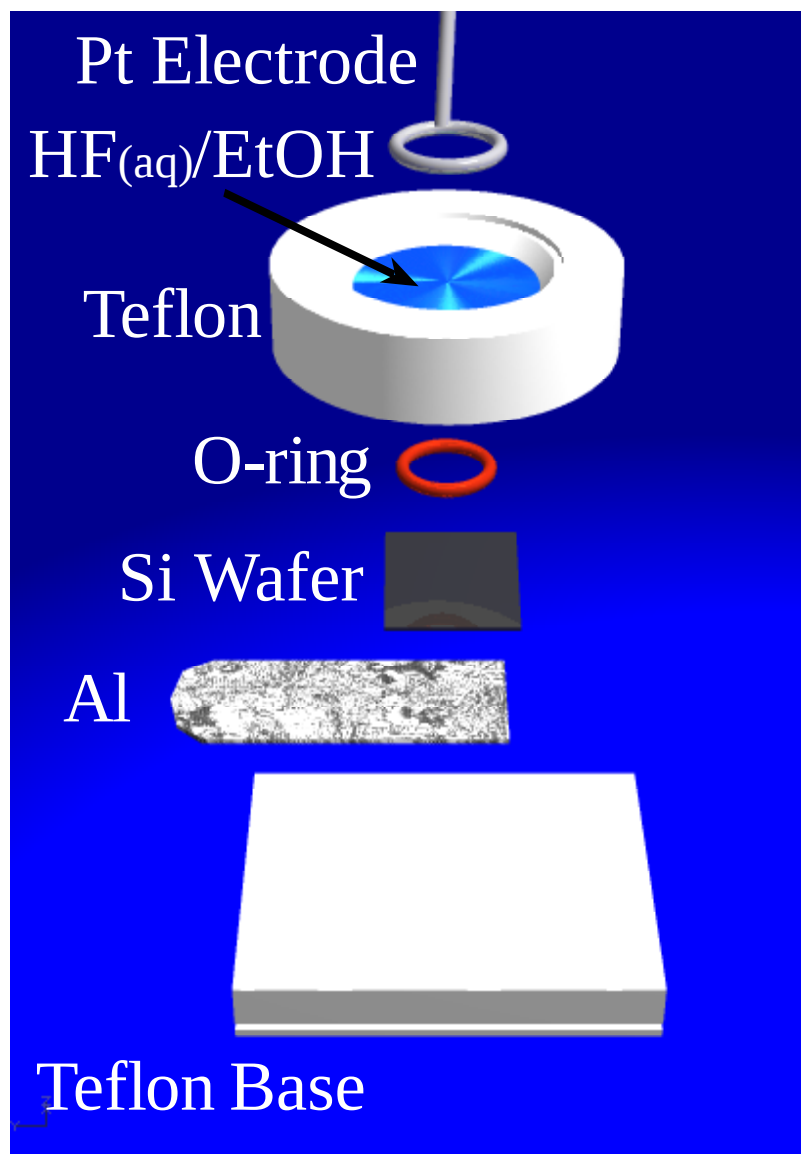


Figure 2.1 Schematic of the components in an electrochemical setup used to prepare porous Si.

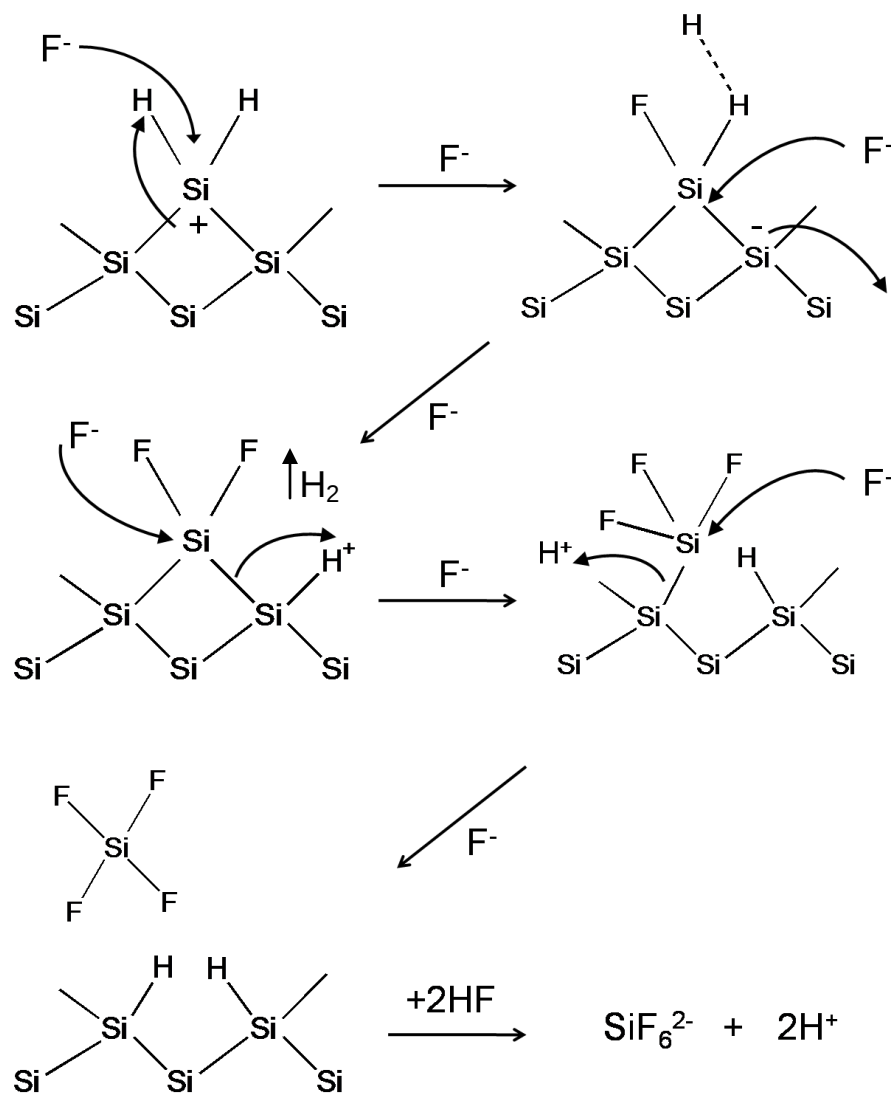


Figure 2.2 Mechanism of porous Si pore formation, adapted from reference. ^[93]

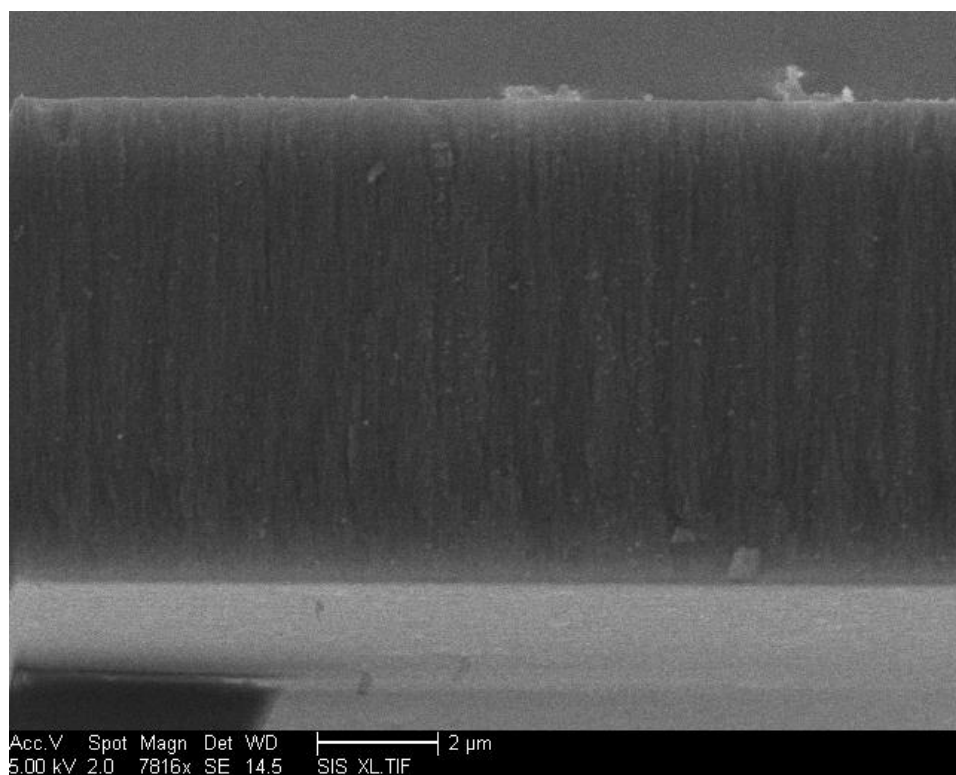


Figure 2.3 Scanning electron micrograph of a single current density etched porous Si film observed from a cross-sectional angle. (Image acquired by author).

2.2.2 Stain-etching

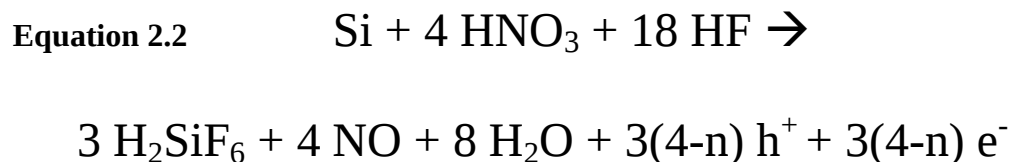
Although the electrochemically-etched preparation of porous silicon is definitely the more common method, a less frequently utilized stain-etching procedure exists, which involves an open-circuit chemical etch using a solution of HF, nitric acid, and water. The limitations in the method include non-uniform etch profiles, thin porous layers^[23], and possibly lower photoluminescence quantum yields.^[24] Although it is a less popular etching procedure, stain-etched films have been a subject of investigation.

In chemical etching, the aqueous solution contains an oxidizing agent such as nitric acid, and an anion like F^- that can form a water soluble dissolution product with a semi-conductor such as germanium or silicon.^[25] Although there is no external anodic bias, the reaction is essentially “electrochemical” in nature considering that there are anode and cathode sites within the semiconductor upon which local cell currents flow, resulting in dissolution of the material. The dissolution occurs at the anodic positions and the oxidizing agent, nitric acid, is reduced at the cathode. Etching preferentially occurs in highly localized anodic areas such as grain-boundaries or dislocations in the crystal. The average current density between the local anode and cathode sites can be calculated by observing the etch rate (r). Where ϵ is the electrochemical equivalent (coulombs/gram), d is the density (grams/cm³) of the semi-conductor. **(Equation 2.1)** has been modified from the following reference.^[25]

Equation 2.1

$$I = 2r\epsilon d$$

The overall mechanism for stain-etching porous silicon can be described by the following equation (**Equation 2.2**)^[26]:



In the body of this work, stain-etched wafers or films have not been used, but rather the stain-etching procedure was utilized to etch pits into crystalline silicon particles that have been ball-milled and filtered into a specific size regime. The etch depth limitations have been found to be $\sim 4 \mu\text{m}$, thereby limiting the porosification to a depth that only affects the shallow surface of larger diameter particles exceeding $4 \mu\text{m}$. However, in the case of smaller particles, the chemical etch is able to penetrate through the depth of the entire particle. The stain-etched particles used in this thesis were obtained from a commercial source (Vesta Ceramics).

2.3 General Particle-Based Cancer Drug Delivery

Tumor tissue often has a unique architecture in comparison with normal ones. The tissue is divided into three different types: cellular, interstitial, and vascular. Tumor vasculature is much more heterogeneous than non-cancerous tissue and has characteristics that are highly proliferative, has larger tortuosity, and acquires an altered basement membrane conformation. There is an enhanced

permeability of the blood vessels present in tumors. Although the mechanism for enhanced permeability is not well understood, there are models suggesting that the transport size through the pores is between 380 and 780 nm. [27-28]

Tumor interstitial tissue is composed of collagen, interstitial fluid, hyaluronic acid, and proteoglycans which make up a gel-like composition. There is also the presence of a high interstitial pressure that leads to an outward flow of nutrients. The transport properties of any drug within this part of the tissue will be affected by physiological characteristics such as interstitial pressure, physical composition of the matrix elastic fiber network, and the properties of the drug itself based on size, charge and hydrophobic nature.

One of the reasons drugs are incorporated into particles is because there seems to be a benefit with these systems to overcome cellular resistance and physical barriers that can prevent the drugs from entering into the tumor tissue. Particles might be able to somewhat shield the drug until it gets into the tumor. The transport of a larger particle into the tumor would depend on passive diffusion across a leaky vasculature based on an enhanced permeability and retention effect. Retention is said to occur because of the decreased clearance by the lymphatic drainage systems in tumors. Active targeting would involve ligands that can specifically bind to the site of interest.

There are some examples of particle-based systems that have successfully demonstrated increased anti-tumor efficacy. By those that haven't worked, more

insight has been spread into the factors that contribute to improvements in particle-based anti-tumor targeting.

One example consists of small particles (50-60 nm size) that were loaded with taxol in polymer nanospheres composed of polyvinylpyrrolidone.^[29] In this study, the mice were injected intravenously with taxol infiltrated nanospheres and compared with free taxol and the results showed significant regression of the tumor in subcutaneously implanted tumors.

First, it is known that high curvature < 100 nm size and a hydrophilic surface is needed to avoid opsonization reactions that clear foreign bodies by macrophages. This is the body's first line of defense and typically hydrophobic, non-surface treated particles are faced with rapid clearance from the body by macrophages.^[30] The specific characteristics of small size, hydrophilicity, and the ability of the particulates to provide a sustained release of drugs made for the successful delivery of therapeutics into the tumors.

One approach to creation of long circulating polymers is through the use of a coating consisting of a hydrophilic polymer, polyethylene glycol (PEG) on the nanoparticle surface. The PEG helps to shield the particle from opsonization because it acts as a hydrophilic barrier that appears to be like water. In several studies the in-vivo biodistribution and pharmacokinetics did not seem to be altered when coupled with the hydrophilic polymers.^[31-32] The next section will discuss some of the recent advances in polymer-based delivery systems.

2.3.1 Polymeric Delivery Systems

For polymeric delivery systems the problems aimed to be addressed are poor drug solubility, short in-vivo circulation time, and fast clearance by macrophages. For awhile, polymer-based therapeutics had a difficult time making it through clinical trials due to the heterogeneity in molecular weight, and parameters that were difficult to characterize, such as number of drug molecules present per particle. In 1990, the FDA changed the regulatory process of polymer-drug composites into its own separate division. Although they still require a rigorous series of characterization steps, detailed analysis of the manufacturing process, and determination of dosing, by defining it as a separate entity, got rid of unrealistic regulatory hurdles that were initially preventing such materials to be accepted for clinical use.

The example of a block copolymer built with a PEG shell has emerged as a versatile model for drug delivery. For example, a class of polymers known as poloxamers was constructed from PEO-b-PPO-b-PEO structure that forms a core-shell micelle and is known by the trade-name, Pluronic®. Improved delivery of doxorubicin was exhibited towards tumors. However, delivery was induced by separation of the drug between the hydrophobic inner core and the external environment. Similar amounts of doxorubicin were found in non-tumor tissue for the particle system as in comparison to free drug.^[33] Another system used a PEG-b-poly(aspartic acid) di-block copolymer and showed improved efficacy against C-26 colon carcinomas compared with free drug.^[34]

2.3.2 Porous Inorganic Materials

Porous inorganic materials have been used as coatings or material for implants or in-vivo devices. A comprehensive review of three commonly used materials, aluminum oxide (Al_2O_3), titanium oxide (TiO_2), and porous silicon has recently been reported. ^[35]

2.3.2.1 Porous Alumina

Porous Alumina is fabricated by exposing an aluminum film to sulfuric, oxalic, phosphoric acid or a combination of the three. The acidic solution instigates the formation of an oxide and anodization leads to pore formation because of a balance between rate of oxide formation and rate of dissolution. ^[36-37] Pores sizes between 5nm-10 μm can be achieved. ^[38]

These materials have been tested for biocompatibility and as a material for sustained release. One study demonstrated that the addition of poly (ethylene glycol) (PEG) modification on aluminum oxide surfaces inhibited pore-clogging and surface fouling due to binding of in-vivo matter. ^[38] One in-vivo drug release study was performed with 316L stainless steel stents coated with porous alumina. The stents were loaded with tacrilimus, an immunosuppressive drug, and inserted into the common carotid artery of New Zealand white rabbits. The tacrilimus concentration hit maximum levels by 1 hr and receded to un-detectable limits after 48 hrs while never exceeding the therapeutic limits. This drug eluting stent resulted in a milder inflammatory response showing that porous alumina could be used as a drug release coating for cardiovascular stent applications. These are few

of the examples of how porous alumina can be used as a general drug delivery material.

2.3.2.2 Porous Titanium

Titanium and its respective alloys have been one of the most ubiquitously integrated of orthopedic and dental materials and implants because of its mechanical strength and inert properties in-vivo. Porous titanium is formed also via an anodic reaction with hydrofluoric acid (HF), organic electrolytes containing fluoride ion, or chlorine containing electrolytes. ^[39-40]

The reason for the wide-spread use of titanium and titanium alloys in biomedical applications has been due to its strength and its anti-corrosive, non-tissue reactive properties. A thin oxide layer of several nanometers does form upon exposure to air.

One study attempted to improve the compatibility of titanium implants for biological use by coating the surface with antibacterial and anti-inflammatory drugs.^[41] The drug was loaded by simple physical adsorption and compared to simulated body fluid (SBF) assisted drug loading. There was shown a prolonged release in the case of the SBF-assisted loading. When these two surfaces were exposed to osteoblasts, a higher survival of cells was observed on the SBF assisted templates.

2.4 Porous Silicon for Drug Delivery

The aim of drug delivery devices is to provide a means by which drugs can be delivered in a sustained fashion or directed to the site of interest. ^[42-43]

Sustained or local delivery may eliminate some of the side effects present in systemic doses as drugs are that are circulated throughout the body potentially induce toxicity in healthy tissue. Designing delivery modalities has been an active area of research in modern medicine and thereby a gamut of materials has been tested to demonstrate their feasibility. Traditionally materials such as polymers have been a popular choice as previously described. However, more recently porous Si has emerged as a feasible material due to its physical and chemical characteristics.

2.4.1 Biocompatibility

In the literature particularly that pertaining to the field, porous Si is generally referenced and regarded as biocompatible due to a number of preliminary studies done by Leigh Canham in the 1990's.^[19, 44-47] These studies specifically found that when the material is in contact with biological tissue, it degrades into a biologically relevant product, silicic acid. Porous silicon was essentially implanted sub-cutaneously in guinea pigs and it was found that the material degrades over a series of time-lapsed images taken by scanning electron microscopy (*pSiMedica, Inc.*). Following that, a series of studies observed the interfacial behavior and toxicity of porous Si with mammalian cells.^[48-52] The first study to utilize porous Si as a drug delivery reservoir sought to deliver insulin across a Caco-2 cell monolayer.^[53] Another instance where a biocompatible surface might be desirable would be that of a cell-based biosensor. In both cases, although the application might be different, the focus of research was to

chemically modify an inorganic surface such as Si to make it more compatible with a biological interface. ^[54-55]

The benefit to using porous Si is that it has been demonstrated that the final degradation product of the material is a bioresorbable ortho-silicic acid, $\text{Si}(\text{OH})_4$. However, it is possible that during degradation the material can occasionally generate short-lived silane gas (SiH_4) which is chemically reactive and highly toxic. For the most part, the freshly-etched, un-modified version of porous Si contains Si-H, SiH_2 , and SiH_3 , which readily oxidizes and degrades into silicic acid. It is even known from various studies that Si is an essential element for normal bone and tissue growth.

Few studies have probed the in-vivo biocompatibility of porous Si ^[56-57], yet the material is generally characterized in the community as biocompatible. In one in-vivo study, porous Si was implanted into the abdominal wall of a rat and the tissue reaction was observed after 1 week, 6 weeks, and 12 weeks compared to non-porous Si and oxidized, non-porous titanium. ^[58] The results showed that porous Si does not have a higher toxicity by means of cell density and capsule thickness when compared to the titanium control.

One thing to consider is that even though the final product may indeed be biocompatible, it is still true that silicon is a relatively insoluble material in aqueous environments (~120 ppm). Therefore, even though in infinite supplies of fluid the material would completely dissolve, the process of replenishment may be slow in the case of a saturated environment where silicon levels end up being at

the level of saturation in the body. This could be true in a beaker or reactor, but likely could be as equally true in an open physiological system where there is a net flow of fluids throughout. In the body, there is a relatively well defined homeostatic flow rate that fixes the amount of water that can replace a given solute. In the metabolic process most metabolites are processed through the liver or the kidneys. In the case of silicon it seems to be metabolized through the kidneys. We will discuss the implications to the limits of biocompatibility in the case of an intraperitoneal delivery system for ovarian cancer in the body of this thesis.

2.4.2 Surface Chemistry

The surface chemistry of porous Si plays a role in the stability of the surface in physiological environments. Immediately after etching, the surface is terminated by Si-H groups. The freshly-etched surface can be modified by oxidation in high temperature conditions or by covalently linking an organic group by hydrosilylation chemistry.^[59-61] In addition, an oxidized surface can be modified by silane chemistry. Modifying the surface chemistry often has effects on the hydrophilicity as well as the degradation properties of porous Si.

2.4.3 Tunable Pore Sizes and Surface Area

One of the unique properties of porous Si is the ability to tune the pore size in a facile manner. Pore sizes of electrochemically-etched Si in particular can be modified by adjusting the etch current density. As a general trend, the larger the current density, the larger the pore size becomes. In addition the dissolution rate

in vitro is correlated to the pore size.^[62] Smaller pores have higher surface area and therefore display an increased rate of degradation, but larger pores are more accessible for bio-molecules to be incorporated.

2.4.4 Optical Properties of Porous Si

Optical properties of porous Si are quite interesting, but will not be a major topic of this thesis until the Appendix. However, a background of this subject is included for reference to this portion. Optical properties of porous Si are such that the etch current can be set as a constant or a sine or cosine modulated current density to yield a signal that can be monitored by reflection of white light off of the film. In the case of a constant current etch, a Fabry-Pérot interference pattern can be observed with the help of a CCD spectrometer (**Figure 2.4**) where the peaks and valleys in the fringes represent constructive and destructive interference with the light that reflects off of the air-porous Si and porous Si-silicon interfaces. When a sine or cosine etch waveform is applied to the wafer, the resulting porous layer alternates in layers of high and low porosity. The resulting interference pattern is a single peak that can be tuned to a specific wavelength (**Figure 2.6**).

These properties can be harnessed to detect a change in signal thus using the film as an interference-based optical sensor. For example, with a Fabry-Pérot film, there is a governing relationship described by (**Equation 2.3**). In this equation m is the spectral order of the fringe at wavelength λ , n is the composite (porous Si + [air or other analyte]), and L is the thickness of the porous layer. The

value 2 represents reflectance geometry, where the optical path is twice the physical path.

Equation 2.3
$$m\lambda = 2nL$$

As a given drug, bio-molecule, solution, or gas flows into and out of the porous Si film, the refractive index (n) of the film changes, affecting the wavelength value of the interference pattern. In a Fabry-Pérot film, it is possible to take a Fast Fourier Transform (FFT) of the interferogram, resulting in a single peak that is directly related to the optical thickness, or ($2nL$) (**Figure 2.5**). In the sine or cosine etched porous Si it is possible to directly monitor the change in peak position.

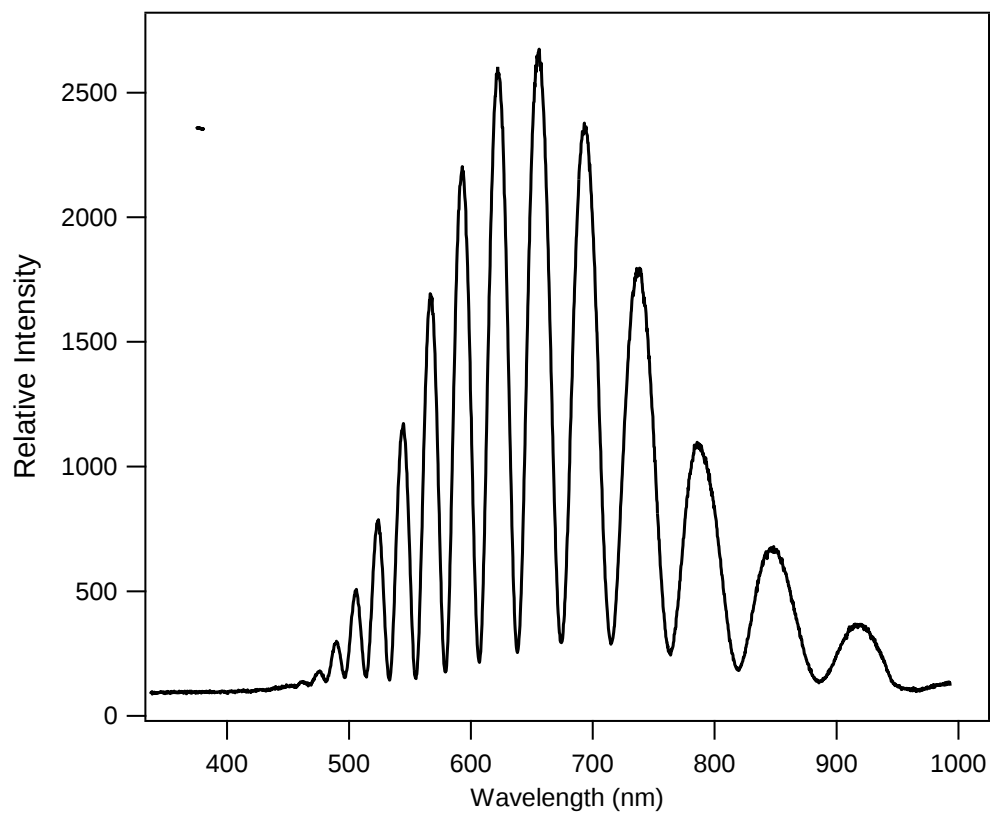


Figure 2.4 Reflectance Spectrum of Fabry-Pérot film etched galvanostatically.

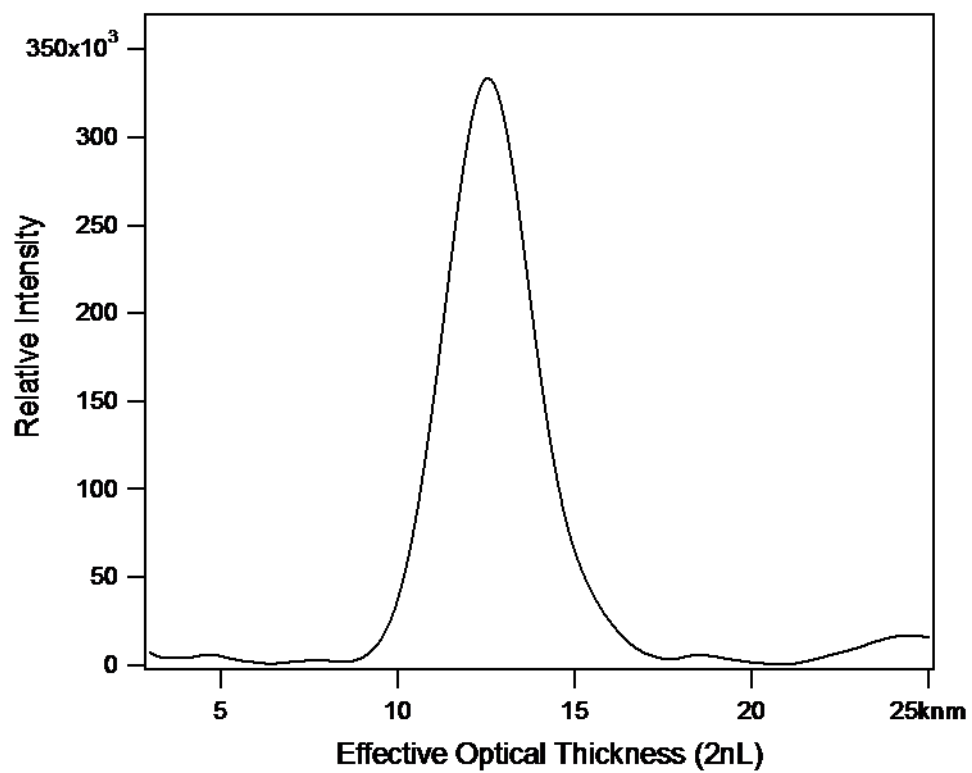


Figure 2.5 Fast Fourier Transform of a reflectance spectrum is taken to generate a plot of relative intensity vs. effective optical thickness.

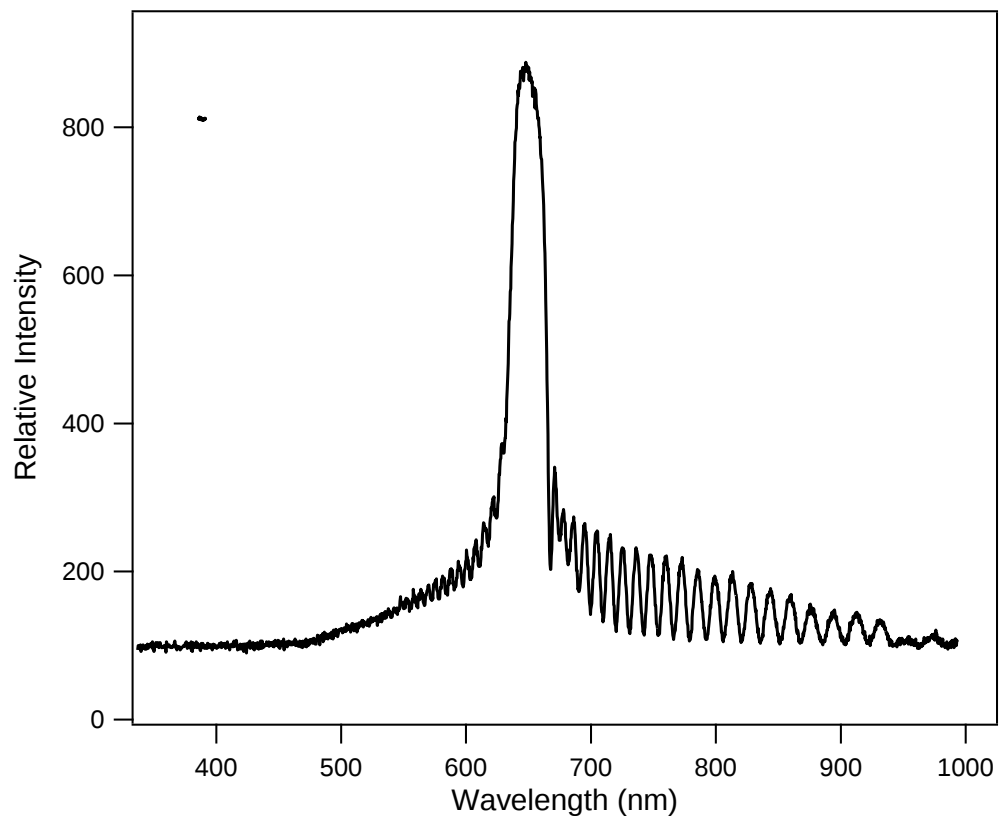


Figure 2.6 Example of a reflectance spectrum of a rugate (sine or cosine) etched film.

2.4.5 Previous Delivery Systems

A number of drug delivery systems involving porous Si have been studied. Herein a review of the various chip-based delivery modes and the more recent particle-based systems will be covered. Although these are the two main sub-sets of delivery systems included here, other effective drug delivery devices include implants, composite materials, and micro-needles.^[63]

2.4.5.1 Chip-Based Delivery Modes

Various types of drugs have been loaded into porous Si chips. One study demonstrated the release of a steroid, dexamethasone from a film where the pores were expanded with HF and DMSO to incorporate a higher degree of loading.^[17] In addition, the surface chemistry was modified with 1-dodecene to shield the surface from water and subsequently slow the release rate of the drug in comparison to a freshly-etched one. Drug loading and release was monitored by optical reflectivity measurements.

Another study aimed to use a porous Si layer as an implant in the peritoneal cavity for sustained release of drug. The porous Si was etched as a double-layer, the first one being macro-porous (2 μ m) and the second one smaller nanometer regime pores.^[64] Porous Si was fully dissolved in a Krebs solution while not readily dissolved in the same time frame for water and ethanol. Doxorubicin, a chemotherapeutic, was also released and dosed on human colon adenocarcinoma cells, where the system showed an increase in drug concentration for a five hour period and a simultaneous decrease in cell viability.

Lastly, one group utilized hydroxyapatite impregnated with transition metal compounds ($\text{Ru}(\text{bpy})_3^{2+}$, $\text{Ru}(\text{phen})_3^{2+}$, and cisplatin) to demonstrate loading and delivery from a chip-based system.^[65-66] This will be discussed again in the section describing cisplatin delivery with porous Si.

2.4.5.2 Particle-Based Systems

Particle-based systems are beneficial because they offer a much higher surface area for delivery and, if synthesized in the nanometer size regime, they can be circulated within the body. Several groups have explored the use of nano- or micro-particles for the delivery of therapeutics.

One of the first studies with drug delivery using porous Si particles was the delivery of insulin across a Caco-2 mammalian cell monolayer as described previously in the biocompatibility section.^[53] Following this, another group demonstrated the loading and release of five model drugs in mesoporous Si microparticles for use in oral delivery.^[67] In addition, recently a study was done on the release of doxorubicin from porous Si microparticles.^[21] A fluorescent dye or fluorescent drug, doxorubicin was covalently attached to the particle. Fluorescence of the attached molecule is quenched by the Si until the silicon undergoes oxidation, upon which the fluorescence is recovered. The toxicity of the released drug has been demonstrated over HeLa cells.

Finally, the luminescence properties of porous Si were utilized in a study to synthesize nanoparticles that can be imaged and deliver doxorubicin in-vivo.

The nanoparticles are relatively non-toxic and degradable in comparison to such materials as Cd-Se quantum dots, but still possess imaging capabilities in-vivo.^[68]

2.5 Ovarian Cancer Therapeutics

2.5.1 Ovarian Cancer

Ovarian cancer appears in the form of three sub-categories of epithelial, germ-cell, and stromal tumors. The class, epithelial is the most prevalent, and all three types are distinct in their aetiology. In many cases, the disease progression is asymptomatic and thereby detected in the advanced stages. In most cases (>80%) the disease has metastasized beyond the ovary into peritoneal and serosal surfaces. Typically the disease is treated by surgical de-bulking in combination with chemotherapeutic treatments.

2.5.2 Historical Overview of Therapeutics

The history of first-line ovarian cancer therapeutics has undergone major modification since the 1970's.^[69] In the 1970's it was common practice to use a combination therapy of anthracycline doxorubicin with cyclophosphamide. When cisplatin was introduced into clinical treatment for cancer, it was introduced into the previous cocktail as a combination of cisplatin, doxorubicin, and cyclophosphamide. In the 1990's paclitaxel was used as a drug to treat patients who relapsed from first-line treatments with platinum drugs. Finally, paclitaxel was studied in combination with cisplatin and carboplatin and found to be the more effective treatment in comparison to cyclophosphamide and cisplatin. Carboplatin was FDA approved in the 1980's and studies have found that there is

no evidence that the carboplatin + paclitaxel first-line therapy is more effective than cisplatin + paclitaxel.

First-line therapy with a platinum drug + paclitaxel combination is usually administered every three weeks for six cycles and results in a clinical efficacy of 80% with 40-60% response in advanced stage disease.^[70] Cisplatin is typically injected at a dose of 50-120 mg/m².^[71-73]

2.5.2.1 Platinum Compounds

Platinum chemotherapeutics were discovered serendipitously in the 1970's. Although the structure and existence of cisplatin has been known since 1845, it was not known for its widespread application towards medicine. Barnett Rosenberg and co-workers conducted an experiment studying the effects of electric field on the elongation of *Escherichia coli* cells.^[74] In the process, they noticed that something was halting cell division and causing bacterial elongation. After a process of elimination, they realized that it wasn't the electric field, but a compound formed by the platinum electrodes reacting with the ammonium chloride solution. They tested that compound with tumors and found that it has anti-tumor activity.^[75] After FDA approval was acquired in 1978 for cisplatin it has been used to treat a wide variety of human cancers. It was the treatment that was used to treat Lance Armstrong when he battled testicular cancer. The cure rate for testicular cancer is > 90% and has been effective in other types of cancers including ovarian, head and neck, non-small cell lung cancer, breast, and bladder cancer as well.

Although cisplatin has been the first of the platinum compounds to display activity towards cancer, quickly following that other platinum molecules were synthesized to overcome toxic side effects, cellular resistance and other limitations. Carboplatin was synthesized with a bidentate carboxylate leaving group that is less toxic than chlorine from cisplatin.^[76] Oxaliplatin has a 1, 2 diaminocyclohexane group in place of the two ammine groups and a bidentate oxalate group on the adjacent side. Nedaplatin has been approved for use only in Japan for esophageal, lung, and ovarian cancers.^[77] Satraplatin (hormone refractory prostate cancer), and Picoplatin (small cell lung cancer) are both compounds that are currently being tested in clinical trials.

2.5.2.2 Cisplatin Mode of Action

Cisplatin is capable of attaching to a number of different intracellular sites within cellular components especially those containing thiol groups. Therefore, when a molecular mechanism of action was being clarified, studies were done to determine where in the cell the drug was being targeted to. In one study, DNA, RNA, and proteins within the cells were fractionated and analyzed for platinum content after incubation with cisplatin. It was found that 1 in 30,000-300,000 proteins and 1 in 10-1000 RNA molecules contained platinum. On the contrary, 9 platinum molecules were bound to each DNA likely indicated it be the intracellular target.^[78]

It was then found from NMR structural analysis that cisplatin can bind to DNA in a number of conformational patterns. A study done by enzymatically

digesting salmon sperm DNA then performing NMR analysis on the separated products showed that the major results were 1, 2-intrastrand cross-links with adjacent guanine bases. The adduct of the form $\text{cis-}[\text{Pt}(\text{NH}_3)_2(\text{dGpG})]$ results in 47-50% of the groups that are formed. Another group makes up 23-28% with the structure $\text{cis-}[\text{Pt}(\text{NH}_3)_2(\text{dGpG})]$. Then, 8-10% of the digested products contained 1,2-intrastrand cross-links involving non-adjacent guanines $\text{cis-}[\text{Pt}(\text{NH}_3)_2(\text{dGNG})]$, and the remaining 2% was a result of a monofunctional binding to a single guanine. ^[79] **(Figure 2.7)** shows a 1, 2 intrastrand cross-link, a 1, 3-intrastrand cross-link, and an interstrand crosslink of cisplatin with GG sequences on DNA. ^[80] Cisplatin has also been found in a few studies to bind to mitochondrial DNA. ^[81] Mitochondrial DNA is unable to form nucleotide excision repair and thereby the binding may be more permanent contributing to cellular toxicity.

Binding of cisplatin on DNA obviously has deleterious effects because it can interfere with the normal cellular functions of cell division and protein production. A hampering of these processes could be detrimental to cell health eventually leading to cytotoxic effects.

One of the processes by which cisplatin interferes with cellular function is DNA replication. Replication involves a process facilitated by DNA

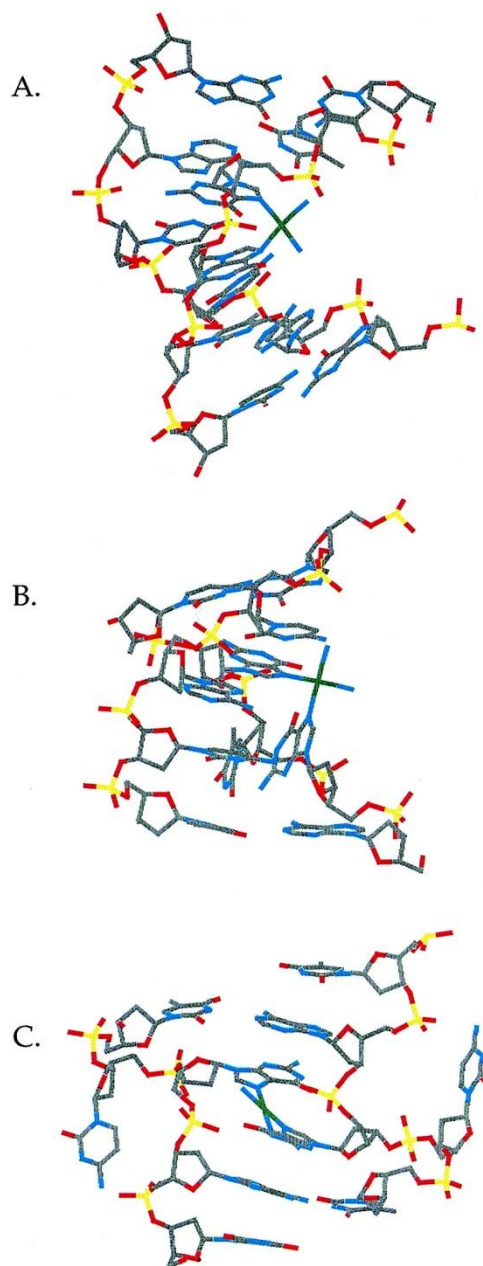


Figure 2.7 Diagram of cisplatin-DNA adducts (from reference Jamieson E.R. and Lippard, S.J., Chem. Rev. (1999)). It shows a structural representation of a 1, 2 intrastrand cross-link (A), a 1, 3 intrastrand cross-link (B), and an interstrand cross-link (C).

polymerases where the DNA is unwound from chromatin, the double-helix accessed, and the two strands are separated and the strand duplicated using one side as a template. In one study the replication of salmon sperm DNA by human DNA polymerases α and β (and a leukemia virus reverse transcriptase) were inhibited by the presence of cisplatin and transplatin.^[82] However, it was found that it required 2-7 fold fewer cisplatin molecules bound to a nucleotide to inhibit 50% binding in comparison to transplatin. Many other studies have confirmed that cisplatin has an effect on the DNA replication process.^[83-84]

Transcription is the process by which mRNA is produced from a DNA template and then eventually results in protein synthesis. This process is vital for cellular replication as well. A series of studies found that transcription was inhibited for wheat germ RNA polymerase II and E. coli RNA polymerase because of the presence of cis-GG, cis-AG, and cis-GNG, and interstrand crosslinks. Transplatin and monofunctionally bound molecules did not completely block the polymerase action.^[85-86] One final intracellular target for cisplatin that has not been mentioned yet, is in the telomere region of DNA that is rich in G base sequences. Telomeres exist on the ends of DNA strands to protect the ends from degradation and thereby insuring sequence integrity from one generation to the next. But it is still controversial as to whether these groups play a major role in cell death.

In addition it is interesting how the cell processes DNA-cisplatin adducts. The repair is done in cells primarily through the nucleotide excision repair

pathway (NER) where proteins will repair lesions caused by damaging factors. First there are proteins that identify the damaged DNA (XPA, RPA, and XPC) and bind to the sequence. Then, transcription factors are recruited, the damaged DNA sequence is cut out, and DNA polymerases and ligases come in and fill in the cut area.

The understanding of the mechanism for cisplatin action at the cellular level has been studied extensively; however still there is no overarching mechanism that has yet been decided to be the dominating theory to tie it all together. It seems like a number of different pathways including DNA replication, transcription, possibly telomere binding, and recruitment of repair proteins could eventually contribute to apoptotic pathways.

2.5.2.3 Limitations of Cisplatin Therapies

Treatment with cisplatin can induce resistance in tumors. Resistance can be classified as either intrinsic resistance specific to the cell or acquired resistance upon exposure to the compound. Specifically in ovarian cancer, often there is an initially positive response to the treatment, and then over time resistance to the drug builds up.

Resistance could be caused by accumulation of the drug inside the cell, an increase production of intracellular thiols in defensive response to cellular toxicity, and a heightened ability of the cells to respond to DNA damage. This is a process that is still not well elucidated as studies have had contradictory results.

2.6 Studies with Porous Si and Cisplatin

An initial study was done in combination with porous silicon and cisplatin, where silicon wafers were etched and cisplatin was deposited onto the wafer through a hydroxyapatite layer.^[65-66] Cisplatin release was monitored by UV-Vis absorption spectroscopy by reporting the raw absorption spectra over time. The limitations to this method are that cisplatin can change conformation in solution and the molecular absorption pattern will change as measured by UV-Vis absorption. The quantity of the cisplatin complex released was additionally not characterized.

2.6.1 Surface Reactivity of Porous Silicon

One paper reported in 2006 that the mechanism of certain cellular viability assays react with porous Si in a way that causes the signal to read a false positive.^[87] For example the Alamar Blue viability assay depends on the intracellular reduction of resazurin, a phenoxazin-3-one dye. An enzymatic reduction alters the blue resazurin to pink resorufin by intercellular dehydrogenases. Resazurin is an electron acceptor that can be readily reduced by metabolic changes such as that of NADPH or NADH coenzymes. MTT, MTS, and XTT are additional cellular assays that rely on reduction pathways to generate a response. In MTT, tetrazolium salt reduces to an insoluble formazan. XTT and MTS are additionally two different types of tetrazolium salts that will reduce into formazan. It is known also that porous Si oxidizes and hydrolyzes in aqueous solutions ultimately forming silicic acid. While this is the known biodegradation

pathway that is amenable to use of porous Si as a biomaterial, we also realize that assays dependent on enzymatic reduction can yield false positives in the presence of reducing agents, like porous Si. Oxidation occurs at the locations where Si-H terminated groups still have yet to be oxidized and remain. Thereby it is interesting to note that porous Si has unique reactivity characteristics under certain conditions.

2.6.2 Discussion of Reductive Potential

An interesting property of porous Si is that it can act as a modest reducing agent. Any aqueous metal ion system that has an oxidation state higher than zero has the ability to reduce in the presence of porous Si. A number of studies have confirmed this behavior and others have sought to better understand the mechanism. Dry metal deposition processes such as chemical vapor deposition are widely used to deposit metals in the field of Si technology. In the case of porous Si, however, the metal is often unable to penetrate into the pores due to pore mouth blockage. There are also methods such as electrodeposition^[88-89], electroless plating^[90] and immersion plating^[91] that can be used to deposit metals. Each of these other methods seems to not be limited by diffusional transport in the pores.

The presence of the anodic reaction on the surface of porous Si is important for the progression of the reaction to occur. Metal ion reduction is promoted by the simultaneous oxidation of Si in the presence of water. So in the case of non-aqueous solutions, porous Si does not oxidize and therefore the metal

oxidation cannot occur. In one study, the growth of metal nodules on the surface after a series of SEM images after different incubation periods (5 s, 30 s, and 5 min) in a 0.01 M $(\text{CF}_3\text{SO}_3)_2$ MeOH solution, it shows that deposition appears as nodules of Cu on the surface, first starting off to be small (120-180 nm) after 5 s, then growing to 500 nm after 30 s, and reaching a near monolayer coverage after a period of 5 minutes of growth.^[92]

It appears that the nucleation develops from a finite number of nucleation sites which are dependent on the properties of porous Si. Nucleation progresses as the electrons are supplied by the underlying porous Si, which is oxidized in the presence of water. Overall, the mechanism is described as an open cell model rather than a general corrosion type because the reaction is short lived and halts when the porous Si is no longer in contact with the solution. This process is explained in the reference by Harraz FA, Tsuboi T, Sasano J, Sakka T, and Ogata YH, *J. Electrochem. Soc.* (2002).^[92]

Metal deposition occurs at a significantly higher rate on porous Si than on Si surfaces due to the high surface area and the greater presence of silicon hydrides on the surface. The presence of surface defects or contaminants generally increases the potential for nucleation to occur at that site. Therefore, the morphological difference of the porous Si versus polished silicon can explain the driving potential for metal deposition.

2.6.3 Reductive Deposition of Platinum into Porous Si Particles

One of the major themes of this thesis emphasizes the fact that porous Si is reactive under certain conditions. In some cases, the reactivity can purposefully be used to load a drug into the pores. In this case, we have demonstrated the reaction of cisplatin with porous Si by reduction into platinum metal and have utilized this chemistry to trap viable drug into the pores to effectuate a higher toxicity than free drug.

2.7 Summary

Many materials including inorganic substrates, polymers, iron oxide to name a few have been used for sustained and localized drug delivery applications. In this thesis we will focus on some work done with porous Si, to demonstrate its feasibility for use as an intraperitoneal microparticle carrier for cisplatin. Some strategies include utilizing the intrinsic reactivity of porous Si to load drug as well as modifying the reactivity to curb toxicity.

2.8 References

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Chapter Three

IMPROVED BIOCOMPATIBILITY OF CHEMICALLY-MODIFIED POROUS SI MICROPARTICLES WITH OVARIAN CANCER CELLS

3.1 Abstract

Nanostructured porous Si microparticles are tested for their suitability as drug delivery carriers towards intraperitoneal (IP) ovarian cancer. In a previous study involving IP delivery of a large quantity of silicon-containing particles in rats, acute toxicity was exhibited in the kidneys as threshold doses were exceeded.

^[1] We hypothesize that the sheer volume of silicon byproduct generated by a large quantity of material causes toxicity because the kidneys cannot tolerate Si beyond a threshold flux. In this work, particles are synthesized by two different methods: (1) electrochemical-etching and (2) stain-etching; then surface chemistry is modified and a post-processing rinse step is introduced to reduce the toxicity in-vivo. The relative degradation rates of the particles are measured by quantifying the soluble Si(OH)_4 released in aqueous buffer. Biocompatibility of the particles with cells by sulforhodamine B viability assay show no variation in toxicity, while in-vivo intraperitoneal toxicity studies in Balb/c mice result in an improved compatibility upon addition of the surface modification and rinse procedure.

3.2 Introduction

The unique properties of porous Si such as tunable pore structure, large surface area, degradability into bio-resorbable products, and ease of surface modification make it an attractive candidate as a carrier for the controlled release of drugs. Porous Si has been applied to a number of diagnostic and therapeutic uses including biosensor ^[2, 3], drug delivery ^[4, 5], and as an interfacial material for

tissue regeneration ^[6, 7]. Biocompatibility has been tested for these uses in the presence of cells and living tissues. ^[8-12]

As various classes of materials that possess nano-sized properties emerge with novel applications and heightened interest, so do the precaution towards potential toxic effects the particles could exhibit in their interaction with biological systems. Thus, there is motivation to further characterize the toxicity of these materials even though preliminary screening classifies it as biocompatible. In addition, depending on the form of silicon and the location of exposure, the body can react with a different response.

Si is an essential element for normal mammalian development particularly in structures that require strength and elasticity such as bone and cartilaginous tissue. ^[13-16] Conversely, in particulate form by inhalation, silica dust is a commonly known occupational hazard that can form inflammation in the lungs leading to fibrosis and scarring, known as silicosis. ^[17] According to IARC (International Agency for Research on Cancer) this form of silica is a known carcinogen. Silica has been FDA approved and has been considered safe for use as a food or an animal-feed ingredient. ^[18] Surface modifications with a silica-coating over structures such as toxic cadmium quantum dots, have been a known route to enhance their biocompatibility. This goes to show that biocompatibility has a diverse consequence across the board even for the same material and it depends on size, surface charge, composition, and site of delivery.

Silica gel has been injected intraperitoneally at a dose of 0.15-0.2 mg/g in rats and as a result a degenerated epithelium was seen in the proximal tubule of the nephron. Deposits of silica aggregates were found to be concentrated to the mitochondria. After 2 days the deposits were eliminated from the cell and the silicon content in the kidney returned back to normal.^[19] In another study, dogs received a daily oral dose of 0.8 mg/g of silicon dioxide or 2.4 mg/g sodium silicate. No renal lesions showed up when given silicon dioxide, but renal lesions were present in animals given sodium silicate.^[20] Settle and Sauer demonstrated that 300 mg of sodium metasilicate delivered to guinea pigs orally and intraperitoneally, resulted in siliceous deposits in the kidneys.^[21] Finally, the intraperitoneal delivery of silicon-containing ceramic particles delivered to mice at 1-5 mg/g body weight induced acute nephrotoxicity.^[1] We aim to explore the ways that the biocompatibility of silicon-based microparticles can be improved for use in intraperitoneal delivery.

Size, shape, porosity, degradation rates, and surface chemistry all play a role in compatibility. Due to its high surface area and porous morphology, porous Si readily undergoes hydrolytic oxidation because water can access the pores.^{[22,}
^{23]} The degradation pathway is related to this process, where upon oxidation to SiO_2 , the particles can degrade to soluble forms of ortho-silicic acid that is the bio-resorbable form. One aim is to decrease toxicity by covalently modifying the surface with a hydrophobic moiety to reduce that rate of particle degradation. The second aim is to introduce a post-processing rinse procedure that can minimize the

initial burst release of silicic acid. Micron-sized particles are too large for systemic delivery, but feasible for use in intraperitoneal delivery by accessing the high effective surface area for better therapeutic loading compared with nano-sized particles.

3.3 Materials and Methods

3.3.1 Sample Preparation

Porous Si particles were electrochemically-prepared from highly-doped single crystalline p-type Si (resistivity between 0.009-0.0012 Ω -cm, <100> oriented, boron-doped). Anodization of Si wafers was performed in an ethanolic HF solution (3:1 (v/v) 48% aqueous HF: ethanol) using a two-electrode configuration with a platinum mesh counter-electrode. Samples were etched in a Teflon etch cell that exposes 8.04 cm² area of Si wafer to the electrolyte. Wafers were etched at 124 mA/cm² current density for 4 minutes. After removal of etching solution, resulting porous Si layer was rinsed three times with ethanol and dried under a stream of nitrogen. The thin porous layer was lifted off by electro-polishing at 8.7 mA/cm² for 2.5 minutes in dilute HF solution (1:10 (v/v) 48% aqueous HF: ethanol). The free-standing film was rinsed three times with ethanol to remove residual HF. The etching and electro-polishing steps were repeated numerous times to provide a sufficient quantity of porous Si. Free standing films were fractured into micron-sized particles in ethanol for 45 minutes by ultrasonication (Model 50T, Fischer Scientific). Size distribution of particles was refined by passing through two nylon mesh filters with nominal pore sizes of

80 μ m and 5 μ m (Millipore). The resulting micro particles were further rinsed with ethanol and allowed to dry in air overnight. The ethanol rinse step is used to sterilize the particles from endotoxins and is a solvent that minimized oxidation.

Two variations of stain-etched particles have been used. The samples have been obtained from Vesta Ceramics Ltd. Due to the limitations of the etch depth, the 4 μ m particles are porous throughout and the 20 μ m samples retain a solid Si core with a 4-6 μ m porous shell.

3.3.2 Surface Area and Porosity Characterization

Nitrogen adsorption-desorption isotherms of porous Si particles were recorded at 77K using a Micromeritics ASAP 2010 volumetric apparatus. Prior to the adsorption experiment, the samples were degassed in situ at 323 K until a static vacuum of 5×10^{-5} Torr was reached. Nitrogen doses were admitted, and the adsorbed amount was registered as a function of the equilibrium pressure. The surface area of the samples was measured by the BET method, which yields the amount of adsorbate corresponding to a molecular monolayer.^[24] Pore dimensions were determined by using the BdB method from the nitrogen adsorption curve for the electrochemically-etched particles.^[22] The BJH model was used to determine pore size for the stain-etched materials. Particle structure and morphology was studied using an FEI Quanta 600 environmental SEM and Philips XL30 environmental SEM, at an accelerating voltage of 20keV.

3.3.3 Post-Process Rinse and Fluorescent Dye Conjugation

Two types of post-processing procedures, fluorescent dye conjugation and oxidative rinsing have been applied to the porous Si microparticles. For the rinse procedure, particles are soaked in PBS for 12 hours, and the initial burst of silicic acid byproduct is removed. The particles are air-dried. The fluorescent dye conjugation involves first modifying the surface with undecylenic acid, then attaching the dye molecule. The un-modified particle surface is composed primarily of Si-H. Porous Si particles are dispersed in undecylenic acid in a small glass beaker and thermally hydrosilylated in a microwave oven.^[23] The dispersion is heated for 1 minute intervals, re-dispersed, and the process repeated 3 times. The resulting particles are rinsed 3X with hexane and 3X with ethanol to removed physisorbed species. Modification with fluorescent dye was accomplished by adding 100 μ L of 1 mg/mL Alexa Fluor 594 hydrazide dye solution in ethanol, 100 μ L of 10mg/mL N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride in ethanol, and 800 μ L of ethanol to 50 mg of particles. The reaction was agitated in the dark for 2 hr at room temperature. After the reaction, the dispersion was centrifuged; the particles pelletized and rinsed with ethanol 3X, then allowed to dry at room temperature.

3.3.4 Monitoring Degradation Rates of Microparticles in PBS

Effects of particle size, mode of etch, and surface chemistry (dye modification and rinse step) on their dissolution rate were studied by measuring the amount of soluble, elemental Si released into solution. Particles were

dispersed in 2mg/ml quantities in conical vials in PBS. Aliquots of 2mL were taken out at time intervals of 2 hours and 5 days. Total soluble Si content was measured by inductively coupled plasma optical emission spectroscopy. A standard Si calibration curve was created from a known silicon standard solution. Samples were measured in triplicate.

3.3.5 Determination of Toxicity by In-vitro Cell Viability Assay

SRB viability assay was used to test the compatibility of the porous Si particle preparations with 2008 human ovarian cancer cell line. As determined by cell doubling time, 3000 cells were inoculated into each well 24 hours prior to the addition of particles. The plates were incubated at 37°C at 5% CO₂, 95% air, and 100% relative humidity. After a 24 hr incubation period, a plate from each cell line was fixed in situ with trichloroacetic acid (TCA) to establish the cell population at the time of particle addition. The particles were suspended in RPMI-1640 reduced serum media and added to a 96 well micro titer plate with progressively increased doses of particles per well. Microparticles tend to have a high density that favors them to settle in solution in comparison to nanoparticles. To provide an even dispersion during dilutions, the particles were re-suspended by vigorously pipetting prior to distributing 100µL in each well. The particles are incubated with the cells over a 5 day growth period under the described incubation conditions. Surviving cells were then fixed using 50% TCA for 1 h at 4°C and stained using 0.4% sulforhodamine B dye. After rinsing away unbound dye with 1% acetic acid, bound dye was solubilized using 10mM Tris-HCl and read at 515

nm on a Versamax absorbance microplate reader (Molecular Devices, Sunnyvale CA). The optical density data was processed as previously reported.^[25]

3.3.6 In-Vivo Toxicity

Particles were administered into Balb/c mice by intraperitoneal delivery. Increasing doses were applied to find the maximum tolerated dose. Four particles types were initially tested for their maximum tolerated dose: 1. Electrochemically-etched, un-modified, 2. Electrochemically-etched, dye-conjugated, 3. 4 μm Stain-etched, un-modified, and 4. 4 μm Stain-etched, dye-conjugated. It was found that stain-etched, un-modified particles could not be delivered due to their hydrophobic surface that resulted in severe aggregation. The deliverable dose was suspended in 850 μL PBS and delivered through a 26 $\frac{1}{2}$ G syringe. Weight was monitored prior to particle delivery and every 24-48 hours for weight loss or indication of toxicity.

3.4 Results and Discussion

3.4.1 Porous Si Etching and Characterization

Three different types of porous silicon particles have been used for this study. It is of interest to characterize the effects of etching procedure, size, porosity, and surface modification. The first particle type was prepared by an electrochemical-etch of a P^{++} type silicon wafer. The second and third particle types were prepared by stain-etching in a hydrofluoric acid / nitric acid etchant, starting with a solid, crystalline silicon powder with a narrow size distribution

(Vesta Ceramics Ltd). The stain-etched particles consisted of two size regimes, 4 μm and 20 μm . The 4 μm powders are porous throughout and the 20 μm samples retain a solid silicon core with a 4-6 μm porous shell.

The different morphology and sizes of the three particle types have been verified by scanning electron microscopy (**Figure 3.1**). The electrochemically-etched particles maintain a flat, uniform surface because they have been fabricated from a porous Si film and fractured by ultra-sonication. The stain-etched particles possess an irregular surface from mechanical grinding of the original non-porous, substrate material. The characteristic pore structure obtained by electrochemical-etching or chemical stain-etching is shown in the SEM inset.

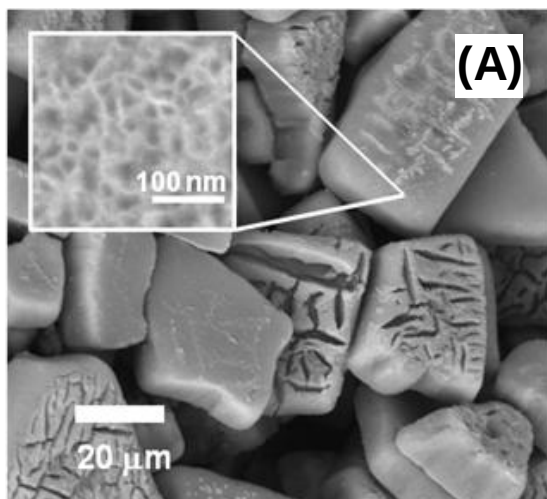


Figure 3.1 Three types of electrochemically-etched and stain-etched porous Si microparticles used in this study have been analyzed by scanning electron microscopy. **(A)** The electrochemically-etched particles have an average diameter of 30-50 μm and were prepared by ultra-sonic fracture of thin porous Si films.

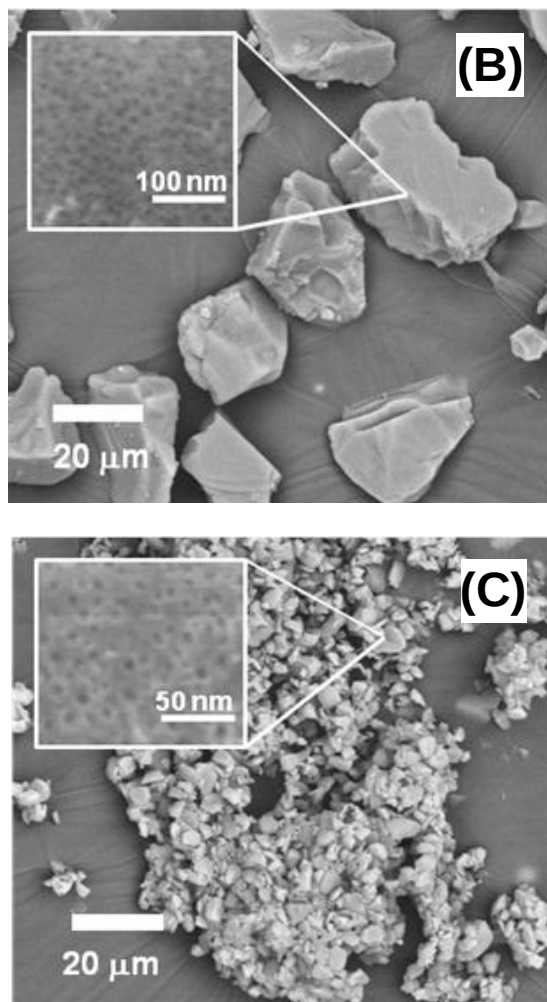


Figure 3.1 cont. (B-C) The two stain-etched particles have average diameters of 4 μm and 20 μm . They have been prepared by a chemical etch of solid crystalline silicon powders and have been obtained from Vesta Ceramics Ltd. Pore size and morphology is depicted in the inset.

3.4.2 Preparation of Fluorescent Dye Modified Porous Si Particles

Particles were hydrosilylated in neat undecylenic acid to achieve a surface carboxyl group for facile coupling to a fluorescent dye. The particles were thermally hydrosilylated in a microwave oven in 1 mL undecylenic acid for 1 min for 4 cycles. The conjugated particles were then rinsed in 1-hexane (3X) and ethanol (3X), centrifuged and decanted between rinses. A fluorescent dye was then reactively coupled with N-(3-dimethylaminopropyl)-N'-ethyl carbodiimide hydrochloride to the modified surface (**Figure 3.2-3.3**). The dye was initially attached for the purpose of imaging the location of a drug delivery carrier in-vivo. Un-modified particles exhibit an intrinsic luminescence that can be utilized for imaging, however the photonic intensity is greater for the dye coupled particles. The resulting surface modification combinations tested for biocompatibility are shown in (**Figure 3.4**)

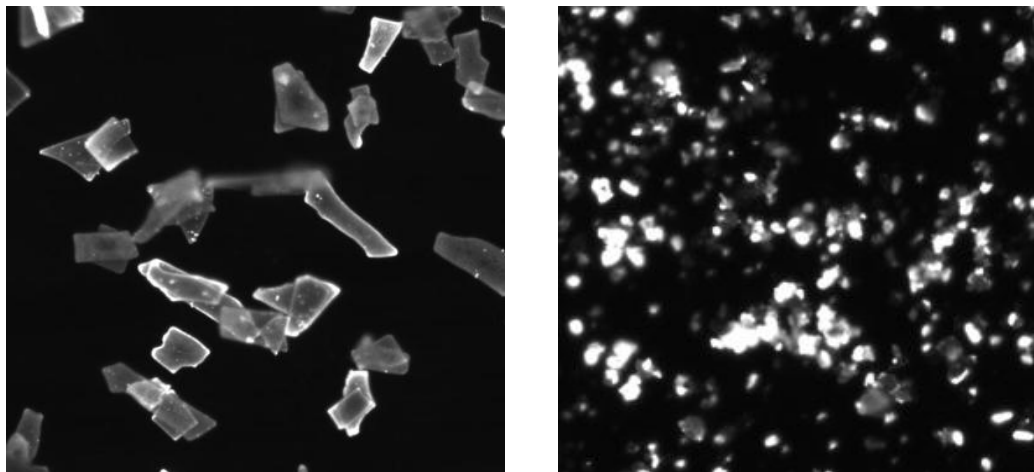
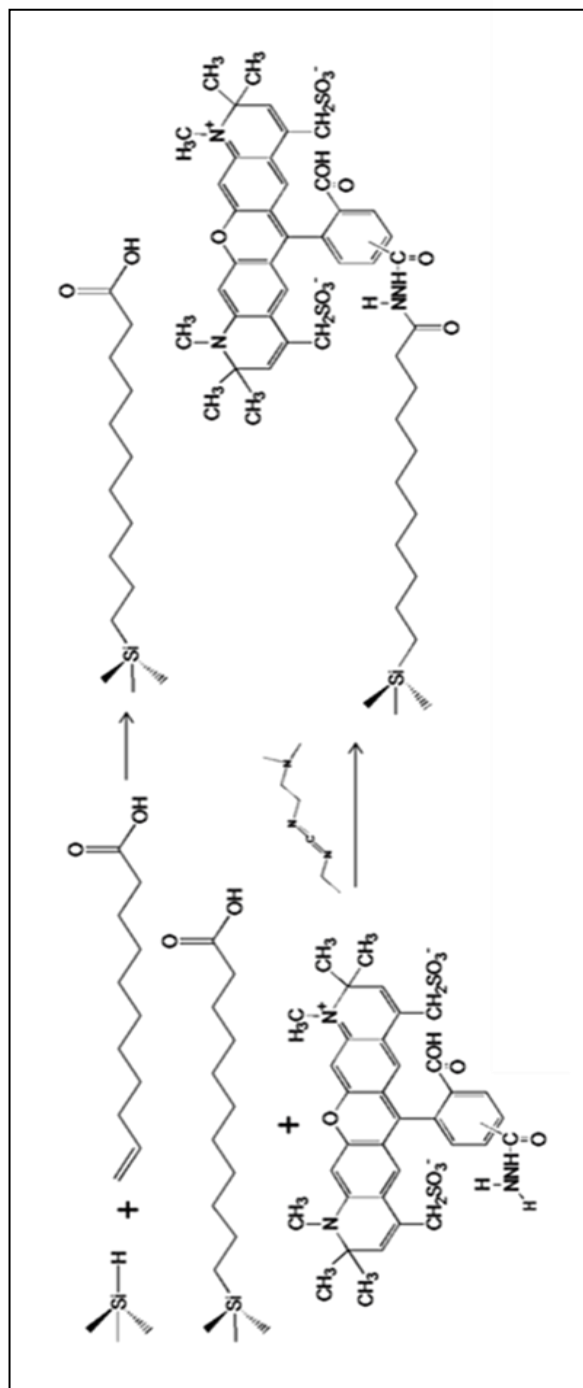


Figure 3.2 The attachment of fluorescent dyes to porous silicon microparticles has been verified by fluorescence microscopy. (A) Electrochemically-etched porous silicon microparticles have been imaged with Alexafluor 594 hydrazide dye attached to the particle surface. (B) 4 μ m stain-etched porous silicon microparticles are shown with the same dye attached via identical coupling chemistr

Figure 3.3 The chemical scheme shows the process of the fluorescent dye attachment to the silicon surface. The freshly-etched surface is modified with a carboxyl terminated, hydrocarbon linker, undecylenic acid. Alexafluor 594 hydrazide dye is then linked by ethyl-3-(3-dimethylaminopropyl)-carbodiimide mediated amine bond formation.



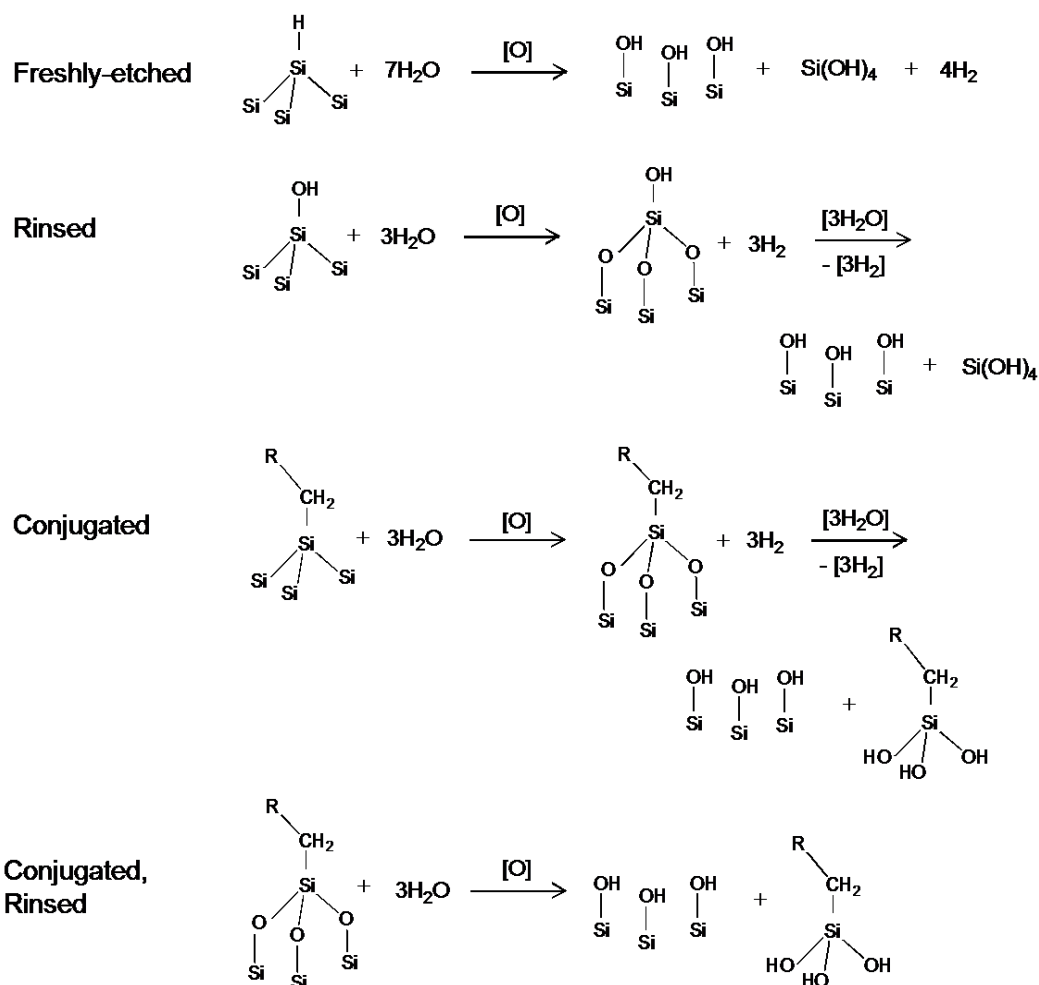


Figure 3.4 Four surface modifications have been applied to electrochemically-etched, 4 μm stain-etched and 20 μm stain-etched microparticles. The schematic diagram shows the possible degradation pathways. The rinsed particles have undergone a 12 hour soak in phosphate buffered saline to remove excess silicic acid and to provide an oxidized surface. The conjugated particles have been modified with a hydrocarbon linker, undecylenic acid, and then coupled to a hydrazide reactive dye Alexafluor 594. [O] denotes oxidation of surface by water or air.

3.4.3 Surface Chemistry Analysis by FTIR

Fourier transform infrared spectroscopy verifies the chemical modification of the surfaces and is shown in **(Figure 3.5)**. The spectrum was taken of only the electrochemically-etched particles, but the spectrum is also representative of the stain-etched particles. The un-reacted, freshly-etched surface is characterized by silicon hydride bonds. Infrared absorption peaks assigned to SiH, SiH₂, and SiH₃ $\nu_{(\text{Si-H})}$ stretching modes are present at 2085, 2110, and 2140 cm⁻¹.^[26-28] A SiH₂ scissor mode is present at 915 cm⁻¹, and a minor surface oxidation peak beginning to form due to oxidants in the air. The freshly-etched sample was then introduced to a 12 hour rinse treatment in aqueous phosphate buffered saline solution in order to try to decrease the toxicity by eliminating the excess silicic acid released from the particle surface. Afterwards, the particles were rinsed (3x) in ultrapure water and ethanol for rapid drying. The surface was oxidized, displayed by the appearance of $\nu(\text{Si-O})$ stretching vibrations at ~1175 cm⁻¹. In addition, $\nu_{(\text{OSi-Hx})}$ vibrations appear at 2260cm⁻¹. These are higher energy Si-H_x vibrations that are representative of Si-H bonds that are back-bonded to oxygen.^[28] The fluorescence dye conjugated samples show the appearance of amide I and amide II vibrational peaks at 1654 cm⁻¹ and 1578 cm⁻¹ and a doublet from the hydrocarbon at 2857cm⁻¹ and 2928 cm⁻¹. A peak at 1716 cm⁻¹ confirms the presence of the C=O bond from the undecylenic acid linker. The major change between the un-rinsed, dye conjugated samples and the rinsed, dye conjugated samples is the appearance of $\nu_{(\text{OSi-Hx})}$ at 2260cm⁻¹. The FTIR results conclude the fluorescent dye to be

covalently coupled via amide bonds, and shows that the rinsing step introduces surface oxidation in both conjugated and un-conjugated, rinsed samples, but more prominently in the non dye-modified sample.

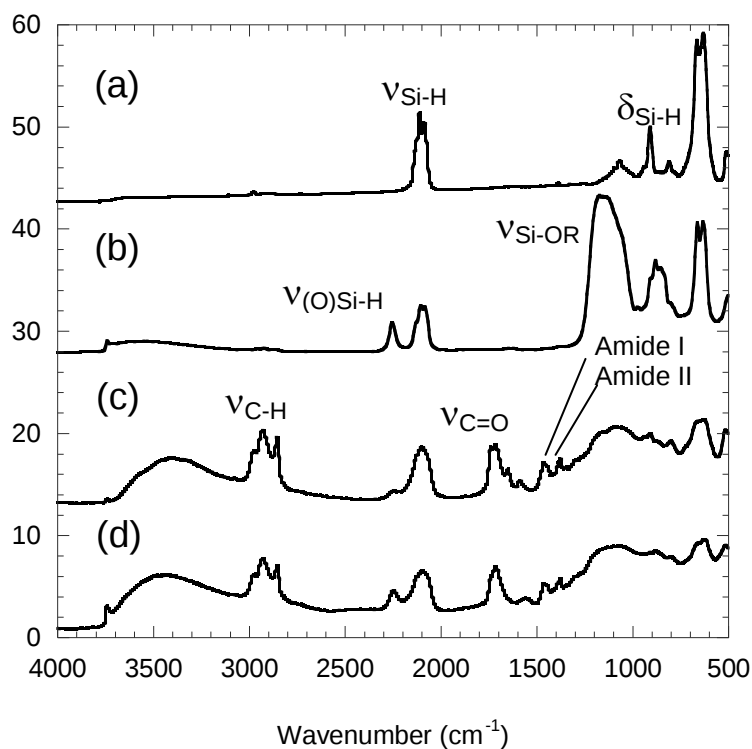


Figure 3.5 Fourier transform infrared spectroscopy verifies the chemistry of dye conjugation and the effects of the rinsing protocol. (a) The freshly-etched sample spectrum shows the presence of Si-H bonds. (b) The rinsed surfaces have the presence of silicon dioxide. (c) The dye conjugated surface indicates the presence of an amide I and amide II bond and presence of carboxylic acid. (d) The rinsed, dye conjugated surface shows the appearance of an oxygen back-bonded to Si-H.

3.4.4 Cellular Toxicity of Porous Si Microparticles with Ovarian Cancer Cells

A sulforhodamine B (SRB) viability assay was used to test the compatibility of the porous silicon particle preparations with 2008 human ovarian cancer cell line.^[29] The results were generated in the form of a dose response curve where increasing concentrations of particles were incubated in a 96 well tissue culture plate (density of 5000 cells per well) for a five day period. After five days, wells were treated with a dye that binds to basic amino acids and colorimetrically tests for total cellular protein, which corresponds to the number of live cells. Porous silicon particles at increasing doses were compared against a no particle control. The mean IC50 values shown in **(Figure 3.6)** give an indication at which dose toxicity occurs. The data provides a comparison of relative compatibilities of the various surface chemistries, the benefit of the rinsing procedure, and the relative in-vitro dose range for the different particles types.

(Figure 3.6) shows the mean toxicities of the three different particle types electrochemically-etched (ECE), stain-etched – 20 μm (SE20M), and stain-etched – 4 μm (SE4M). Each of these particle types has been modified with a dye and rinsing procedure for a total of four distinct modifications per particle type. The four types are freshly-etched, un-modified rinsed, dye conjugated, and dye conjugated rinsed. Toxicity exhibited between particles types was not too significant with the exception of the electrochemically-etched, rinsed particle set. This will be described in more detail later.

The rinse step involved soaking the particles in PBS (without Ca^{2+} or Mg^{2+}), in an effort to reduce toxicity in-vitro and in-vivo. Ortho-silicic acid produced during degradation of porous silicon has been shown to be non-toxic in macrophages up to certain doses.^[30] However this and a study by Kawanabe et al. have identified toxicity at high concentrations in-vitro and in-vivo.^[1] The purpose of the rinsing step is to induce some surface oxidation and to reduce the amount of free silicic acid or silane that might be generated during porous silicon degradation. Silane can be produced by Si or porous Si but not by SiO_2 (**Figure 3.7**).^[31]

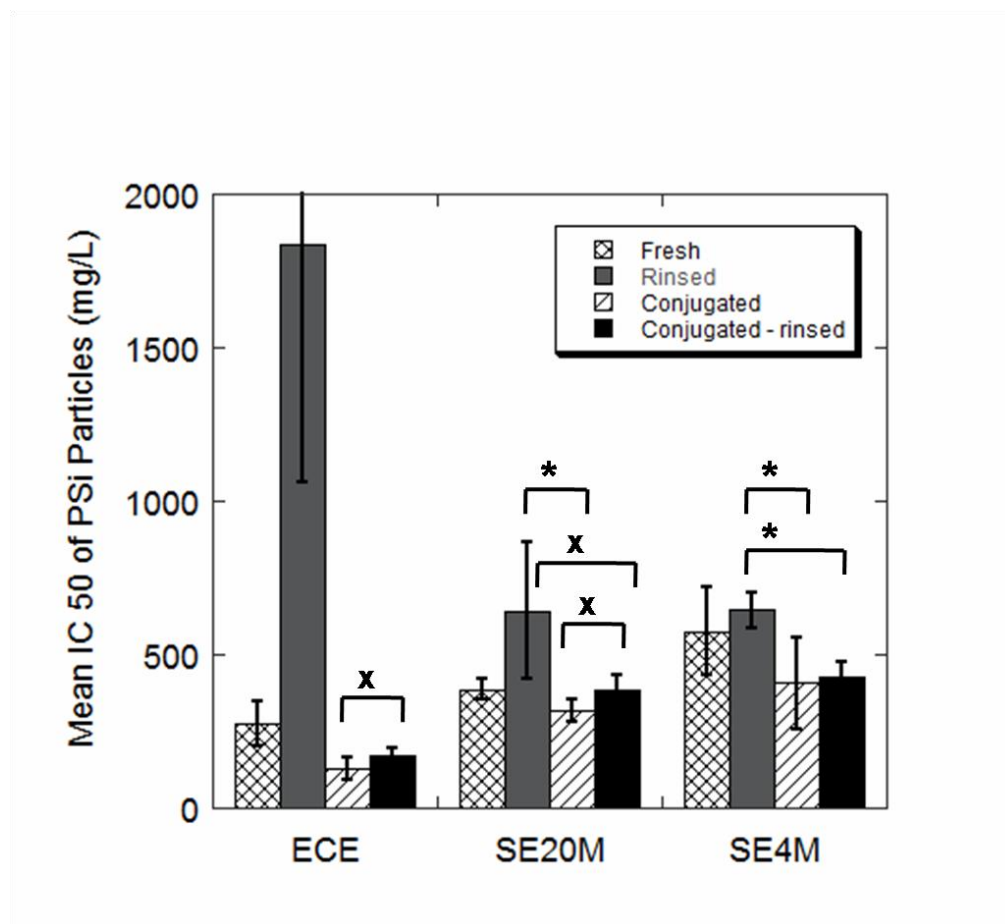


Figure 3.6 Cellular toxicity has been assessed by sulforhodamine B viability assay with three variants of porous silicon microparticles applied with four distinct surface chemistries. The assay was screened in a 96 well plate with 5000 cells per well (2008 human ovarian cancer line). Wells were dosed with increasing concentrations of particles and a dose response curve was generated. The average IC₅₀ value was calculated, which is the dose that generates 50% cell survival. Statistical significance is noted for p-value < 0.05. [X denotes no statistical significance for the given data set, and * indicates a statistically significant group].

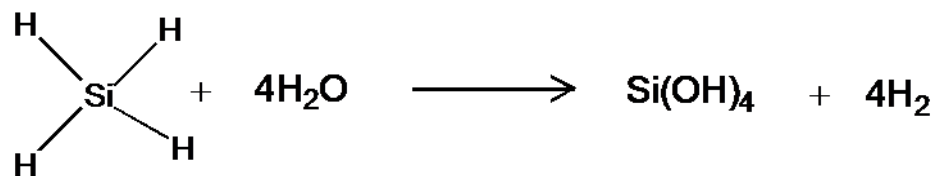


Figure 3.7 Chemical reaction of silicic acid generated from silane, a degradation byproduct of a hydride terminated porous silicon surface. Although short-lived, silane can produce toxic effects within cellular environments. Silane cannot be generated from a SiO_2 surface.

The addition of the undecylenic acid and dye did not statistically increase toxicity ($p\text{-value} > 0.05$) in any of the groups but the electrochemically-etched, when compared with the freshly-etched particle. The rinsing did not improve toxicity in any of the groups with the exception of the electrochemically-etched particle set, both for the un-modified and the conjugated particles. It is interesting to note that the covalently attached undecylenic acid moiety may attribute some toxicity as it is statistically more toxic in comparison to the least toxic (un-modified, rinsed) in all three particle types. This may be prominent only in the combined effect of rinsing reducing the toxicity, and conjugation adding to it, while each factor individually does not contribute significant change. The data indicates that when using a linker such as undecylenic acid to attach fluorescent dyes, it may be necessary to consider the additional contribution to toxicity. This aspect is relevant to a previous study that used an identical linker chemistry to demonstrate covalently drug attachment.^[32]

Rinsing in comparison adds an extra element of biocompatibility. It is hypothesized that the surface oxidation that occurs upon rinsing corresponds to improved compatibility with cells due to a preferential adherence to a negatively charged surface. Across the various sized particles we saw that both stain-etched particles (20 μm and 4 μm) in all surface modification categories were less toxic than the corresponding electrochemically-etched. There is no noticeable distinction between the two stain-etched materials; therefore it seems that size does not play a role in toxicity within this size regime. The electrochemically-

etched particles may have added toxicity due to their mode of synthesis by using a hydrofluoric acid based etchant. Although the particles were rinsed thoroughly, there may be trace fluoride compounds that remain on the particle surface that contributes to toxicity.

The prominent difference in decreased toxicity in the electrochemically-etched, rinsed particle set compared to the two stain etched materials could be explained perhaps by the relative pore size and surface area. **(Table 3.1)** shows the relative particle surface areas and porosities. A rinse procedure would induce oxidation more readily in a freshly-etched surface compared to a conjugated one due to the inability of water to access a hydrophobic surface. As expected, the dye conjugated surfaces did not see much difference in toxicity between the rinsed and the un-rinsed samples. Pore characteristics may have an effect on ability of water to effectively penetrate a freshly-etched surface. The electrochemically-etched samples have a higher porosity and thus may be able to penetrate water more readily and oxidize the surface extensively. The stain-etched particles have a lower porosity that may exclude water from entering the pores.

Table 3.1 Surface area and porosity measurements of electrochemically-etched (ECE), 4 μm stain-etched (SE4M), 20 μm stain-etched (SE20M) porous silicon microparticles, and non-porous, solid silicon powder, by BET surface area analyzer are shown.

Sample	Surface Area/ m^2g^{-1}	Porous Volume/ cm^3g^{-1}	Mean Porosity/%	Mean pore width/nm	n
ECE	429 +/- 59	1.15 +/- 0.106	73 +/- 1.9	8.01 +/- 3.56	3
SE20M	57.5 +/- 7.64	0.0776 +/- 0.018	15.3 +/- 3.0	7	2
SE4M	166 +/- 3.731	0.183 +/- 0.0119	29.9 +/- 1.1	7	3
Si powder	2.043 +/- 0.624	no porous volume	no porosity	no porosity	2

3.4.5 Porous Si Microparticles Dissolution Rates

The relative degradation rates have been determined for each of the particle sets and are shown in **(Figure 3.8)**. Degradation rates have implications in determining rate of covalent drug release, rate of soluble silicic acid generation, and dissolution of the particles to be cleared from the body in a reasonable time frame for therapeutic delivery. Previous studies have found that the rate of silicon generation contributes to siliceous deposits in the renal tubules; therefore, ascertaining the rate of silicic acid generation is of interest. We hypothesize that if we are able to control the degradation rate of the porous silicon into silicic acid by covalent modification of the surface, the rate of silicon entering the kidneys can be regulated. A lower flux of silicon in the kidney could contribute to a reduction in toxic response. As a general frame of reference, the aim of this study is to test what extent chemical modification and rinsing can change the particle degradation rates.

Each particle set was suspended to a final concentration of 2 mg/mL in phosphate buffered saline in triplicate. Aliquots of 2 mL were collected, and then assayed for soluble silicon content using inductively coupled plasma optical emission spectroscopy at 2 hour and 5 day intervals. The amounts of silicic acid generated during this time were reported.

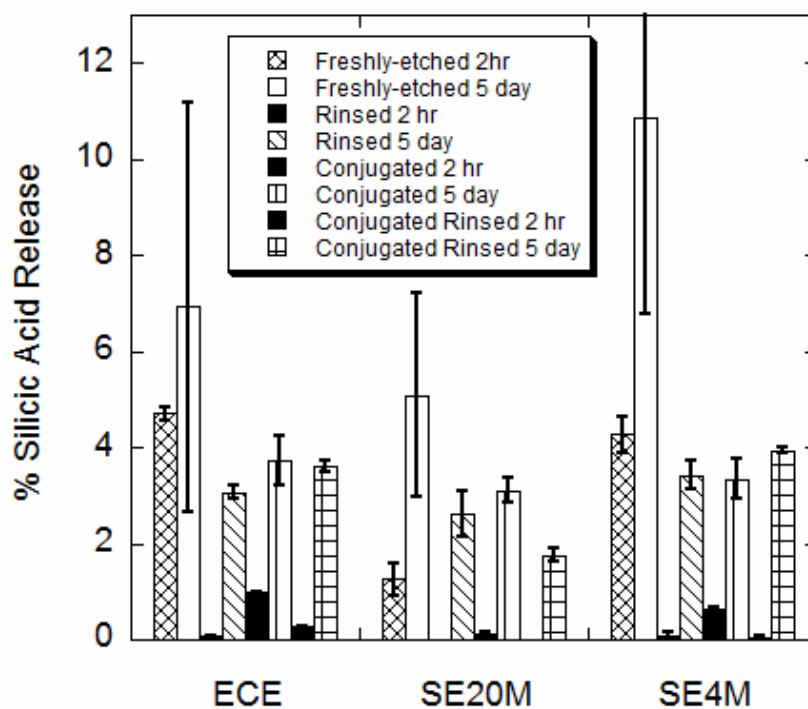


Figure 3.8 Relative degradation of surface-modified porous silicon microparticles into silicic acid by-product has been demonstrated. The particles were soaked in phosphate buffered saline at a concentration of 2 mg/ml. Aliquots of the soluble silicon released in solution were taken at 2 hour and 5 day intervals. The samples were measured for silicon content by inductively coupled plasma optical emission spectroscopy. Statistical significance is measured for $p\text{-value} < 0.05$.

It was found that silicic acid release is lowest for the un-conjugated, rinsed particles. The dye conjugation also decreases the release rate of silicic acid. The freshly-etched particles have the greatest release of silicic acid and the conjugated, un-rinsed particles release the second most. After a five day period, the rinsing does not affect the conjugated particles, but does affect the un-modified particles. The limitation to the method used is that it is a static system that does not have a continuously flowing exchange of fluid. In the in-vivo system, a more accurate model would mimic the rate of exchange of fluids in the peritoneal cavity to replenish Si saturated fluids. The data was measured with a statistical significance of $P \text{ value} < 0.05$.

We wanted to test what amount of silicic acid would be toxic to the 2008 human ovarian cancer cells. If the amount of silicic acid produced by the release data corresponds to the amount of toxicity seen in the viability studies, we can conclude that the response is likely caused by silicic acid. However, our results show that at an excess dose of commercially obtained silicic acid beyond the saturation limit, we are still unable to show toxicity in the cells. Toxicity was tested by sulforhodamine B viability assay with 5000 cells per well and dosed with > 300 fold excess of silicic acid than the amount released in the degradation study (**Figure 3.9**). This could indicate that the response to toxicity of silicic acid in cells is different than that in-vivo. Toxicity in the cell is likely to be caused by an agent alternative to silicic acid.

Previously, it was found that hydride-terminated porous Si can generate silanes that produce toxicity within cellular environments. We propose that the transient silane gas species could generate a toxic response.^[31]

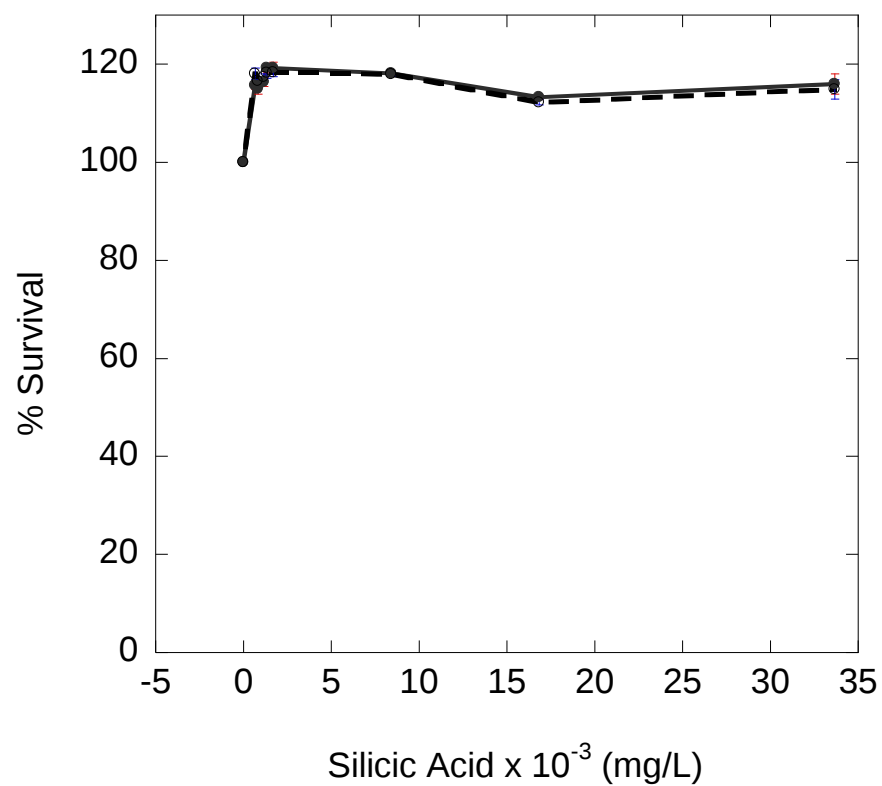


Figure 3.9 Silicic acid has been dosed at high concentrations on 2008 human ovarian cancer cells to ascertain the toxicity profiles. Sulforhodamine B viability assay was used to test for toxicity. It has been found that with exceedingly high concentrations of silicic acid, the cells still do not exhibit toxicity.

3.4.6 In-Vivo Toxicity Determination of Maximum Tolerated Dose

The maximum tolerated dose as defined by the National Cancer Institute is, *the highest dose of a drug or treatment that does not cause unacceptable side effects*. The maximum tolerated dose is determined in clinical trials by testing increasing doses on different group of people until the highest dose with acceptable side effects is found. Maximum tolerated dose has been studied in Balb/c mice to see if conjugation and rinsing could improve toxic effects. The mice were injected intraperitoneally with increasing concentrations of particle suspensions in an injection volume of 0.8 ml. Body mass, behavioral activity and general healthiness of appearance were used as indicating factors to monitor their vitality. Lethal toxicity was usually manifested within 48 hours of injection. Four sets of particles were attempted to be delivered including: electrochemically-etched, freshly-etched; electrochemically-etched, conjugated rinsed; 4 μ m stain-etched, freshly-etched; and 4 μ m stain-etched, conjugated rinsed. The stain-etched 4 μ m freshly-etched particles were not able to be delivered because of aggregation in the syringe due to the hydrophobic repulsion of the Si-H surfaces. The range of maximum tolerated dose for each of the tested particle types is listed in **(Table 2)**. The maximum tolerated set was the 4 μ m stain-etched conjugated, rinsed particle. From the electrochemically-etched group, it has been shown that conjugation and rinsing make ~ 2 fold improvement in in-vivo toxicity.

Histopathological sections of mice injected with electrochemically-etched particles with toxic effects showed the main source of toxicity to appear in the

kidneys (**Figure 3.10**). Microscopic infarcts blocked capillaries within the kidneys, and there was a greater presence of irregular cells with apoptotic, condensed nuclei. The kidney appears to have focal areas of necrosis in roughly 1/3 of the organ. The other organs including the lung, liver, and heart did not seem to be affected. Our findings of kidney malfunction as the probable cause of toxicity in this study is supported in literature.^[33-35] Through the MTD analysis we have found the dose that is tolerated intraperitoneally within Balb/c mice for three different surface variants of porous silicon microparticles. This study is of interest as a basis for future work that aims to determine the suitability for the micro particle to be used as a potential drug carrier.

Table 3.2 Maximum Tolerated Dose (MTD) of porous silicon microparticles injected in Balb/c mice by intraperitoneal delivery

Experimental groups	MTD (mg/g)
ECE (freshly-etched)	0.23
ECE (conjugated, rinsed)	0.45-0.68
SE4M (freshly-etched)	Not deliverable
SE4M (conjugated, rinsed)	0.77

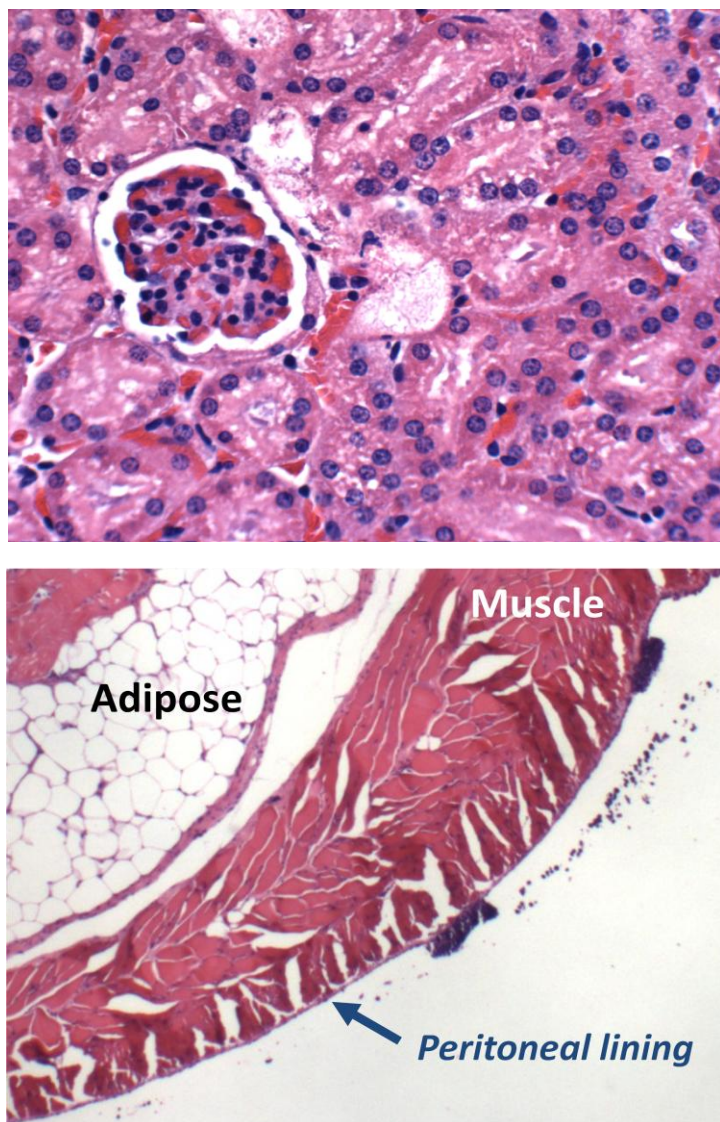


Figure 3.10 Focal areas of necrosis in the glomeruli of the kidney tissue, shown by condensed cellular nuclei (top). Histological cross-section of the peritoneal lining of a nu/nu mouse. Toxicity was exhibited from an injection of a high dose of particles (bottom).

3.5 Conclusions

The goal of this study was to ascertain the improvement in cellular and in-vivo compatibility of porous silicon microparticles, upon post-processing procedures such as oxidative rinsing and surface chemistry modifications, delivered intraperitoneally for therapeutic applications. We have found that the most pronounced reduction in toxicity within the in-vitro viability studies were the electrochemically-etched, rinsed set. Within the groups tested in-vivo for maximum tolerated dose, the 4 μm stain etched, conjugated rinsed particles were tolerated at the highest levels compared to the un-modified, un-rinsed set and the electrochemically-etched counterparts.

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Chapter Four

LOCALIZED DELIVERY OF CISPLATIN, ANTI-CANCER DRUG

4.1 Abstract

Cisplatin and other platinum anticancer drugs are commonly used to treat a number of different metastatic diseases including ovarian, testicular, head and neck, breast, and bladder cancer. Although cisplatin and its second generation cousins (carboplatin, oxaliplatin, nedaplatin, satraplin) are now a gold standard for chemotherapy treatments, it is known that there are hurdles to overcome due to nephrotoxicity, cellular resistance, and deactivation of the drug by plasma proteins. In this study we introduce the construction of porous silicon (Si) microparticles that provides localized delivery of Pt compounds with the aim to provide an alternative treatment for intraperitoneal ovarian cancer. The reducing power of Si nanophased materials is used to demonstrate that upon incubation with cisplatin, the microparticles partially reduce the compound to metallic platinum, locking the drug in confined pores. Platinum metal is not known to show toxicity. We have found the toxicity of the drug loaded particles to be greater than free cisplatin therefore concluding that the drug is locally confined within the vicinity of the material, rather than being freely eluted to the surrounding media. This is advantageous because the drug is remains within the carrier until it reaches its target tumor tissue or cell, resulting in a potentially high local concentration and low systemic concentration. Unloaded particles exhibit no toxicity. Release of the drug into physiologically relevant media has been thoroughly tested to confirm the hypothesis of localized therapeutic delivery. A microparticle has been used in preference to the more common nanoparticle

because it can hold higher volumes of drug and will have a similar mass and flow pattern within the peritoneal cavity due to its larger size.

4.2 Introduction

Platinum anti-cancer drugs have been widely used to treat a number of different cancers including ovarian, testicular, lung, head and neck, and bladder.^[1] Platinum drugs putatively exhibit their potent activity by forming intrastrand or interstrand cross-links to N7 sites on purine base pairs, forming unwinding and bending of DNA, resulting in recognition by cellular proteins ultimately leading to apoptosis.^[2-5] Evidence suggests that cisplatin is activated to the DNA binding conformation by an intracellular aquation process through the loss of one of its chloride ligands forming a monoaqua molecule in the presence of low chloride environments.^[6] Cisplatin is the original of this class of compounds and remains a gold-standard for many treatments. Design of the next generation platinum drugs have been introduced to the clinic (carboplatin, oxaliplatin) or have entered FDA clinical trials (nedaplatin, picaplatin, and satraplatin) in an attempt to reduce nephrotoxicity or provide a means of oral delivery.

Another approach is to improve the therapeutic efficacy with simultaneous reduction of systemic toxicity by the design of effective drug carriers. The free drug is particularly susceptible to deactivation upon contact with serum proteins containing nucleophilic sulfur or oxygen rich groups.^[7] This hurdle could be overcome by shielding the therapeutic from the external environment by loading it within a drug carrier. This concept has been established clinically in such drug

delivery systems for doxorubicin (liposomal vehicle, Doxil®) and paclitaxel (albumin-based nanoparticle, Abraxane®).^[8, 9] Cisplatin delivery systems such as liposomal particle formulations (SPI-77, Lipoplatin) and hydroxypropylmethacrylamide (HPMA) co-polymers are in Phase II clinical trials.^[10-16] Other cisplatin drug delivery systems have been exploratory with polymers such as polylactic acid (PLA)^[17], poly(acrylic acid)-co-methyl methacrylate^[18], hyaluronic acid^[19], chitosan^[20], and gold nanoparticles.^[21] Mesoporous silica particles (MCM-41 and SBA-15) built up from bottom-up surfactant templated sol-gel synthesis methods in the range of 0.5-1 μm have been loaded with cisplatin.^[22] Not many studies exist for larger micron-scale particulates loaded with platinum therapeutic compounds. Mesoporous silicon, both electrochemically-etched and stain-etched (top down methods where pores are dug into non porous crystalline silicon particles or films) have demonstrated potential as a drug delivery material due to its tunable pore size^[23], high surface area^[24], facile access to surface modifications^[25], and the key parameter that it can be biodegradable^[26] and bioresorbable^[27-31] in-vitro and in-vivo.^[28, 32-36] Its first drug delivery applications demonstrated to delivery of insulin across Caco-3 cell monolayers^[37], loading and release dexamethasone by optical interferometry^[38], and delivery of doxorubicin from micro- and nano-particles.^[39, 40]

The initial discovery of cisplatin was accidental. During an experiment where they sought to measure the effect of electric potential on elongation of E.coli they found a platinum bi-product generated from the electrode and the Cl^-

electrolyte that formed a cytotoxic agent. Eventually this compound was found to have anti-tumor effects and is widely used in clinical trials for the treatment of various carcinomas. In this study we have used this chemistry to sacrifice cisplatin, by reducing it to metallic platinum in order to trap and concentrate the drug in the pores through the metallization reaction. Ogata et. al. demonstrated that porous Si can be a current-free inducer of metallization reactions.^[41] In this work, platinum was plated onto porous Si particles modeled by a nucleation reaction generated by a local cell current flowing from the anode (oxidation of porous Si) to the cathode (reduction of metal). It is interesting how the chemistry upon which the drug was initially discovered has come full circle, and we are now reversing it to thereby attempt to trap the drug into the pores for sustained drug delivery applications.

The reducing power of porous Si has demonstrated the ability to trap cisplatin into the pores by platinum metal. Platinum metal is not known to show toxicity and this has been verified in the results (**Figure 4.7**). The particles release cisplatin at expected rates in solution. However, the toxicity of the particles is much greater than the equivalent dose of free cisplatin indicating a local confinement effect. One advantage of containing drug within the particle is that the therapeutic remains in the particle until it reaches its target site, tumor tissue. Microparticle systems may have an advantage over nanoparticles because of potential for higher loading volumes. Perhaps one clinical idea would be to use these particles intraperitoneally as several clinical trials have demonstrated

improved efficacy of cisplatin IP compared with systemic IV delivery. To expound upon this design, a microparticle system is made to mimic the size of free floating ovarian cancer cells to treat early stage ovarian carcinomas where drug loaded particles can track the flow pattern of the cells. Additionally this can have potential applications where a targeting group can be applied and a toxic compound delivered to the site of interest.

4.3 Materials and Methods

4.3.1 Materials

Stain-etched porous Si microparticles with an average diameter of 4 μm were obtained from Vesta Ceramics Ltd. Cisplatin, platinum (hydrogen hexachloroplatinate (IV), 1 g/L Pt) and silicon (10,000 $\mu\text{g/ml}$ Si) ICP grade standards, sodium cyanide, hexane, undecylenic acid (99.9% purity), and sodium chloride were purchased from Sigma-Aldrich (St. Louis, M.O.). Phosphate buffered saline without Mg^{2+} and Ca^{2+} was obtained from Mediatech (Manassas, V.A.). Fetal bovine serum is from Gibco (Carlsbad, C.A.). AgNO_3 was supplied from Mallinckrodt (St. Louis, M.O.). Trichloroacetic acid and glacial acetic acid were purchased from Fisher Scientific (Pittsburg, P.A.).

4.3.2 Stain-Etched Microparticle Characterization

Immediately after the electrochemical etch and subsequent removal of the films from the crystalline silicon wafer, the particles were hydrosilylated with 1-dodecene. The particles were placed in a microwave safe vial, submerged in ~ 10 mL of 1-dodecene, and loosely capped with a rubber stopper to avoid pressure buildup during the procedure. The vial was placed in a commercial microwave oven (Sears Kenmore model #66319) located in a fume hood. The sample was heated by microwave on full power for a total of 10 min at 1 min intervals. After each 1 minute interval, the vial was removed from the microwave and pressure was released from the system. After hydrosilylation, the remaining 1-dodecene supernatant was decanted, the particles were rinsed extensively with

dichloromethane and ethanol and then dried in ambient atmosphere and temperature.

4.3.3 Surface Modification

Si-H terminated freshly-etched particles were thermally hydrosilylated with neat undecylenic acid. A batch of 50mg of particles were placed in a 10mL Pyrex beaker, immersed in 2ml of undecylenic acid (99.9%, Sigma-Aldrich), then subsequently heated in a commercial consumer microwave oven (Sears Kenmore 700W) for 4 x 1 minute intervals at 280W. The resulting conjugated particles were centrifuged, rinsed with hexane (3X), rinsed with ethanol (3X) to remove un-reacted reagent. Oxidized particles were prepared from S-H terminated freshly-etched particles by thermally oxidizing in tube furnace for 800°C for 2 hr. The particles were cooled to room temperature and stored in a desiccator.

4.3.4 Cisplatin and Hydrophobic Pt (IV) Pro-Drug Loading

Aquated cisplatin has been prepared by precipitating out labile chloride ligands from $\text{AgNO}_{3(\text{aq})}$ as $\text{AgCl}_{(\text{s})}$. Equimolar (7mM) solutions of $\text{AgNO}_{3(\text{aq})}$ and cisplatin were prepared, mixed together and allowed to equilibrate for 1h. A white, cloudy $\text{AgCl}_{(\text{s})}$ precipitate formed and was separated from the loading solution by centrifugation and filtration through a 0.1 μm Durapore® PVDF membrane sterile syringe filter. The resulting solution consists of an aquated cisplatin in the form of $\text{Pt}(\text{NH}_3)_2(\text{H}_2\text{O})_2$. Particles (75mg) were loaded with 20 ml of 3.5 mM aquated cisplatin loading buffer. The non-toxic analogue, transplatin,

was loaded into the particles using the same procedure. The procedure for synthesis and preparation of a platinum (IV) pro-drug compound $[\text{Pt}((\text{NH}_3)_2(\text{Cl})_2)(\text{CH}_2)_9\text{COOH}]^{4+}$ was obtained from.^[45-47] The ligands are conjugated to the axial positions of cisplatin. Cisplatin (Sigma-Aldrich) (200mg) was allowed to react with 7mL (30% peroxides), 5 ml water, for 50°C for 1 h. The resultant solution is chilled, run through a Whatman #2 filter by vacuum filtration, and the remaining sample is rinsed with cold water, ethanol, then dried. The compound $[\text{Pt}(\text{NH}_3)_2\text{Cl}_2(\text{OH})_2]$ is reacted with DMSO and 2 molar equivalents of decanoic anhydride for 48 h at room temperature. The resulting compound is crashed out of solution with cold H_2O , filtered, and rinsed with ethanol, and diethyl ether. The mass of the resulting compound was analyzed by HPLC-MS (**Figure 4.8**).

Hydrosilylated particles (75mg) are mixed with hydrophobic pro-drug (20ml, 2.5mM) and the solvent was evaporated off. In the second set, the particle-drug solution was capped off and loaded for 48 h at 37°C, and then rinsed 3X with chloroform and fully dried.

4.3.5 Platinum Loading Characterization

The drug loading was measured by two separate methods. Firstly, amount of Pt in the initial loading buffer and after a 48 hr loading period is quantified. The second method uses a 1M KOH solution at a particle concentration of (1mg/ml) to dissolve the total drug out of the particles. The sample characterization is done by correlating the intensity of Pt emission at wavelength

214 nm by inductively coupled plasma optical emission spectroscopy (ICP-OES) and fitting it to intensity vs. concentration calibration curve. The samples are diluted to 3% (v/v) nitric acid and measured in triplicate.

4.3.6 In-Vitro Drug Release

The drug loaded particles release their drug content in three different physiological buffers (PBS, hypertonic PBS, and fetal bovine serum) by placing 0.5 mg/ml particles in solution. The particles released their drug contents over a period of 192 hr in a 37°C incubator. At 192 hrs, the solution was separated from the particles by centrifugation, passed through a 0.1 µm Millipore syringe filter, and contents analyzed for Pt emission at wavelength 214nm by inductively coupled plasma optical emission spectrometer (Perkin Elmer, Optima 3700DV). The samples are diluted to 3% (v/v) nitric acid and measured in triplicate. Particles were aliquoted (0.5mg) into each 3,500 MWCO dialysis tube in triplicate. The tubes were placed in a Styrofoam floater and stirred with a magnetic stir-bar in 300mL of fetal bovine serum. At each time point, particles in the dialysis tubes were collected, dissolved in 1M KOH and measured for Pt¹⁹⁵ content using inductively coupled plasma mass spectrometry by using a 1ppb In¹¹⁵ internal standard, and 3% (v/v) nitric acid. The total platinum that was released at each time point was collected over time.

4.3.7 Cellular Toxicity with Cisplatin-loaded Microparticles

Drug loaded microparticles are assayed for cellular toxicity using sulforhodamine B cellular viability assay. The 2008 human ovarian cancer cell

line was grown in RPMI 1640 media with 10% heat inactivated fetal bovine serum at 37°C in 5% CO₂. Media was obtained from Thermo Fisher. Sensitivity of the cell line to drug loaded particles was determined by seeding 5,000 cells per well into 96-well plates. After 24 hr, increasing concentrations of particles were added to the media and cells were allowed to grow for five days. Particles were thoroughly rinsed from the plate with PBS. The surviving cells were then fixed using 50% trichloroacetic acid for 1 hr at 4°C and stained using 0.4% sulforhodamine B dye. After rinsing the unbound dye with 1% acetic acid, bound dye is solubilized using 10mM Tris-HCl and read at 515 nm on a Versamax absorbance microplate reader (Molecular Devices). The optical density data is process as reported in other literature.^[48]

4.4 Results and Discussion

4.4.1 Preparation of Stain-etched Microparticles

Porous Si microparticles can be prepared from either an electrochemical-etch by applying a current to an ethanolic hydrofluoric acid etchant or by stain-etching with a combination of HF and HNO₃ with no bias. The synthesis has been described in the methods section. In a previous study we have determined the surface area of the porous Si microparticles to be 166 +/- 3.73 m²/gram by B.E.T. nitrogen adsorption. The particles have an average pore width of 7 nm and a mean porosity of 29.9 +/- 1.1% Scanning electron microscopy indicates an average particle diameter of 4 µm.

4.4.2 Surface Functionalization for Drug Loading

Ease of surface functionalization by access to modifiable sites is one benefit to the use of porous Si as a biomaterial for drug delivery. Immediately after etching, the particles have a surface primarily composed of water repellent Si-H groups. Exposure to oxygen in ambient air oxidizes the Si-H porous Si surface to O-Si-O in the order of tens of minutes.^[25, 42] In this study we aim to modify the surface with two chemistries: hydrosilylation with undecylenic acid and thermal oxidation to SiO₂, to test for loading efficiency and achieve a sustained release profile.

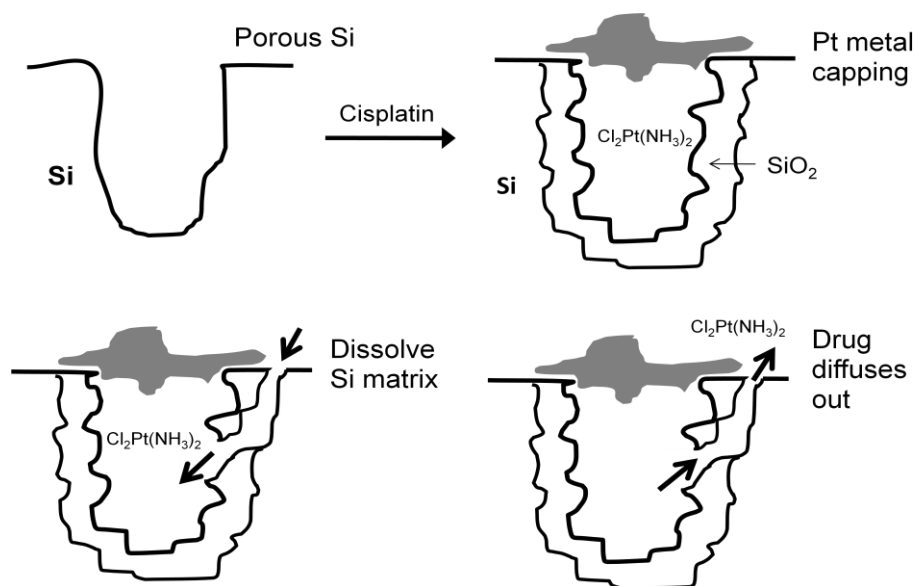
Hydrosilylation with undecylenic acid terminates the surface with carboxylic acid groups. Yan X.L. and Gemeinhart R.A. proposed aquated cisplatin could covalently bind to the surface of carboxyl groups on a poly(acrylic acid-co-methyl methacrylate) microparticle.^[18] We attempted to explore this mechanism of attachment by first forming aquated cisplatin by removal of the chlorine groups with AgNO₃ induced precipitation reaction to form insoluble AgCl_(s).^[43-44] Carboxylic acid terminated microparticles were incubated with 3.5mM cisplatin in water to infiltrate the drug. We found that very little platinum was present in solution after a 48 hour loading period at 37°C. Upon performing similar loading experiments with particles surface modified with 1-dodecene (non-carboxylic acid terminated), a similar amount of platinum was deposited on the particle. In conclusion, a large amount of platinum was deposited during the

drug loading procedure, but the mechanism of loading was not dominantly due to covalent attachment of cisplatin to carboxyl chains.

Secondly, an oxidized surface was formed by thermally oxidizing porous Si at 800°C in a tube furnace for 2 hours. This procedure fully oxidizes the surface throughout the 4 µm particle diameter. Aquated cisplatin was loaded with the identical procedure as the first surface modification. It is hypothesized that the net positively charged drug may adhere by electrostatic attraction to the net negatively charged silica surface. A much lower quantity of total platinum was deposited on the particle compared with 1-dodecene and undecylenic acid modified surfaces. Later it will be explained how this is not necessarily indicative of poor drug loading.

Finally, it has been the strategy of several groups to improve the therapeutic delivery and efficacy of Pt anti-cancer drugs by synthesizing Pt (IV) pro-drug compounds that can be tethered with pendant molecules and can be easily conjugated with surfaces.^[45-46] For example, one group has demonstrated that a Pt (IV) compound can be concentrated into a gold nanoparticle by covalent attachment.^[21] The synthesis of a Pt (IV) pro-drug $[\text{Pt}((\text{NH}_3)_2(\text{Cl})_2)(\text{CH}_2)_9\text{COOH}]^{4+}$ is described in the methods section. A hydrophobic surface such as undecylenic acid may be more amenable to loading with a drug that is primarily hydrophobic in structure. The structure of the molecule has been verified by HPLC-MS (**Figure 4.8**). This compound is known

to reduce down to cisplatin in reducing environments such as the cell cytoplasm. The drug is suspended in chloroform and then submerged with the particles for 48 hours. Drug loaded particles were rinsed three times with 10ml chloroform and dried before analysis. A schematic diagram for the mechanism of cisplatin loading onto porous Si microparticles is depicted in **(Scheme 4.1)**.



Scheme 4.1 Cisplatin has been loaded into surface-modified stain-etched porous Si microparticles have been modified with undecylenic acid. Aqueated cisplatin is then loaded onto the microparticles for 48 hours at 37°C in an aqueous loading buffer. The drug undergoes reductive chemistry from cisplatin to metallic Pt while porous Si surface Si-H oxidizes to SiO_2 . The silicon matrix dissolves releasing the trapped drug from the matrix.

4.4.3 Drug Loading

Two methods were utilized to quantify drug loading. In the first method, the concentration of platinum was quantified in the loading buffer after a 48 hour loading period at 37°C and compared to the initial concentration at time zero. The Pt removed from solution was correlated to the amount of platinum deposited on the microparticles. In the second method, the particles were degraded in a 1M KOH base (pH 14) at room temperature. It is feasible that if the efficiency of hydrosilylation is very high, there could be no oxidation or degradation in a boiling solution at pH 13.^[25] The amount of platinum trapped in the particles and released due to degradation has been quantified. Initially we expected the loading values from the two methods to be identical. Rather, we found that cisplatin undergoes a reaction upon which large quantities of drug deposit on the particle surface and is unable to be released. The total mass of drug released per mass of particle by the two methods of quantification has been reported in **(Table 4.1)**.

Table 4.1. Drug Loading for Aquated Cisplatin, Pt(IV) pro-drug $[\text{Pt}((\text{NH}_3)_2(\text{Cl})_2)(\text{CH}_2)_9\text{COOH}]^{4+}$ into various surface chemistries including undecylenic acid, 1-dodecene, and porous SiO_2 . Drug release has been characterized by two different methods. The first method involved measuring the concentration of the loading buffer before and after incubation with the particles. The second method uses 1M KOH to degrade the remaining cisplatin off of the particles. Drug loading efficiency is improved by altering the surface chemistry which affects the hydrophilicity of the particle.

Therapeutic	Surface Chemistry	Platinum Loading [$\mu\text{g}/\text{mg}$]	1M KOH degradation [$\mu\text{g}/\text{mg}$]
Cisplatin	Undecylenic Acid	231.3 +/- 0.2	1.48 +/- 0.79
Cisplatin	1-Dodecene	241.3 +/- 0.0	0.062 +/- 0.022
Cisplatin	Porous SiO_2	0.680 +/- 0.253	0.169 +/- 0.0079
Transplatin	Undecylenic Acid	267 +/- 0.15	1.003 +/- 0.45
Pt(IV) pro-drug (Evaporation)	Undecylenic Acid	N/A	17.83 +/- 1.713
Pt(IV) pro-drug (Capped)	Undecylenic Acid	5.292 +/- 0.054	4.401 +/- 0.038
Pt(IV) pro-drug (Capped)	1-Dodecene	11.107 +/- 0.039	0.669 +/- 0.014

The first drug, aquated cisplatin, has been loaded into the particles modified with three different surface chemistries. The first surface is modified with undecylenic acid to result in $\text{Si}-(\text{CH}_2)_{10}\text{COOH}$. The second surface has been modified with 1-dodecene to yield $\text{Si}-(\text{CH}_2)_{11}\text{CH}_3$. The third is thermally oxidized to yield a surface primarily composed of $-\text{SiO}_2$. According to the first analytical method by assaying for the amount of total platinum deposited on the particles during the loading procedure, we found that loading was significant and similar for undecylenic acid ($231.3 \pm 0.2 \mu\text{g/mg}$) and 1-dodecene particles ($241.3 \pm 0.0 \mu\text{g/mg}$). The porous silica surface yielded only $0.680 \pm 0.253 \text{ mg/mg}$ platinum loading. Of the amount deposited on the particle, after complete degradation in 1M KOH the platinum was extracted and analyzed for the undecylenic acid ($1.48 \pm 0.79 \mu\text{g/mg}$), 1-dodecene ($0.062 \pm 0.022 \mu\text{g/mg}$), and porous silica ($0.169 \pm 0.0079 \mu\text{g/mg}$) surfaces.

It is likely that undecylenic acid and 1-dodecene modified particles undergo similar deposition chemistry. Details of the deposition chemistry will be explained later. We can note that by KOH degradation, more cisplatin was extracted from the undecylenic acid modified particles. In a supplementary experiment, both surface modified particles were submerged in aqueous buffer. It was found that the 1-dodecene particles were quite resistant to water penetration and remained floating on the air-water interface. On the flipside, undecylenic acid modified particles absorbed water more quickly and sank to the bottom of the tube (data not included). From this conclusion we can determine that the carboxyl

group on the surface of undecylenic acid particles increases hydrophilicity and facilitates improved drug loading.

The second drug, $[\text{Pt} ((\text{NH}_3)_2(\text{Cl})_2)(\text{CH}_2)_9\text{COOH}]^{4+}$ has been loaded into undecylenic acid modified surface. At first it was loaded into the pores with a chloroform solvent, the solvent evaporated, and rinsed three times with chloroform. In order to make a direct comparison to relative amounts loaded with aquated cisplatin, the particle solution was capped for 48 hours (with same drug and particles), then rinsed three times with chloroform. Due to the hydrophobicity of the surface, there was achieved a higher loading (4.401 ± 0.038) compared with the aquated cisplatin counterpart (1.48 ± 0.79). Thirdly, the platinum pro-drug was loaded in the hydrophobic 1-dodecene surface with 10% oxidizing agent H_2O to achieve loading of (0.669 ± 0.014). The hydrophobic drug does not reduce to metallic platinum without the presence of H_2O .

4.4.4 Discussion of Reactivity of Porous Silicon

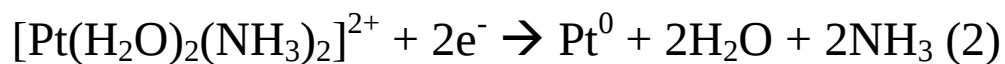
Cisplatin presents a unique challenge in that its working range of concentrations often borders the limits of detection of various analytical techniques whether o-phenylenediamine (OPDA) is bound to cisplatin and measured by UV-VIS spectrophotometric detection or inductively coupled plasma optical emission spectroscopy (ICP-OES) is used. The molecule itself absorbs in the UV-VIS spectrum with a peak maximum at 304nm ^[41-43] but is limited by a rather low molar adsorptivity. The benefit to porous silicon materials is its versatile access to chemical functionalization, but it also poses challenges in that

the surface is highly tenable to reduction of various molecules making it incompatible for use with tests that rely on a reduction pathway for single detection. One such common example of a cellular assay that interferes with porous silicon is the MTT cell viability assay which depends on the reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide to formazan. However, in this study we explore the use of the reactive surface for drug loading applications.

4.4.5 Platinum Reduction Mechanism

Porous silicon particles were analyzed for elemental platinum by scanning electron microscopy (**Figure 4.1**). The overall particle size distribution and deposition of the platinum on the particle surface has been demonstrated. A high resolution micrograph shows the relative pore morphology and size (6-8 nm) compared to the platinum nodules (10-200 nm). Energy dispersive x-ray analysis maps the distribution of elemental silicon and platinum. The images indicate that platinum nodules are large enough to occlude the flow of fluid in and out of the pores.

In the process of drug loading, the drug gets reduced from cisplatin to Pt^0 in conjugation with the process of silicon being oxidized from Si to SiO_2 (**Equation 4.1**).

Equation 4.1:

The metallic form of Pt partially caps off the pores, effectuating the trapping of active drug inside the matrix. As the silicon structure dissolves, the drug will diffuse out. Porous silicon will oxidize in aqueous and oxygen-rich environments and the platinum will thereby be reduced. The reduction reaction can occur for other metals such as Cu and Ag and has been demonstrated in other studies.^[41] In this work, there is evidence that the drug does not get entirely transformed into the metallic form and some remains as toxic cisplatin and remains trapped within the pores.

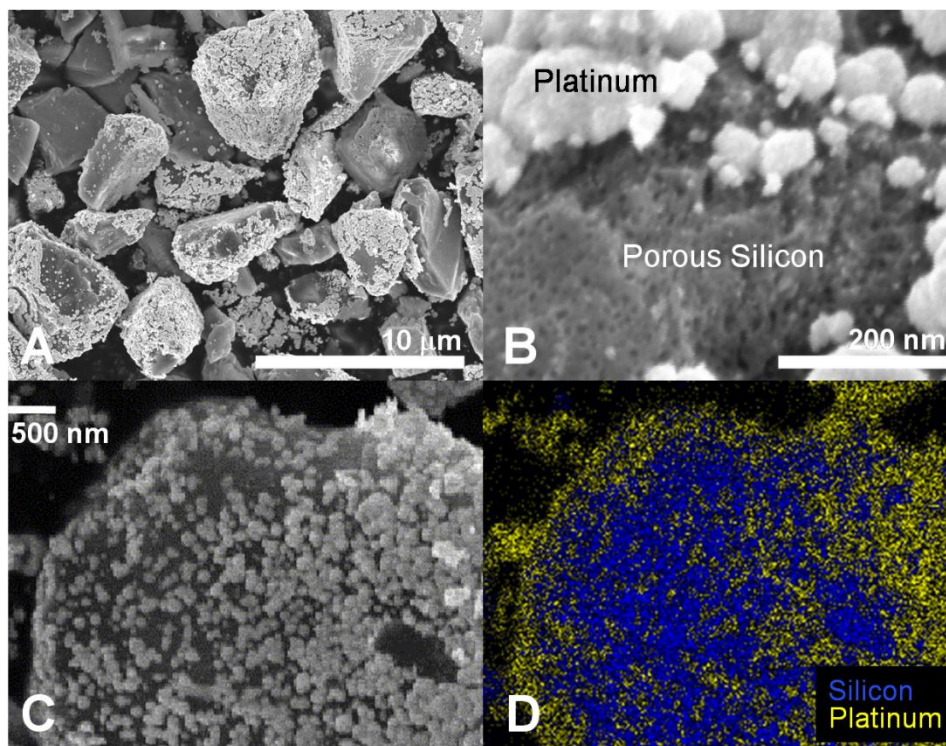


Figure 4.1 Porous silicon microparticles are analyzed for elemental platinum by scanning electron microscopy and energy dispersive x-ray. (A) The overall particle size range and distribution of overall platinum nodules on the surface are shown. (B) The pore distribution is shown in comparison to the nodule size. (C-D) An SEM image of the overall platinum distribution and the elemental dot-map scan indicates both Pt and Si are present.

XPS analysis of cisplatin loaded samples, transplatin loaded samples, and a non-drug loaded sample show that both of the platinum loaded samples contain elemental platinum while the non-drug loaded control shows no level of platinum present. The other elements that were expected to be shown are also present (N, O, Si, and C). The survey spectrum of the cisplatin particles indicates the position at which each elemental peak appears as well as the relative peak area that can be converted to atomic mass %. Hi-resolution spectrum of cisplatin particles in the 4f region shows two peaks: Pt 4f_{7/2} at 71.1 eV and Pt 4f_{5/2} at 74.4 eV (**Figure 4.6**). The peak energy of the Pt 4f_{7/2} signal of 71.1 eV is in the accepted range for a Pt⁰ compound (70.8-71.4 eV), but is below the energy for an oxidized Pt species, Pt²⁺ (> 72 eV). Further analysis has been done to show that although there is not a comparatively high level of Pt²⁺ (cisplatin); according to the detection limits of the instrument (0.1 atom %) there could still be cisplatin present. In addition the sampling depth for XPS is limited to 6-8 nm.

4.4.6 Drug Release Characterization

Platinum drug loaded particles were released in three physiologically relevant buffer solutions over a 192 hour release period (**Figure 4.2**). Phosphate buffered saline (no Ca²⁺ or Mg²⁺) was used as a buffer that mimics the pH conditions and neutral saline concentrations (0.9% w/v). Hypertonic saline at (5% w/v) NaCl_(aq) was used to mimic an environment with high chloride concentrations to see if the release of drug could be triggered by displacement of the hydroxyl groups with chlorine moieties. Results show that hypertonic solution

does increase the release of drug, but the highest release was induced by fetal bovine serum. The healthy peritoneal cavity of a human contains ~50 ml of serous fluid for lubrication. In the diseased state, the cavity can be filled with excess fluid, a condition known as ascites. Fetal bovine serum likely degrades the particles more rapidly by method of enzymatic activity.

By a period of 15 hours ~ 40% of aquated cisplatin is released from undecylenic acid functionalized particles by fetal bovine serum. The data was acquired by dissolving the amount of platinum left on particles that were suspended in dialysis tubes in a large bath of fetal bovine serum. Each data point was taken from a single dialysis tube and analyzed in triplicate. The drug released was calculated by subtracting the initial drug content from the amount remaining on the particle. The drug was measured by analyzing the samples with inductively coupled plasma mass spectrometry for total elemental platinum content. Similar traces with hydrophobic Pt (IV) pro-drug shows that very little drug gets released over the same period (<10%) which indicates that hydrophobic drugs get confined within the particles and precludes the drug from being released into the aqueous environment.

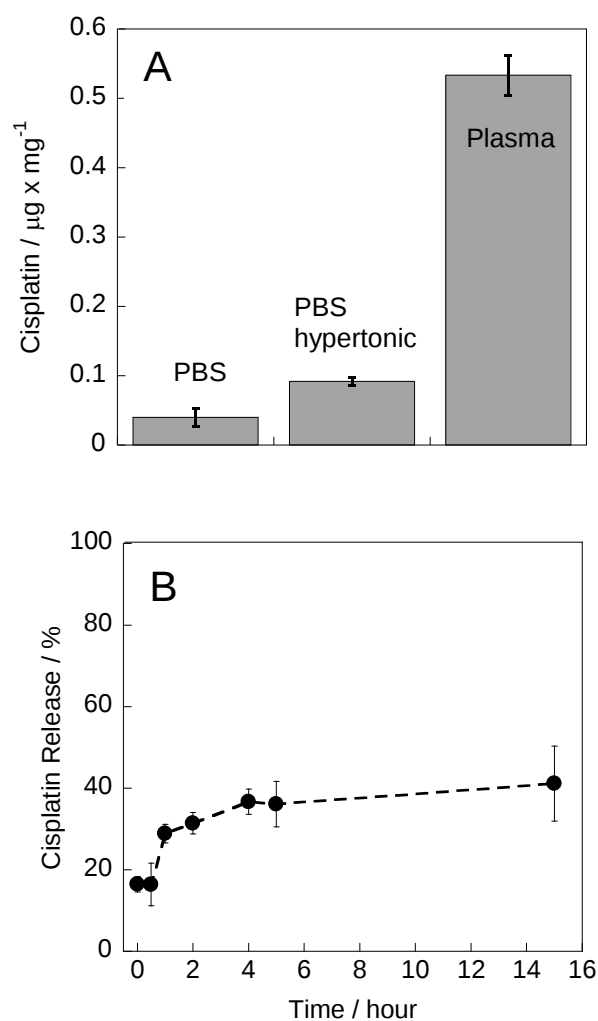


Figure 4.2 (A) Comparison of 192 hour drug release in phosphate buffered saline (0.9% w/v), hypertonic saline (5% w/v) and fetal bovine serum physiological buffers. Undecylenic acid surface modified particles loaded with aquated cisplatin were analyzed for total drug released into solution and expressed as the amount of drug per mass of particle ($\mu\text{g}/\text{mg}$). (B) These same particles are released in fetal bovine serum over a shorter time course (15 h) to determine their time-dependent release characteristics.

4.4.7 Localized Delivery of Cisplatin-loaded Microparticles to Human Ovarian Cancer Cells

Cellular toxicity of 2008 human ovarian cancer cells with porous Si particles has been assayed using sulforhodamine B viability assay with free cisplatin, free transplatin, empty porous Si particles, transplatin loaded porous Si microparticles, and cisplatin loaded microparticles (**Figure 4.3**). A range of toxicities are shown up to 5 μg dose for cisplatin and transplatin in (**Figure 4.6**). Free transplatin and the equivalent dose of empty porous Si particles (data not shown) result in low toxicity (>80% survival) with 48 μM of drug dosed. Transplatin loaded particles demonstrate moderate toxicity which remains fairly level across the board between 8 μM and 48 μM of drug concentration. Free cisplatin exhibits higher toxicity with an $\text{IC}_{50} \sim 33\mu\text{M}$ and < 40% survival with 48 μM of drug dosed. Cisplatin loaded particles exhibit the highest level of toxicity. 1-dodecene conjugated, aquated cisplatin loaded particles have an $\text{IC}_{50} \sim 0.018$ μg cisplatin. Undecylenic acid conjugated, aquated cisplatin loaded particles have an $\text{IC}_{50} \sim 0.006$ μg cisplatin. Porous silica particles loaded with aquated cisplatin have an $\text{IC}_{50} \sim 0.004$ μg cisplatin.

Compared to free cisplatin and transplatin, the particles exhibit a higher toxicity. This result seems counter-intuitive because if the particles with the same amount of drug (as free drug) have been loaded, the toxicity should be equal if not lower. The maximum amount of drug that can possibly leach out should be equal

to the toxicity of the free drug. However, in the case of our studies, we have seen a much higher toxic effect with particles that contain drug than free drug.

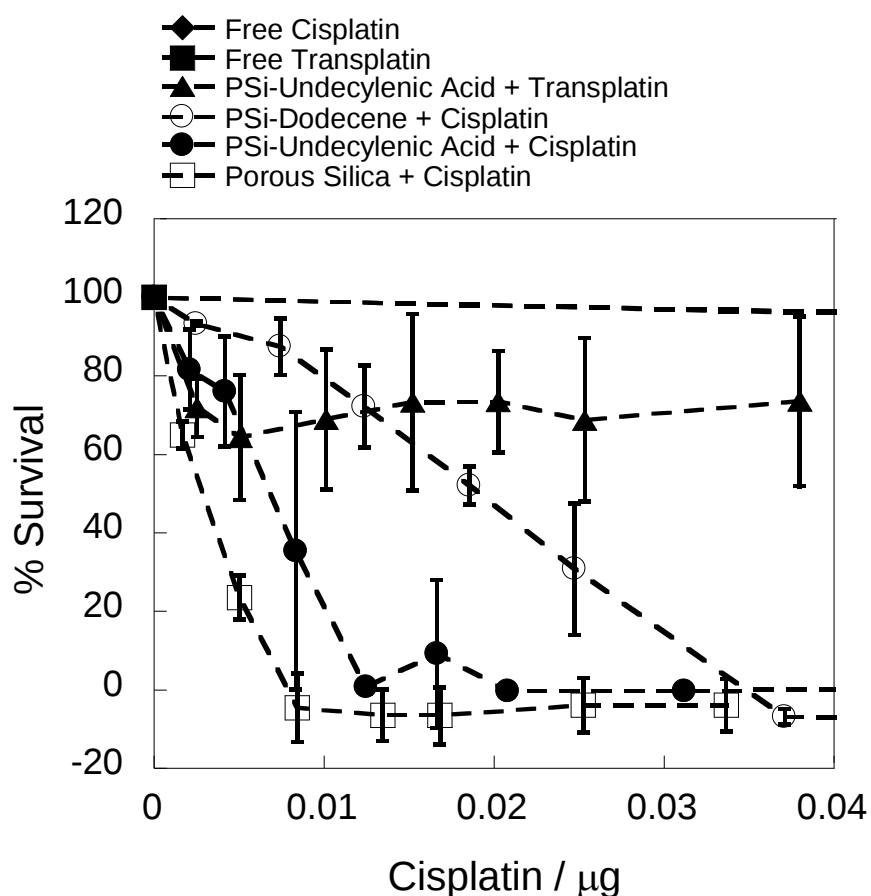


Figure 4.3 Cellular toxicity of ovarian cancer cells with cisplatin loaded porous Si microparticles has been tested with sulforhodamine B viability assay over a growth period of 5 days. Surface modified particles [undecylenic acid (solid triangle), dodecene (open circle), porous silica (open squares)] have been loaded with aquated cisplatin and compared against free cisplatin (solid diamond), free transplatin (solid square), and control particles modified with undecylenic acid and loaded with transplatin (solid triangle). The results show a higher effective toxicity with drug loaded particles in comparison to free drug and control particles.

We propose the mechanism to be a localized delivery effect. The microparticles retain the drug within its vicinity rather than preferring to release the drug and diffuse it throughout the cell culture well. If the drug were diffused throughout the well, it could in effect have toxicity equal to that of free drug. In our case we can assume the particles to retain toxicity to within 4-10 nm film in immediate proximity to the cells. Therefore the drug is highly concentrated and the dose that affects the cells is quite high. A potential benefit to this application is that drugs can be concentrated within the particle and remain there until delivery towards the site of interest. The next step would be to target these particles with tumor targeting ligand to specifically home them towards cancer cells.

Hydrophobic pro-drug $[\text{Pt}((\text{NH}_3)_2(\text{Cl})_2)(\text{CH}_2)_9\text{COOH}]^{4+}$ has also been tested for cytotoxicity with 2008 human ovarian cancer cells (**Figure 4.4**). Free drug was determined to have an IC₅₀ of $\sim 0.06 \mu\text{g}$ drug. The drug loaded particles exhibit a much lower toxicity which is consistent with the release data discussed previously.

Toxicity of 0.5-1 μm solid platinum particles were obtained from Sigma-Aldrich chemicals and assayed with the same method with sulforhodamine B viability assay (**Figure 4.7**).

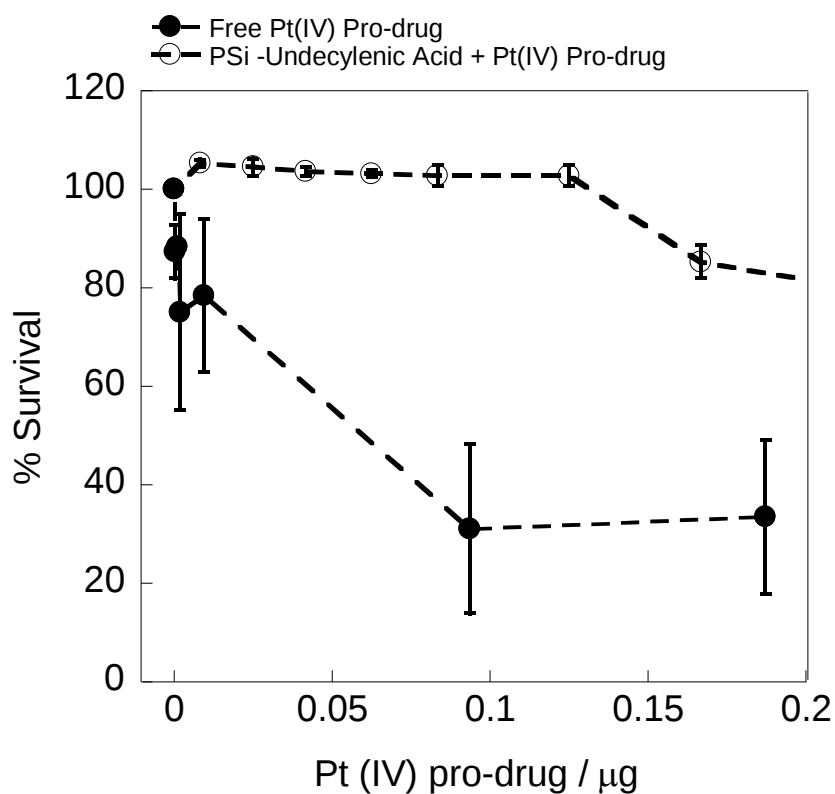


Figure 4.4 Hydrophobic Pt(IV) pro-drug $[\text{Pt}((\text{NH}_3)_2(\text{Cl})_2)(\text{CH}_2)_9\text{COOH}]^{4+}$ is loaded in undecylenic acid modified microparticles and the free drug has been tested for cytotoxicity on 2008 human ovarian cancer cells with the sulforhodamine B viability assay.

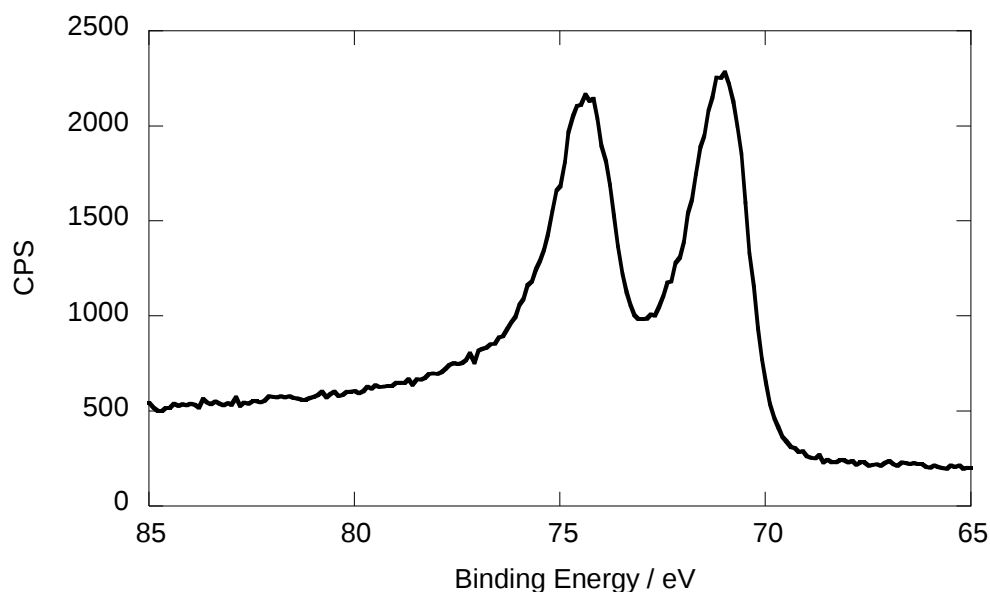


Figure 4.5 High-resolution spectrum of drug loaded microparticle sample of the Pt 4f region. Graph depicts total intensity (CPS) versus binding energy (eV). The microparticles have been surface conjugated with undecylenic acid and loaded with an aquated cisplatin drug. The spectrum in the 4f region shows two peaks: Pt 4f_{7/2} at 71.1 eV and Pt 4f_{5/2} at 74.4 eV. The peak energy of the Pt 4f_{7/2} signal of 71.1 eV is in the accepted range for a Pt⁰ compound (70.8-71.4 eV), but is below the energy for an oxidized Pt species, Pt²⁺ (> 72 eV). Further analysis has been done to show that although there is not a comparatively high level of Pt²⁺; according to the detection limits of the instrument (0.1 atom %) there could still be Pt²⁺ present.

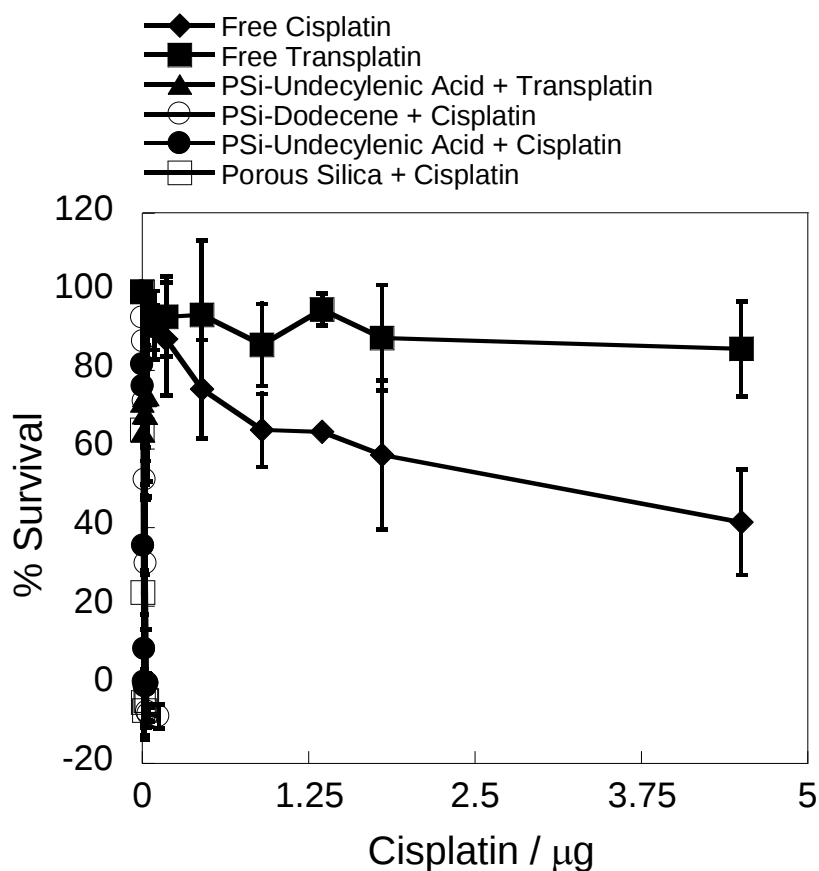


Figure 4.6 Cellular toxicity of ovarian cancer cells with cisplatin loaded porous Si microparticles has been tested with sulforhodamine B viability assay over a growth period of 5 days expanded to 5 μg dose region. Surface modified particles [undecylenic acid (solid triangle), dodecene (open circle), porous silica (open squares)] have been loaded with aquated cisplatin and compared against free cisplatin (solid diamond), free transplatin (solid square), and control particles modified with undecylenic acid and loaded with transplatin (solid triangle). The results show a higher effective toxicity with drug loaded particles in comparison to free drug and control particles.

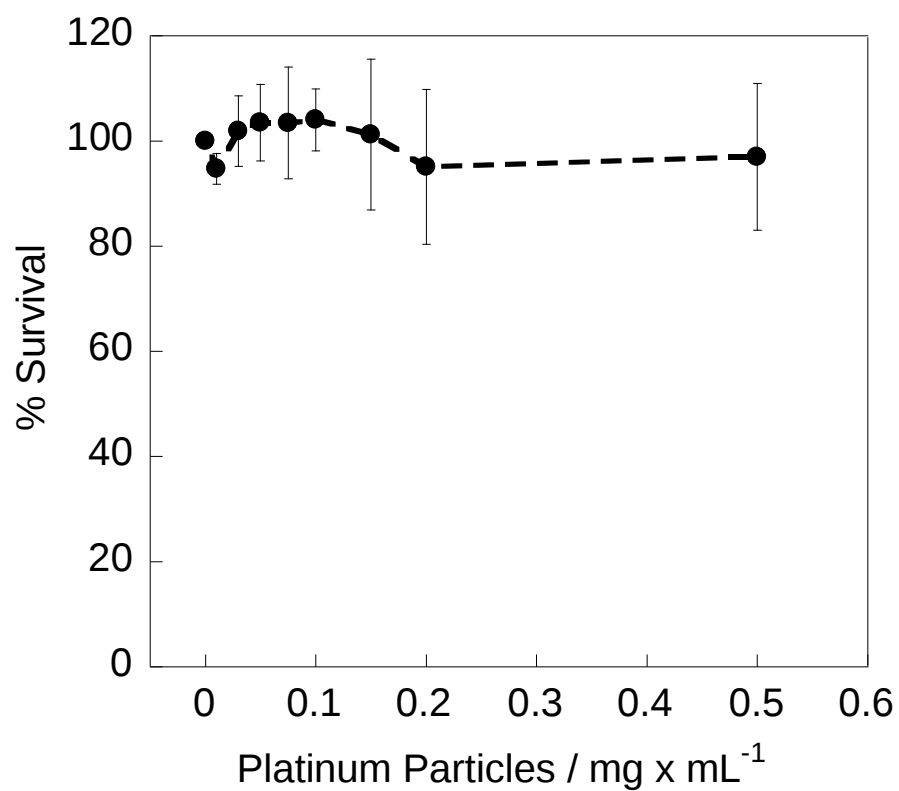
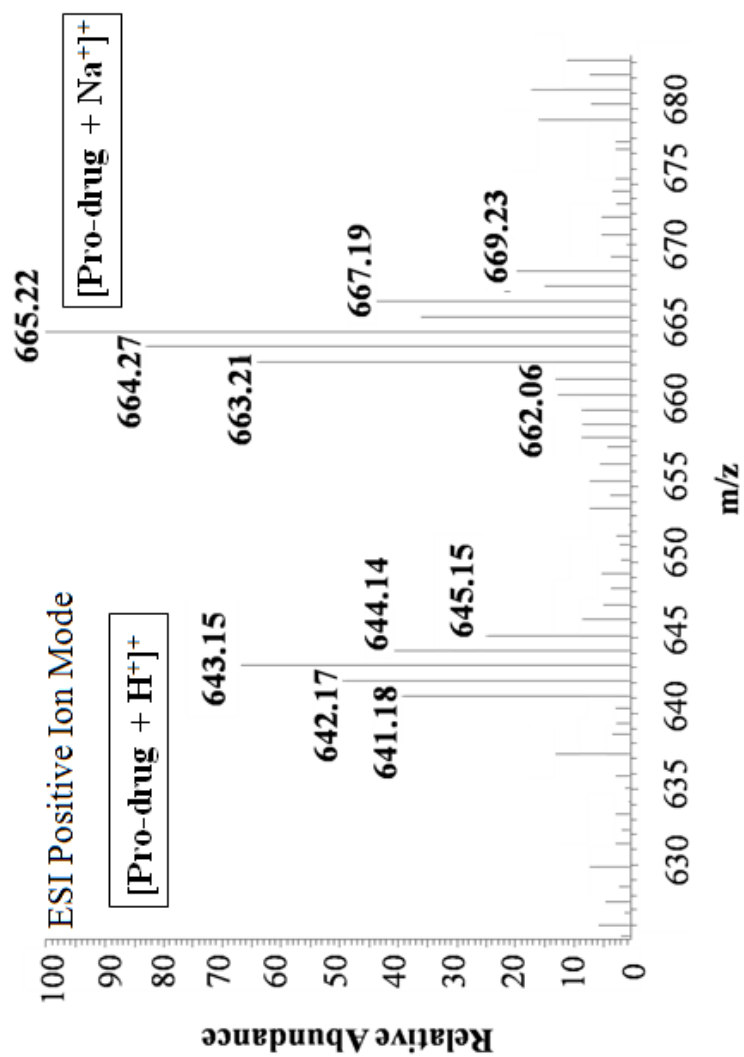


Figure 4.7 Cellular toxicity on 2008 human ovarian cancer cells with solid platinum particles 0.5-1 μm . Up to a concentration of 0.5 mg/ml (150 μg particles) there is no toxicity.

Figure 4.8 HPLC-MS of hydrophobic Pt(IV) pro-drug, $[\text{Pt}((\text{NH}_3)_2(\text{Cl})_2)(\text{CH}_2)_9\text{COOH}]^{4+}$ was measured in positive ion mode in a (1:1) methanol and water mobile phase on a C18 column (2mm I.D., 100mm length) with a retention time of 2.12-2.19 min.



4.5 Conclusions

Porous Si microparticles are loaded with an anticancer drug, cisplatin, via a method that traps the drug into the pores using the inherent reactive properties of the material. Freshly-etched porous Si terminated with Si-H functionality undergoes oxidation to SiO₂ in aqueous environments. This generates a local cell current that promotes the reduction of cisplatin to metallic platinum, trapping the drug in the pores. Cellular toxicity indicates that particles with an equivalent concentration of free drug is more toxic, indicating that drug is confined to the particle providing a means for local delivery to cancer cells. The drug loading results indicate that chemically modifying the hydrophobicity-hydrophilicity of surfaces incorporate higher concentrations of drug.

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Chapter Five

LYP-1 CONJUGATION TO POROUS SI MICROPARTICLES

5.1 Abstract

Platinum or gold – thiol bonds are well known for their robust chemical bond strength. In tumor treatment studies targeted particle-based drug delivery systems have demonstrated positive anti-cancer effects in animal models. One methodology is to isolate short peptide sequences that can bind to receptors over-expressed in tumor tissues. In this study we propose to demonstrate a method to attach LyP-1 targeting peptide directly on the surface of a platinum metal – coated, drug loaded porous Si microparticle surface by thiol-platinum chemistry.

5.2 Introduction

In previous studies, the Ruoslahti laboratory has used a combination of ex-vivo and in-vivo bacteriophage display procedures to screen for peptide sequences that recognize over-expressed motifs on specific tissues. Through this methodology, in 2002 Laakonen and co-workers discovered that multiple copies of a phage that displayed the circular peptide sequence, CGNKRTRGC (LyP-1) presented itself on MDA-MB-435 xenograft tumors.^[1] It was found that the binding of LyP-1 was not universal for all tumor types. It bound positively to MDA-MB-435 melanoma, KRIB osteosarcoma, and transgenic mouse prostate and breast carcinomas, but did not bind to HL-60 leukemia or C8161 melanoma xenografts. It was suggested that the LyP-1 was being targeted to lymphatic endothelial cell markers. Some of the peptides internalized into the nuclei of the

vessels stained positive for lymphatic markers, LYVE-1, podoplanin, and VEGFR-3.

The second paper targeted LyP-1 by i.v. injection in orthotopic MDA-MB_435 melanoma tumors and found accumulation of the fluorescently tagged peptide within the tumor. ^[2] They hypothesized that the lymphatics are a common route for metastasis and therefore lymphatic markers as mentioned previously, that have been found in co-location with LyP-1 are highly prevalent near tumor tissues.

In 2008 Fogal discovered the receptor for LyP-1 to be a protein called p32. ^[3] Many human tumor cultured cells over-express p32. When p32 antibodies were used, they emphasized the presence of the receptor in hypoxic areas of the tumor – thereby possibly indicating that stress induced tissues express this peptide more prevalently. Upon its initial discovery, it was purified along with nuclear splicing factor SF-2. ^[4] It was shown to also bind C1q and was thus named the gC1q receptor. ^[5] However, it has been found since then that a number of extracellular and intracellular proteins bind to p32 indicating that its sole match is not singly LyP-1. ^[6-7] The peptide is a doughnut shaped trimer ^[8] and can be present in the mitochondria, cell surface or the nucleus. The role of the peptide is still undefined. **(Figure 5.1)** is from reference [8] and is a depiction of the structure of p32 based on the protein crystal structure.

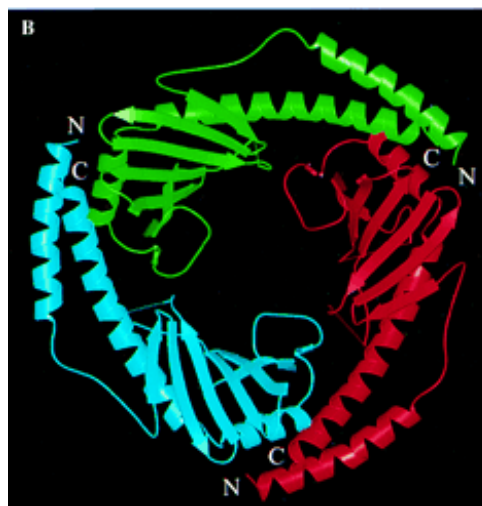


Figure 5.1 Image of receptor to Lyp-1 peptide, p32, based on the crystal structure determined from the reference [8].

Then, Akerman et al. synthesized quantum dot nanocrystals to target three different types of peptides: the first, CGFECVRQCPERC peptide (or GFE) that binds to membrane dipeptidase on the endothelial cells in lung blood vessels; KDEPQRRSARLSAKPAPPKPEPKPKKAPAKK (F3) which prefers to bind to tumor cells and blood vessels; and LyP-1 peptide.^[9] Their idea was that nanoparticles can be used either as a diagnostic imaging device or to deliver therapeutics. ZnS- capped CdSe Q-dots were used and it was shown that the GFE peptide-bound particles accumulated in the lungs, F3 to blood vessels, and LyP-1 to lymphatic tissues. However, the F3 and Lyp-1 peptide-bound Q-dots did not accumulate in the tumor tissue possibly due to the larger size of the particles that could not penetrate. However, these results have shown that it is possible to selectively target inorganic nanomaterials to a specific site, which is of relevance to this chapter.

There is some evidence from previous studies in the Ruoslahti laboratory that LyP-1 peptide binds more strongly to certain types of ovarian cancer cells over others. The list of ovarian cancer cells tested was: 2008, A2780, IGROV-1, SKOV-3, OVCAR-3, and HEY. The targeting peptides tested for binding were: LyP-1, F3, CGKRR, Bld-1, REA, ARAL, and three targeting peptides cited in the literature for binding to ovarian cancer cells from the Lam group at UC Davis.^[10] It was found that LyP-1 (and REA) bound most strongly to A2780 ovarian cancer cell line and fairly to 2008 cells.

Table 5.1 Binding of peptides to cells: +++++ strong, ++ good, + fair, +/- weak, - negative. Peptide resources: (a) peptides from Dr. Erkki Ruoslahti laboratory; (b) ovarian cancer binding sequences from literature. ^[10] This data was acquired not by the author, but by Dr. Lianglin Zhang of the UCSD Moores Cancer Center.

	2008	A2780	IGROV-1	SKOV3	OVCAR3	HEY
FITC-LyP-1 (a)	+	+++++	++	++	-/+	+
FITC-F3 (a)	-	+	-/+	-	-	-/+
FITC-CGKRK (a)	-	+	-	-	-	-/+
FITC-Bld-1 (a)	-/+	-	-	-	-	-
FITC-REA (a)	++	+++++	++	-/+	++	+
FITC-ARAL (a)	++	+	-	-	-	-
FITC-CDGLGDDC (b)	-/+	-	-	++	+	-
FITC-CLDWDLIC (b)	-	+	+	-/+	-	-
FITC-CDGWGPNC (b)	-	-/+	-	++	-	-

One study explored the conjugation chemistry needed to attach LyP-1 peptide to iron oxide nanoparticles in order to achieve a robust, generalizable attachment scheme for in-vivo applications.^[11] In this chapter, we introduce some preliminary work done to explore the use of LyP-1 conjugated porous Si microparticles to target ovarian cancer cells in-vitro. Although future work remains to ascertain further applications of this, we have demonstrated the principle feasibility of using the chemistry of cisplatin-drug loading from the previous chapter to attach LyP-1 to the surface.

5.3 Results and Discussion

5.3.1 Lyp-1 Conjugation to Platinum Surfaces

The LyP-1 peptide is a circular homing peptide with the structure as depicted in **(Figure 5.2)**.

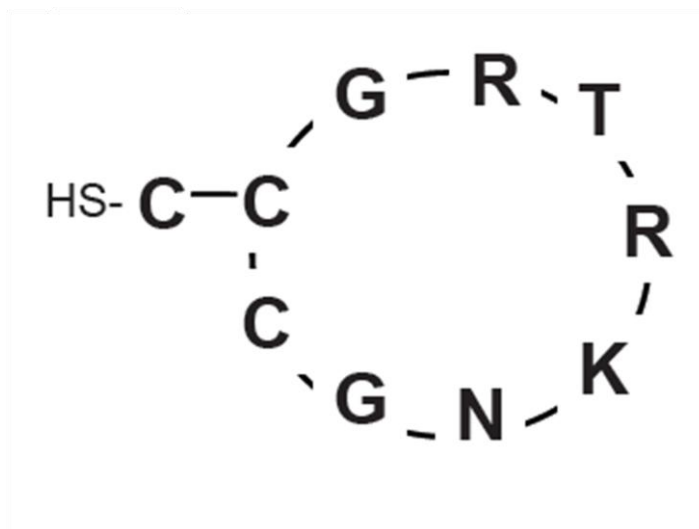


Figure 5.2 Amino acid sequence and structure of LyP-1 homing peptide with terminal cysteine.

The -thiol chemical moiety can readily be bound to gold or platinum surfaces. Porous Si microparticles have been loaded with cisplatin through a route where the oxidation of Si which generates a potential by which metallic platinum can be deposited on the material surface thereby trapping some drug into the pores. LyP-1 peptide contains a terminal cysteine group that is often used in other examples to conjugate to surfaces via a thiol-reactive linker (e.i. maleimide). We have previously explored attaching the peptide via a 5 kD heterobifunctional PEG linker to prevent non-specific binding or aggregation. However, we wanted to explore the possibility of attaching LyP-1 directly to the platinum surface using the robust -thiol / platinum chemistry. **(Scheme 5.3)** shows a diagram of the peptide attachment to the microparticle surface.

The Lyp-1 peptide is covalently-coupled to a fluorescein isothiocyanate dye for imaging purposes **(Figure 5.4)**. The platinum coated particles are incubated with 1 mg/ml Lyp-1 at a particle concentration of 0.5 mg/ml. By fluorescence microscopy (exposure time 500ms, Ex: 480/40 – Em: 535/50) the peptide conjugated particles have verified attachment. Suspending the particles in water diffuses the intensity of the dye. There is no auto-fluorescence from the particles at the identical exposure time of 500ms.

Next, the particles (0.5 mg/ml) were incubated with increasing concentrations of LyP-1 peptide **(Figure 5.5)**. The non-platinated particles served as a non-specific control. In this case, we still obtained non-specific physical adsorption of the peptide to the un-modified porous Si surface. As the porous

structure of the microparticles is less occluded without platinum, more non-specific adsorption could occur. As the peptide concentration was increased, we did not see a relative increase in intensity. The platinated samples were then dosed with increasing concentrations of peptide, and like-wise the amount of peptide bound on the particles increased. The measurements were taken on a 96 well fluorescence microplate reader. The results indicate that peptide binding to platinum is specific, although there is some physical adsorption also occurring from the signal in the control set.

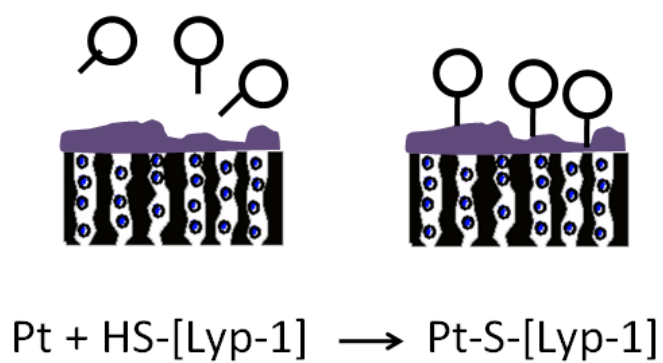


Figure 5.3 Schematic representation of Lyp-1 circular peptide bound to platinum coated, drug-loaded microparticle through the thiol bond on a terminal cysteine residue.

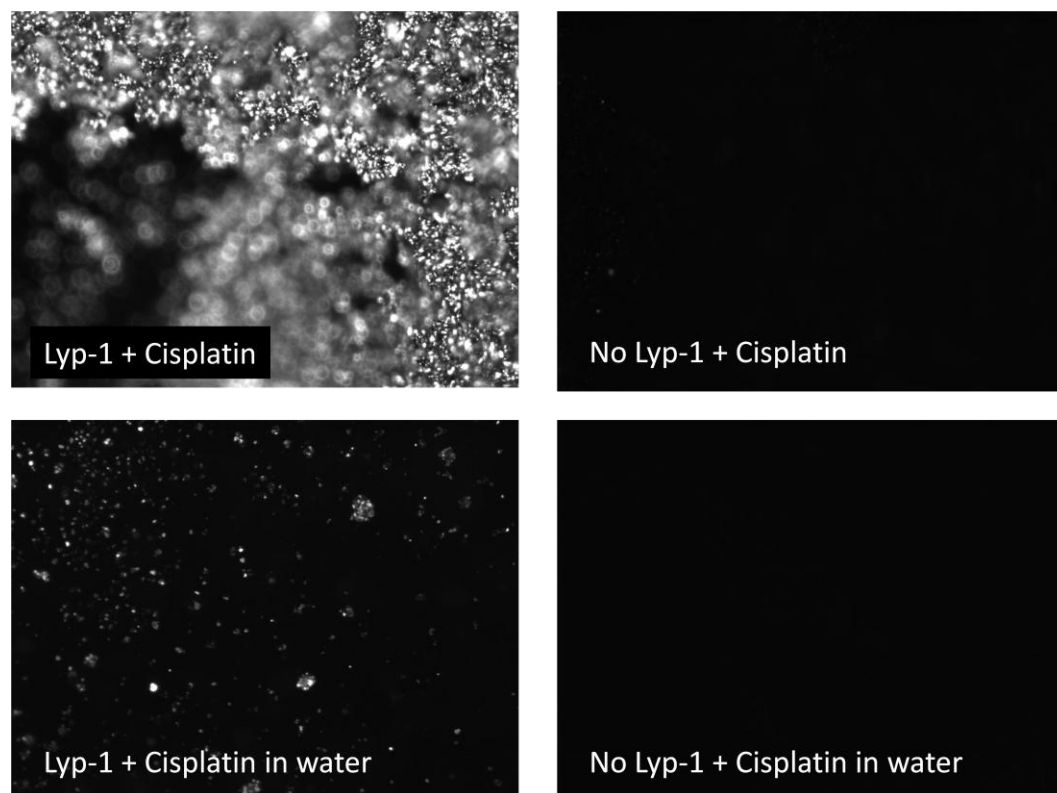
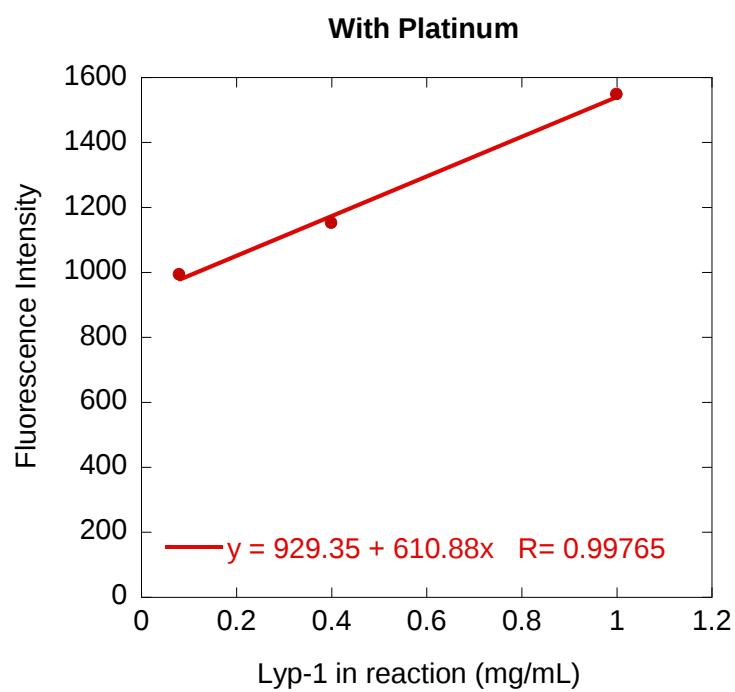
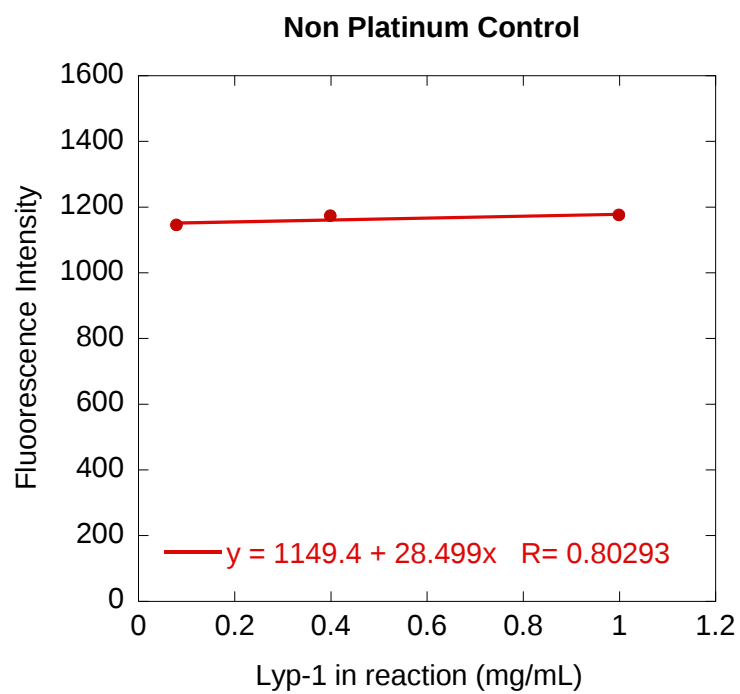


Figure 5.4 LyP-1 binding to cisplatin-loaded microparticles. Drug loading and characterization is thoroughly described in Chapter 4. The top left panel is of LyP-1 bound to cisplatin loaded microparticles in air. The bottom left panel is LyP-1 bound to cisplatin loaded microparticles in water. The top right panel is drug-loaded particles with no LyP-1 indicating no autofluorescence of the particles at this exposure time. The bottom right panel shows drug-loaded particles with no LyP-1 in water.

Figure 5.5 Binding of LyP-1 with increasing peptide concentrations with drug loaded, platinum coated surface, and non-drug loaded, non-platinum coated surface. The results show there is some non-specific binding of the drug to the particle without platinum, which remains constant as you increase the peptide concentration. The fluorescence signal increases linearly for the platinum coated particle as you dose higher concentrations of LyP-1.



5.3.2 Cellular Toxicity with LyP-1 Conjugated, Drug-loaded Microparticles

The last experiment was the testing of how toxic these peptide conjugated microparticles in comparison to non-conjugated ones. Sulforhodamine B viability assay was used with 2008 human ovarian cancer cells. Four particle sets were tested. Cisplatin-loaded particles and the non-toxic counterpart, transplatin-loaded particles were both conjugated with LyP-1 and left without LyP-1. The results show that there is an enhanced toxicity in the particle types with the addition of the peptide. It is interesting to note however, that the increased toxicity also occurs in the transplatin particles which should not be toxic to the cells. Cisplatin particles exhibit higher toxicity than the transplatin set as expected.

From this data, we were able to see a small difference in toxicity due to the addition of a homing peptide. However, future studies will likely determine that perhaps alternative cell-lines such as A2780 or OVCAR-3 may show a higher sensitivity to LyP-1 binding.

Light and fluorescence microscopy images of the live cells with the LyP-1 coated particles indicated that there was a sort of aggregation with the particles around the cell edges. However, it was not included in this chapter, because it was inconclusive whether it was due to affinity, or due to “stickiness” of mammalian cells that are not healthy due to a lack of sufficient O_2 (g) chamber in the microscopy facility. Future work can also be done on the affinity of these particles with alternative cell lines.

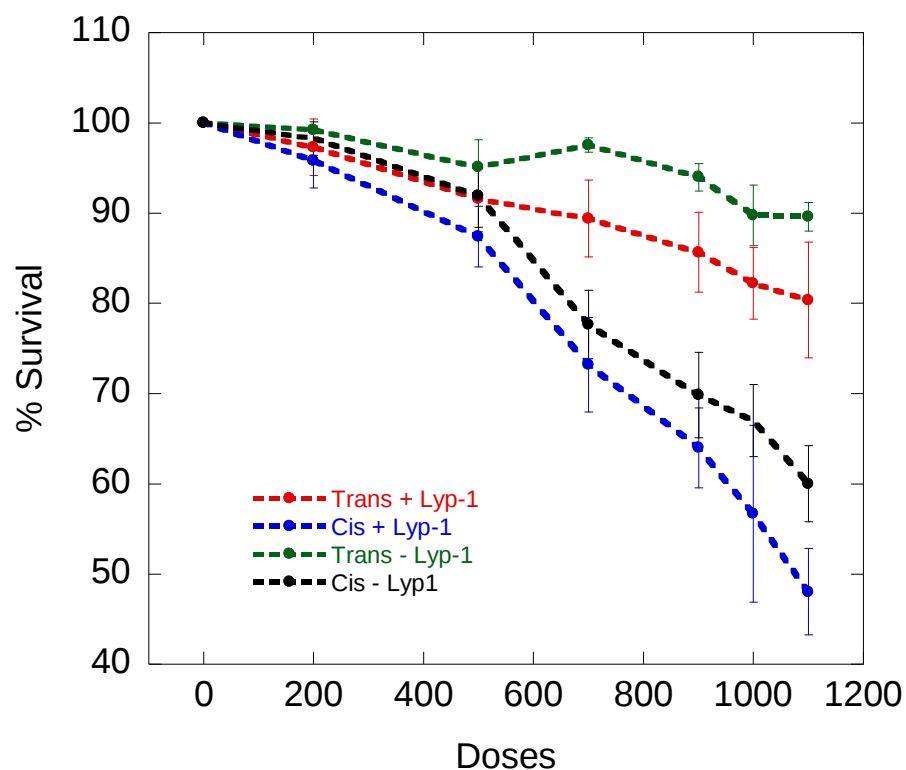


Figure 5.6 Cellular toxicity of 2008 human ovarian cancer cells with LyP-1 bound porous Si microparticles. X-axis depicts dose of particles in (# of particles) and Y-axis is % survival of cells. The toxicity was measured by sulforhodamine B viability assay. (Cis + LyP-1 = Cisplatin loaded microparticles with LyP-1 peptide attached, Cis - LyP-1 = Cisplatin loaded microparticles with no LyP-1. Trans + LyP-1 = Transplatin loaded microparticles with LyP-1 peptide attached, Trans - LyP-1 = Transplatin loaded microparticles with no LyP-1.)

5.4 Conclusions

Porous silicon microparticles can be loaded with cisplatin by partially depositing the metallic platinum on the particle surface. This surface can then be used to directly couple LyP-1 targeting peptide. LyP-1 has shown promising results in terms of its ability to target specific ovarian cancer cells. Further studies will need to be performed as to the binding affinity of these particle types with alternative cell lines such as A2780 and OVCAR-3.

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Chapter Six

FUTURE WORKS

Introduction

The next stage for this study would be to conduct in-vivo maximum tolerated dose experiments with the cisplatin loaded porous silicon microparticles. In Chapter 3, varied types of porous Si particles (etch type, size, surface chemistry) were compared against each other in a toxicity study with non-drug loaded particles. From this set we can draw out results for a control data set: 4 micron, stain-etched conjugated, rinsed experimental group to use as a non-drug loaded control.

The goal of a maximum tolerated dose study done with cisplatin loaded porous Si microparticles would be to find that highest dose at which this drug delivery system would be effective from which a therapeutic dose range can be designed.

Materials and Methods

6.1 In-vivo toxicity studies of cisplatin loaded porous Si microparticles

The study can be performed on Balb/c mice ranging between 19-21 grams. They will be weighed prior the start of the experiment and monitored for changes in weight after every injection and every 12 hours afterwards. Cisplatin loaded porous Si microparticles can be synthesized according to the procedure outlined in Chapter 4. The particles will be injected in increasing doses until either toxicity is observed or the mass of the animals decreased to 10% or greater weight loss

which is also to have considerable toxic effect. The animals that have a toxic effect will be sacrificed with standard operating procedure at earliest possible time to minimize unnecessary pain.

6.2 In-vitro binding of Lyp-1 conjugated cisplatin loaded porous Si microparticles

Several attempts were made to demonstrate the binding of Lyp-1 conjugated particles with several types of ovarian cancer cells (OVCAR-3, 2008, A2780). The methods that were previously used were fluorescence microscopy and flow cytometry.

We were able to explore the general method for data collection using both of these methods. One suggestion is to keep in mind the culture time for the cells to keep them healthy in the process of the experiment. As the cells grew sticky, we found the particles tending to stick to the cells thereby inducing false positive results. Compared to the controls (non-Lyp-1 coated set), the peptide coated particles did not seem to have much higher adherence than non-coated. In fact, both sets seemed to stick to the cells. This is concluded to be due to the sticky cell membrane induced during the process of apoptosis. We were not able to use a CO₂ / heat chamber during the microscopy image acquisition. One future suggestion would be to attain microscopy conditions that would allow live cell image acquisition. Another suggestion would be to acquire a high volume of images to average, because due to the large size and mass of the particles, they tend to disperse in clusters rather than in a uniform, evenly spaced distribution. In

determining the amount of peptide bound, the intensity of fluorescence from Lyp-1 (FITC) can be compared to the average particle surface area.

Several variables for this experiment could be incubation time with the particles and temperature. Temperature was found to have an effect on potential of nanoparticles binding to tumors.

6.3 In-vivo toxicity studies of Lyp-1 conjugated cisplatin loaded porous Si microparticles

Finally, the last step to conclude this study is to determine the effect of reducing tumor mass by the Lyp-1 conjugated cisplatin loaded microparticles compared with free cisplatin. This study would be predicated upon results from the in-vitro Lyp-1 particle binding experiment in section 6.2. However, even if there is not significant in-vitro binding, sometimes the results are more (or less) pronounced in-vivo. This will be the capstone experiment of this study. The drug loaded, peptide conjugated microparticles will be injected at an effective dose comparable to the therapeutic concentrations typically delivered clinically for cisplatin – based on the calculations in Chapter 3 for total drug loaded on the microparticles.

Xenograft tumors based a 2008 ovarian cancer model will be used because the metastatic growth pattern most closely resembles that of the microscopic tumor nodules present in intraperitoneal ovarian cancer. A2780 tumor model is also a possibility, but tends to grow much larger volume tumors and forms ascites.

Both are available with GFP or other fluorescent labels in order to provide a tool for imaging.

Once the tumors are grown for a period of 4-6 weeks, the tumors size and volume will be imaged by Xenogen in-vivo live animal imager. Then, the drug loaded particles will be injected as described in the previous paragraph. An equivalent cisplatin dose will also be injected. The animals will be monitored for their mass every 24 hours. The mass of the tumor will be imaged every 24 hours to observe the effect of tumor shrinkage by the drug loaded microparticles over time.

Appendix A

NOTES ON THE STUDY OF POROUS SI-BASED POLYMER REPLICAS FORMED BY BEAD PATTERNING

A.1 Background

Monodisperse microparticles containing a tunable nanostructure are desired for various high-throughput screening and targeted drug delivery applications.^[1, 2] The applicability of porous silicon-based particles and films has been demonstrated.^[3-13] However, for many uses porous silicon is limited by its chemical and mechanical stability. The use of polymer replicas based on a porous silicon template eliminates these issues and provides robust, flexible materials that are compatible in biological systems and harsh environments.^[14] Previously, we showed that disk-shaped particles composed of a porous Si/polymer composite can be synthesized using a derivative of dry soft-lithography, where a polymer mist is spray coated onto the surface of a porous Si photonic template.^[15] Appropriate modification of the etch conditions in the porous silicon-based template provides a means to adjust the porosity and nanostructure of the polymer/porous silicon composites with a high degree of control. However, the shape and size of the particles is not well controlled with this microdroplet patterning method. In this work, we use a highly uniform dispersion of 8 μm in diameter poly(methyl)methacrylate (PMMA) beads instead of an aerosol spray as a polymer source. It is found that this approach provides much greater uniformity in the resulting nanocomposites and completely evades the use of solvents that could be toxic to a biological environment.

A.2 Materials and Methods

A.2.1 Porous Si Preparation

Porous Si templates were prepared by an anodic electrochemical etch of highly doped P-type Si wafers, with an average resistivity of $1.5 \text{ m}\Omega \text{ cm}$. (Siltronix, Inc.). The etching electrolyte solution contains a 3:1 (v/v) mixture of 48% aqueous HF (EMD Chemicals, Inc.) to absolute ethanol (Aldrich Chemicals). The etch cell consisted of a two-electrode configuration with 1.33 cm^2 of the Si wafer exposed in a TeflonTM chamber. The samples were etched with a time-modulated sinusoidal current density, producing a pseudo-sinusoidal porosity variance through the thickness of the porous layer. Two different current density waveforms were used in this study. The etch waveform consisted of 150 cycles varied between 151 and 301 mA/cm^2 or 60 cycles varied between 90.4 and 377 mA/cm^2 . Periodicity was manipulated to obtain wavelengths of peak reflectivity within the visible spectral range, typically on the order of 1.0-1.5 seconds. The freshly-etched samples were rinsed with ethanol and dried in a stream of nitrogen gas.

A.2.2 Bead Dispersion, Infusion, and Particle Collection

An aqueous dispersion of PMMA microspheres with a coefficient of variance of $< 3\%$ were purchased from Fluka Chemicals. The beads were nominally $8 \text{ }\mu\text{m}$ in diameter. The suspension was diluted to a 1:20 (v/v) concentration of beads to deionized water. The bead dispersion was pipetted onto the porous Si sample and pressed gently with a MylarTM film to homogeneously

disperse the beads on the surface. The chip was placed on a glass coverslip and the beads were softened and infiltrated into the porous Si film using a three-step heating protocol on a laboratory hotplate: 125 °C for 20 min, 200 °C for 55 min, and 250 °C for 55 min. The polymer bead replicas were removed from the porous Si matrix in an aqueous base solution. The base solution was added in aliquots of 200 μL of 3:1 (v/v) 0.5 M $\text{KOH}_{(\text{aq})}$: ethanol, followed by 400 μL of 0.5 M $\text{KOH}_{(\text{aq})}$ until the porous layer has been dissolved. The base mixture was diluted with deionized water and the free-standing PMMA replicates were collected on a 0.2 μm alumina filter (Anodisc) by vacuum filtration.

A.2.3 Characterization

Reflectivity spectra were obtained using a tungsten light source and a Princeton Instruments/Acton SpectraPro 150 spectrometer fitted with a liquid nitrogen-cooled charge-coupled-device (CCD) detector, adapted to a light microscope (Nicolet Instrument, Inc.) at 40X magnification. The source beam and the spectrometer path are coaxial and both normal to the sample surface. Scanning electron microscopy (SEM) micrographs of PMMA particles were obtained using a FEI Quanta 600 environmental scanning electron microscope operated at an accelerating voltage of 20kV. All samples were gold sputtered prior to imaging.

A.3 Results and Discussion

The steps used to fabricate monodisperse, micrometer sized polymer replicas of porous Si domains are illustrated in **(Figure A.1)**. Porous Si films are prepared using an electrochemical-etch with a time-modulated current density. The periodic variation through the depth of the pore creates multi-layered stacks of alternating refractive index, resulting in the formation of an optical structure known as a rugate filter.^[16] A shift in the characteristic reflectivity peak of the rugate filter verifies infiltration of micron-sized polymer domains into the porous Si film.^[14, 15]

To make the nanocomposites, an aqueous dispersion of PMMA microspheres (8 μm in diameter) is spread on the surface of a one-dimensional porous Si photonic crystal. The PMMA beads are heated beyond their glass transition temperature (T_g) such that they soften and flow into the porous matrix. A three-stage heating cycle was employed. At the beginning the beads were heated slightly beyond their T_g (125°C) in order to prevent coalescence during heating. After partial infiltration is achieved the temperature is raised to further lower the viscosity of the polymer and ensure maximum infiltration.

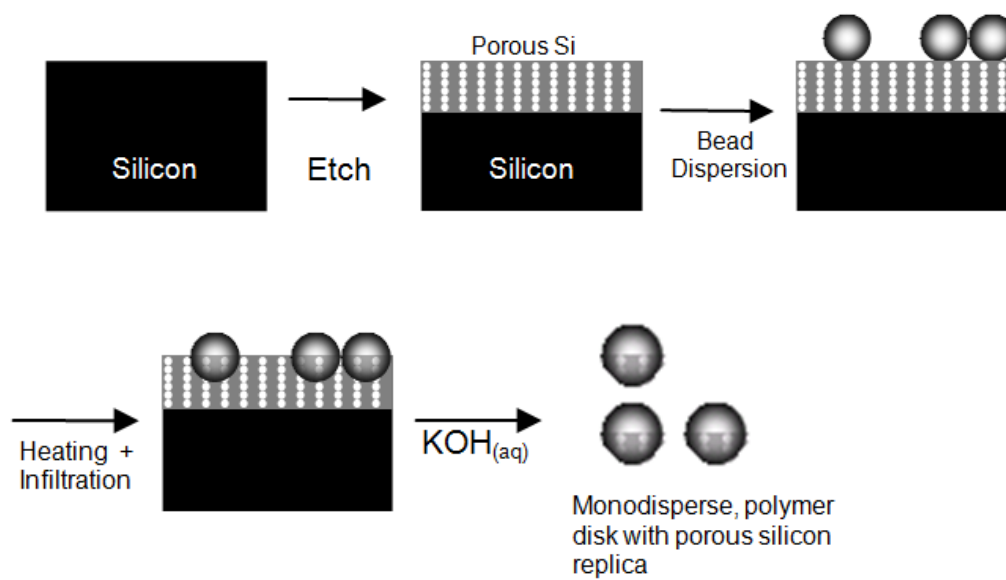


Figure A.1 Scheme employed to produce uniform-sized porous Si-based polymer replicates.

Optical reflectivity spectra of the composite domains verify the replication of the porous silicon photonic nanostructure into the polymer beads. Reflectivity spectra were obtained on a porous Si film immediately after polymer bead infiltration (prior to the KOH-removal step). The spot size was focused over a cluster of several polymer beads. Stark color contrast between the regions of infused beads (orange, $\lambda_{\text{max}} = 590$ nm) and the un-filled porous silicon matrix (green, 510 nm) is apparent in the optical reflectivity spectra (**Figure A.2A**) and in the light microscope images (**Figure A.2B**). The reflectivity spectrum of a ~ 400 μm -diameter region in which bead infiltration has occurred displays two peaks (**Figure A.2B**). The second peak corresponding to the empty porous silicon film ($\lambda_{\text{max}} = 510$ nm) can be observed because the spectrometer spot size includes regions of polymer/porous silicon composite and regions of porous silicon/air. Contribution to the second peak may also arise from the porous Si region immediately beneath the polymer beads, because the polymer beads do not infuse all the way to the porous Si/crystalline Si interface.

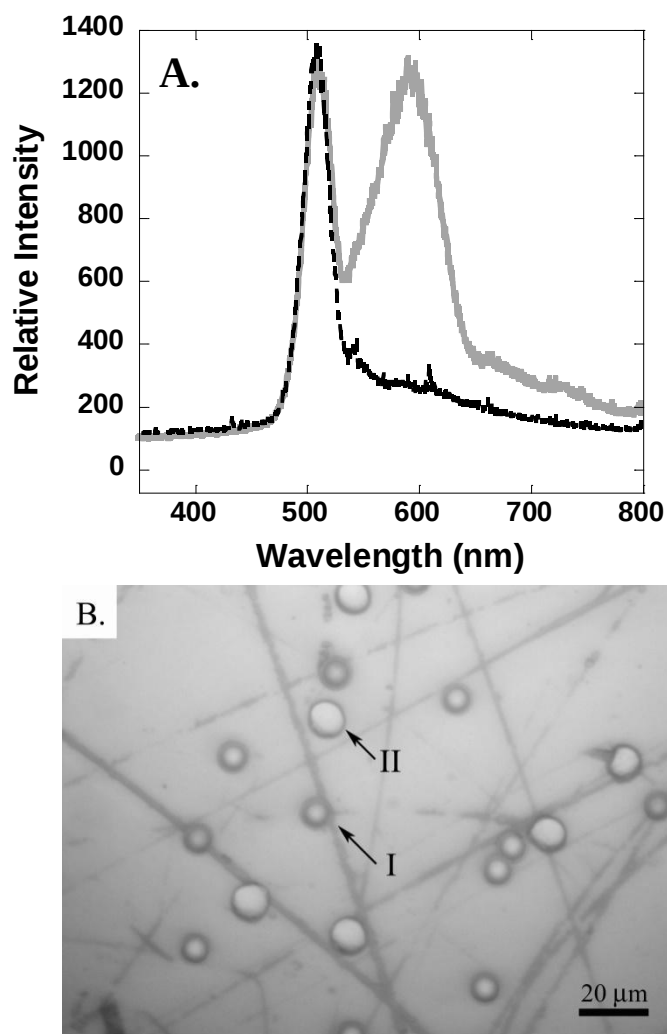


Figure A.2 Polymer Bead Infiltration into a Porous Si Photonic Crystal.

- A. Optical reflectivity spectra of empty regions of a porous Si photonic crystal (dashed line) and regions containing infiltrated PMMA beads (solid line).
- B. PMMA beads (8 μm) on porous Si. I. Un-infiltrated bead, added after the heating step for comparison. II. Infused bead displaying a red-shift in wavelength due to infiltration into the photonic crystal film.

Removal of the polymer beads from the porous Si template can be accomplished by dissolving the template in base. The porous Si film is treated with an aqueous sodium hydroxide solution containing ethanol. The polymer does not dissolve under these conditions; the polymer beads are undercut by the base solution and float free from the substrate. The free particles are then collected and filtered. SEM micrographs of the particles are shown in **(Figure A.3)**. The particles assume a uni-directional convex disk shape with an apparent porous, textured face and an opposing smooth side **(Figure A.3A)**. As depicted in **(Figure A.3B)**, the porous texture of the PMMA replicas extend to a depth of approximately 500nm, indicating partial infiltration of the PMMA into the template. A comparison of the size of the original PMMA bead to the porous silicon replicated polymer disk is shown in **(Figure A.3C)** and **(Figure 3.D)**, respectively. The photonic features are lost upon removal of the polymer beads from the porous Si substrate. Although the porous Si template imparts a textured nanostructure to the PMMA beads, presumably the lift-off conditions used are too harsh to retain the more delicate nanostructure of the original porous Si template. Additionally, the degradation of the photonic structure could also potentially result from the thermal degradation of the polymer. However, PMMA should be fairly stable at this temperature.

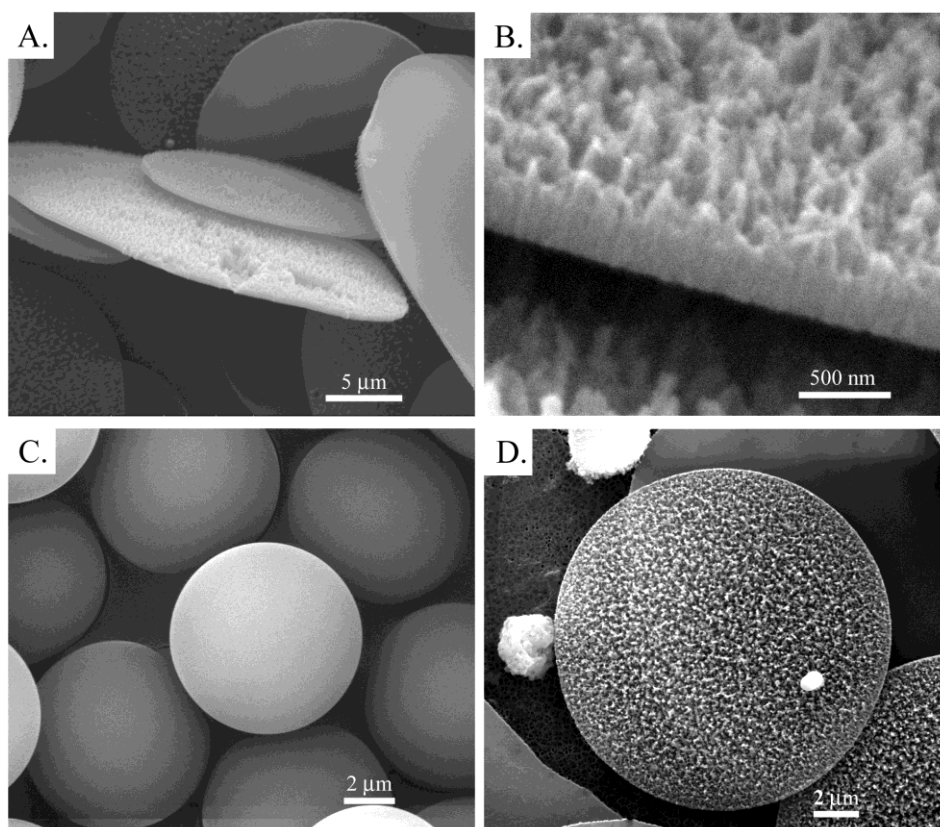


Figure A.3 Scanning electron microscope (SEM) micrographs of free-standing polymer replicas of porous silicon.

- A. Smooth side versus infiltrated porous side of PMMA replicas.
- B. A side-view micrograph of a PMMA replica particle.
- C. Original PMMA microspheres as-received.
- D. A plan-view micrograph of a PMMA replica particle.

A.4 Conclusions

Infiltration of heat-softened monodisperse polymer beads into porous Si photonic crystal templates allows the construction of nanocomposite disks with well-controlled dimensions and optical properties. Removal of the beads from the porous Si template using a basic etch destroys the photonic structure, but some of the nanotexture is retained. Potentially this simple method can be employed to produce nano-structured particles using other polymer systems.

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