PHYSICS OF NANOPLATFORMS AND THEIR APPLICATIONS
IN NANOMANUFACTURING AND NANOMEDICINE

A dissertation presented

by

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to
The Department of Physics

In partial fulfilment of the requirements for the degree of
Doctor of Philosophy

in the field of
Physics

Northeastern University
Boston, Massachusetts
December, 2009
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ABSTRACT OF DISSERTATION

Submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy in Physics in the Graduate School of Arts and Sciences of Northeastern University, December, 2009
Abstract

Nanoplatforms are nanoscale structures designed as general platforms for multifunctional nanotechnology applications. Applications of nanotechnology cover broad spectrum of research fields and require true interdisciplinary and multidisciplinary studies. It also requires a fundamental understanding of physical principles in nanoscale since nanomaterials exhibit different properties and experience distinct forces compared to the materials in macroscale. In this thesis, we studied two different nanoplatforms, namely nanoporous oxide coatings and superparamagnetic nanoparticles. We analyzed their physical properties and illustrated their applications in two different fields, nanomanufacturing and nanomedicine.

The first nanoplatform we studied is ordered nanoporous arrays of aluminum and titanium oxide. We investigated their fabrication as well as their applications in both nanomanufacturing and nanomedicine. We addressed the question of assembling spherical and cylindrical elements into porous holes - all in the same nanoscale. To investigate the assembly of nanoelements, one has to have an understanding of forces in nanoscale. In this length scale, the electronic and magnetic forces are the dominant forces whereas some macroscale forces like gravity has none to little effect. We demonstrated 3D directed assembly of nanobeads as well as single-wall carbon nanotubes (SWNT) into nanoholes by means of electrophoresis and dielectrophoresis at ambient temperatures. For nanobead assembly, SEM images were sufficient to demonstrate 100% assembly of loaded nanobeads. For SWNT, the connection through assembled nanotubes were used to prove the success of the assembly. The I-V measurements clearly showed that strong Si-SWNT interconnects carrying currents on the order of 1 mA were established inside the nanoholes. This assembly technique is particularly
useful for large-scale, rapid, 3D assembly of $10^6$ SWNT over a centimeter square area under mild conditions for nanoscale semiconductor electronics applications.

We also demonstrated that nanoporous oxide coatings can be utilized as non-eroding sustained drug release platforms for up to weeks of elution. The release kinetics was explained in two main regions: burst and sustained release. The sustained release kinetics for nanoporous coatings was characterized as an activated surface density dependent desorption model in form of $df/dt = -\alpha f \exp(-\beta f^2/k_BT)$. Nanoporous inorganic coatings were proved to be well suited to provide improved efficacy and integration of implants in a variety of therapeutic situations like drug eluting stents or antibiotic coated hip replacements.

The second type of nanoplatform we investigated is magnetic nanoparticles. Magnetic properties differ in nanoscale due to the increasing role of the surface spins as the particle size is decreased, leading very interesting phenomenon like superparamagnetism. We studied magnetic properties of nanoparticles, namely superparamagnetic iron oxide nanoparticles (SPION). Magnetic properties of SPION was studied through superconducting quantum interference device (SQUID) magnetization as well as nuclear magnetic resonance (NMR) spectroscopy measurements. We showed that SPION can be successfully encapsulated inside micelle and liposome structures to create versatile theranostic nanoplatorms for enhanced drug delivery and monitoring of cancer treatment. The ability to incorporate SPION cargo renders these nanoplatforms to be highly susceptible to guidance by external magnetic fields, as well as making them exceptional magnetic resonance imaging (MRI) contrast agents, enabling visualization of their distribution in vivo.

The efficacy of using magnetic immuno-micelles (MIM) and magnetic cationic liposomes (MCL) for magnetic targeting and as MRI contrast agents was investigated using in vitro NMR and in-vivo MRI studies. For micelle study, human breast carci-
noma cells were incubated with specific anti-body attached MIM. NMR measurements showed not only MIM affect $T_2$ relaxation time of the sample, but also the cell binding of MIM was higher with specific (2C5) anti-body compared to non-specific anti-body or control groups.

For liposome study, metastatic melanoma tumor was grown in the right flank of SCID mice. Magnetic targeting was employed by placing a disk magnet on top of the tumor of the animal after administration of MCL for one hour. Pre-injection and post-injection MR images were used to assess response to magnetic targeting effects. Hypointense areas in MR images, decreased signal intensity, and lower $T_2^*$ relaxation times all directly correlated to MCL accumulation. We showed that with the help of magnetic targeting the accumulation and retention of MCL was 2-fold higher in the tumor side compared to control groups.
Acknowledgments

The work I presented in this thesis was conducted with great help of my research group and with the collaborations from different groups. Among these people, first I would like to express my gratitude to my advisor Prof Srinivas Sridhar for accepting me in his group and his confidence in me, for creating the projects and establishing the collaborations that made the work presented here possible. I am also thankful to him for having such a lively, friendly and helpful lab which makes research a lot easier.

From our research group, Dr Dattatri Nagesha was almost like a co-advisor to me. He taught me, guide me, supervised me and surprised me with his incredible knowledge/memory of past publications. I thank him for all his help as well as for his friendship throughout my years in graduate school. I thank Dr B. Didier F. Casse for his warm welcome for all my questions, his encouraging guidance in research as well as in professional life and for creating our lunch-pack tradition. I would like to thank my fellow graduate student in our lab, Francisco Reynoso for sharing my frustrations over things we have not yet learned—but sensed that they will cause problems on the way—and more importantly for increasing the fun coefficient of our lab. I would like to thank Dr Wentao Lu for his help on my random questions and on Matlab. I thank Dr Ravinder Banyal for his assistance on optic systems. And I want to thank all the other lab members Yongjian Huang, Salvatore Savo, Dayane B. Tada, Evan Jost, Craig Levy and past members Cory Fantasia and Erin Pritchard for their help. I would like to thank Rita Kaderian, she was so helpful with everything. I am also grateful to Tim Hussy for his help in fabrication processes.

I want to thank all my collaborators for sharing their knowledge and helping with
my experiments. I thank Prof Latika Menon for teaching me the details of nanoporous template fabrication. I would like to thank Prof Don Heiman for showing me how to use SQUID in the line of my research, for understanding what I was missing on magnetism and teaching that. I thank Prof Mansoor Amiji for his help on drug elution studies and, from his lab, Dr Mayank Bhavsar for teaching me preparing standard curves for fluorescent measurements. I am grateful to Prof Robert Campbell for his help on magnetic cationic liposomes and in-vivo studies as well as for being so willing to teach me. I thank Praba Selvasarah for helping me with the clean room instruments and also letting me win the graduation race. I thank Dr Praveen Kulkarni for spending a lot of time to teach me how to operate MRI. I want to thank all other collaborators Prof Ahmed Busnainia, Prof Alain Karma, Prof Vladimir P. Torchilin, Dr Rishikesh M. Sawant, Prof Craig Ferris, Aditi Jhaveri and Prof Mukesh Harisinghani for their assistance.

I received great deal of help from other groups or centers in Northeastern University. I thank Dr Roger Kautz for teaching me how to use NMR spectroscopy and to analyze the results. I thank Scott McNamara and Richard DeVito for teaching and helping me with instruments in Kostas Center. I thank William Fowle for his help on imaging techniques with SEM and TEM. I also want to thank Prof Clive Perry for helping me with the spectrometer and with my optical setup.

I believe that accomplishing a dissertation is not completely limited to the effort or the time spent in the graduate school but well beyond that. For this reason the number of people that I feel obligated to acknowledge is much longer than that I can list, but I try my best to thank here as many of them as I can.

I am grateful for having Utku Kemiktarak on my side all these years. I thank him for supporting me, making me laugh and laughing with me, encouraging me, trusting me and listening to me tirelessly when I have something to say—God knows how
often I have something to say.

I would like to thank my parents, for directing my childish curiosity to scientific questioning and for not keeping all those expensive encyclopedia with pretty pictures away from my catastrophic hands when I was a kid.

I thank my sisters, Esin and Elif, and my brother Abdulhak, for bringing so much joy in my life, for all the precious moments and for their unshaken trust in me.

I thank my professors who have not only thought me physics but advised me on life when I needed it most. I thank Prof Levent Kurnaz for his encouragement and support and Prof Alpar Sevgen for teaching me the H-integral\(^1\). I thank Dr Yasar Safkan for believing me and boosting my self-confidence repeatedly.

I thank my roommate Zeynep D. Ok for bearing with me for more than 3 years. I am grateful for her patience, understanding and her friendship. I also thank Elif Bingol and Aysu Oguz for their unending support from overseas. I thank I. Baris Altunkaynak for his one of a kind personality, being a semi-reliable computer source and his chats that always leave me with a laugh. I want to thank all my friends for their priceless friendship, Susmita, Selis, Sinem, Rasim, Nilay, Cagdas, Burak, Dilek, Guerreiro, Kamanducaia, Faceira and C.M Chuvisquinho.

\[^1\] \(H = \int_{-\infty}^{\infty} dh\), where \(H\) is the total and \(dh\) is the differential happiness.
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<th>Description</th>
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<tbody>
<tr>
<td>AAO</td>
<td>Anodic aluminum oxides</td>
</tr>
<tr>
<td>ATO</td>
<td>Anodic titanium oxides</td>
</tr>
<tr>
<td>Dox</td>
<td>Doxorubucin</td>
</tr>
<tr>
<td>FID</td>
<td>Free induction decay</td>
</tr>
<tr>
<td>FWHM</td>
<td>Full width half maximum</td>
</tr>
<tr>
<td>MCL</td>
<td>Magnetic cationic liposomes</td>
</tr>
<tr>
<td>MIM</td>
<td>Magnetic immuno-micelles</td>
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<tr>
<td>MR</td>
<td>Magnetic resonance</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscope</td>
</tr>
<tr>
<td>SPION</td>
<td>Superparamagnetic iron oxide nanoparticles</td>
</tr>
<tr>
<td>SQUID</td>
<td>Superconducting quantum interference device</td>
</tr>
<tr>
<td>STDC</td>
<td>Standard curve</td>
</tr>
<tr>
<td>SWNT</td>
<td>Single wall carbon nanotubes</td>
</tr>
<tr>
<td>$R_1$</td>
<td>Longitudinal relaxation rate</td>
</tr>
<tr>
<td>$R_2$</td>
<td>Transverse relaxation rate</td>
</tr>
<tr>
<td>$T_1$</td>
<td>Longitudinal relaxation time</td>
</tr>
<tr>
<td>$T_2$</td>
<td>Transverse relaxation time</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscope</td>
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Chapter 1

Introduction

Nanotechnology is a platform technology that utilizes the unique properties of matter in nanoscale. In this length scale, physical phenomena are dominated by the forces such as Van der Walls force, hydrogen bonding, electronic charge, hydrophobicity, hydrophilicity and quantum mechanical tunneling. Some of the everyday forces we meet, however, are excluded from this world like inertia or turbulence. Nanomaterials have broken a size barrier where quantization of energy for electrons in solids becomes relevant. Due to this effect they behave differently, for instance, solids turn into liquids in room temperature (gold), opaque substances become transparent (copper) or insulators turn conductors (silicon).

The platforms designed for applications of nanotechnology are known as nanoplanformms. In this thesis, we discuss two different nanoplanformms, nanoporous oxide coatings and superparamagnetic iron oxide nanoparticles. We investigate physical properties of these nanoplanformms and demonstrate their applications in two different fields, nanomanufacturing and nanomedicine. We begin with introducing nanoporous oxide coatings in forms of anodic aluminum oxides (AAO) and anodic titanium oxide (ATO). We investigate different fabrication techniques to obtain different size porous
templates. We demonstrate that ordered nanoporous templates can be achieved by two-step anodization.

**Nanoporous Templates**

As the first application of nanoporous templates we studied electric field assisted assembly. Controlling the assembly of nanoelements is one of the critical technical barriers in nanomanufacturing. There is a need to conduct fast directed assembly of nanoelements at high rates and over large areas. We discuss feasibility of utilizing AAO templates for high-rate assembly. We demonstrate 3D directed assembly of nanobeads and single-wall carbon nanotubes (SWNT) into porous alumina nanotemplates fabricated on Al films or on Si substrates. 100% assembly of nanobeads via electrophoresis inside the templates is confirmed by SEM images. For SWNT assembly, the current passing over SWNT between top and bottom layers of AAO demonstrates the success of the assembly.

Another application of nanoporous templates are established for sustained drug release. Conventional drug delivery systems, using various pharmaceutical dosage forms, provide prompt administration of the drug. To keep drug concentration in therapeutically effective range, multiple drug administrations are necessary in the conventional methods. With the advancements in technology, controlled drug delivery systems have been developed. These novel systems are mostly polymer based, however in some cases there is a need for mechanically robust systems. We demonstrate utilization of nanoporous templates as controlled drug delivery systems. The drug release applications of inorganic nanoporous materials are at their infancy, especially compared to polymeric coatings. Although the clinical applications of these materials are getting closer, still many more studies and tests are required before their full potential is realized. With further advances in the convergent technologies many
more new applications and/or devices are expected.

Because of the ease of fabrication and controllable pore dimensions, nanoporous templates are used in many in vitro as well as in vivo drug delivery studies. They serve as excellent drug delivery devices for various conditions where systemic therapy might cause serious side effects and repeated administration of the drug might carry risks or is simply unpleasant. We investigate drug elution profiles from nanoporous templates using a custom made in-situ fluorometry. A model drug, Doxorubucin was loaded into nanoporous templates for this study and release kinetics were compared with a polymeric platform. All the platforms show a similar pattern: first burst release and later sustained release. The burst release can be explained by Fickian diffusion. We offer a theoretical model explaining sustained release from nanoporous structures: an activated density dependent surface desorption model in the form of

\[
df/dt = -\alpha f \exp(-\beta f^2/k_B T).
\]

**Superparamagnetic Nanoparticles**

The second nanoplatform we discuss in this thesis is superparamagnetic iron oxide nanoparticles (SPION). SPION have been used widely in various in-vivo applications. Due to their high magnetization and relative biocompatibility, they have been employed as magnetic targeting, MRI contrast or magnetic hyperthermia agents. We explain their fabrication techniques and their superparamagnetic nature. We analyze the effect of encapsulation on the magnetic properties of SPION. We demonstrate the results from SQUID magnetization measurements proving the superparamagnetic nature of the particles. We explain the NMR spectroscopy results indicating effect of SPION on $T_2$ relaxation time. We also show the magnetic hyperthermia results from different concentrations of SPION - liposomes packages addressed as magnetic cationic liposomes (MCL).
In the sixth chapter, we introduce an application of SPION as an excellent theranostic agent. We show in-vivo magnetic targeting applications of MCL and how they also behave as MRI contrast agents. We use melanoma tumor bearing mice as in-vivo model. MR images of the mice are taken before and after the administration of MCL. We show that, MCL can be manipulated by a small disk magnet towards cancer tumor. Through MR images, we demonstrated the effect of MCL as a contrast agent and monitor the magnetic targeting non-invasively.
Chapter 2

Nanoporous templates

2.1 Introduction

Nanoporous materials can be described as materials with pore sizes less than approximately 100 nm[1]. Nanoporous substrates and coatings distinguished by their large surface area have recently attracted attention in different range of applications, including nano-manufacturing, energy harvesting, integrated circuits, biological or chemical sensing, orthopedic implants and controlled elution. These surfaces can be fabricated with precise control on pore size, pore distribution and pore density. Additionally, the surface chemical properties can also be manipulated to suit the application.

2.2 Anodic Aluminum Oxides (AAO)

Nanoporous anodic alumina is an electrically insulating, optically transparent, chemically stable, bioinert and biocompatible material. It has been used widely in various electronic, optoelectronic, sensing devices as well as in dental and orthopedic implants. While studies on fabricating alumina nanotemplates go back to 1960’s [2], they be-
came more common in research and manufacturing following the work of Masuda et al [3]. Well-defined, ordered nano-architectured templates are possible because of the well refined anodization techniques. Moreover, nanoporous alumina can be directly grown on metal implant surface [4]. Alumina nanoporous templates have very broad size range: pore diameter between 5 nm to 10 µm [5] and array thickness over 100 µm [6]. Figure 2.1 shows representative scanning electron microscopy images of nanoporous alumina template.

**Figure 2.1:** SEM images of nanoporous alumina nanotemplate. (a) Planar view image of the template with uniform periodic nanoholes over large length scales, scale bar=100 nm and (b) cross-sectional view of the template showing smooth cylindrical nanochannels about 250 nm in height extending to the aluminum substrate at the bottom, scale bar=100 nm.

### 2.2.1 Fabrication of Anodic Aluminum Oxides (AAO)

Anodic alumina nanotemplates have been investigated extensively since the 1960’s and they have been manufactured by a number of different methods [7, 8, 9]. The common fabrication method starts with the aluminum film being exposed to an acidic medium, generally sulfuric acid ($H_2SO_4$), oxalic acid ($C_2H_2O_4$), phosphoric acid ($H_3PO_4$) or a combination of these acids. Because of the acidic medium an oxide layer forms over the metal. During the anodization, the applied voltage leads
to pore formation and the growth of the oxide layer. While the type and pH of the acid determines the inter-pore distances, the applied voltage during the anodization determines the pore diameter of the templates [10, 11].

Sulfuric acid with the combination of the applied voltage in the range of 10-30 V will produce nanopores with the interpore distance around 60 nm and the pore size around 20 nm. Voltage range used with oxalic acid is between 30-80 V and interpore distances and pore size acquired are around 120 nm and 60 nm respectively. Phosphoric acid is used for the largest hole sizes. The voltage used with phosphoric acid is between 100-200 V; the interpore distance and the pore size then are 300 nm and 120 nm respectively. Generally the longer the anodization, the thicker the resulting porous template is. However, the acidic medium also causes the dissolution of the oxide layer; and eventually the growth and dissolution rates become equal. This determines the ultimate thickness of the oxide layer which varies between 60 – 120 µm. Another important variable in the fabrication is the temperature of the electrolyte. Lower temperatures slow down the oxidation rate which leads more ordered pore formation [12].

The versatility of this method is that one can deposit aluminum on any substrate and then can completely convert it to nanoporous alumina. Aluminum can be deposited using an electron beam evaporator on different materials like silicon substrates [13] or titanium implant surfaces [14]. For more complicated surfaces like cardiovascular stents, physical vapor deposition process can be used for depositing aluminum [4].

Masuda et al. suggested using two-step anodization technique to reach highly ordered pore structure[3]. We followed this procedure for the fabrication of AAO in general, our protocol can be found in Appendix A. A commercially available Al foil was used as anode, a graphite sheet was used as cathode, and 5 wt % oxalic
acid was employed as the electrolyte. A custom made anodization cell was used for anodization. The cell was consisted of a two layer glass tube was compressed between teflon blocks. A graphite sheet was contacted to the inner side of one of the teflon blocks and served as a counter electrode. On the other side, a teflon block contained five 1.5 cm diameter holes for Al disks. Another teflon block with five submerged copper rods was pressed on the block with holes to squeeze the Al disks in place and supply electrical contact. While the inner glass tube was filled with the electrolyte, the outer tube was connected to a water chiller to supply water bath at a specific temperature. The sketch of anodization setup can be seen in Figure 2.2.

![Figure 2.2: Anodization setup for nanoporous alumina, titania and Si fabrication](image)

Anodization was carried out at 4 °C for 6 h under a constant of 50 V direct current (dc) potential. This produced tens of microns thick porous aluminum oxide consisting of an array of vertically arranged pores on the surface of aluminum as seen in Figure 2.3(a).

The thick porous layer was then completely removed by soaking in chromic phosphoric acid solution at 70°C for about 20 h, which leaves behind a highly ordered patterned Al surface Figure 2.3(b). The patterned aluminum surface was then
reintroduced in the acid bath for a second anodization under the same experimental conditions for about 5 min. This second step produced a periodic, hexagonally ordered nanoporous layer with pore diameter on the order of 30 nm and thickness on the order of 500 nm Figure 2.3(c). The porous layer is separated from the bottom conducting aluminum layer by a thin layer of barrier aluminum oxide. For efficient electrophoretic assembly, an important requirement is the presence of a conducting surface below the pores. Therefore it is important to etch the barrier layer below the pores which is done by soaking the sample in 5% phosphoric acid for about 80 min at room temperature. This results in thinning of the barrier layer at the bottom of the nanoholes. Additionally, this leads to widening of the pore diameter of individual nanopores and decrease in the height of the nanoporous film. Typical pore diameter of the templates is on the order of 80 nm and thickness of the order on 250 nm Figure 2.3(d)s.
2.3 Anodic Titanium Oxides (ATO)

Titanium and its alloys have been extensively used in orthopedics and dental implant procedures due to its mechanical strength and excellent biocompatibility. Upon exposure to oxygen titanium develops a layer of oxide $\text{TiO}_2$ or titania on its surface which is responsible for its biocompatibility. However, it is possible to create this layer of titania on an implant surface in a controlled fashion to facilitate localized drug delivery. Ti and TiO$_2$ have been used in orthopedic implants since the 1970s. Vertically oriented titania nanotube arrays have been introduced in 2001 by Gong and co-workers [15] and the following extensive studies have explored the range of dimensions of these nanotube arrays. Today, it is possible to achieve titania nanotube arrays with tube diameter ranging between 20 nm- 150 $\mu$m [16] and tube length up to 200 $\mu$m [17].

Titanium and its alloys are one of the most common implant materials used in human body and the percentage of the population in need of these implants increases every year. Titania nanotubular coatings can enhance the benefits of currently used titanium implants by adding another functionality i.e. drug delivery.

2.3.1 Fabrication of Anodic Titanium Oxides (ATO)

Titania nanotube templates are fabricated using an anodization method similar to that for nanoporous alumina Figure 2.2. Different electrolytes are used for different tube diameters as well as different aspect ratios. Most commonly used electrolytes for titania nanotemplate fabrication are hydrofluoric acid (HF) [5], fluoride ion containing baths with organic electrolytes [16] or chlorine based electrolytes [18]. The tube size and ultimate length of titania template can be controlled by the type and pH of the electrolyte as well as the applied voltage similar to fabrication of alumina.
The morphology of the titania formed by this method consists arrays of nanotubes bundled together. The diameter of the nanotubes formed, nanotube wall thickness and the overall titania film thickness can be precisely controlled through experimental parameters. In addition, in order to improve the mechanical property of the template, synthesized titania nanotube arrays can be annealed at high temperature to convert titania anatase phase to rutile phase.

Other fabrication methods of ATO were also reported in the literature e.g. use of a block copolymer in combination with precursor, \( TiCl_4 \) [19]. This method could be used for fabricating extremely small pore sized templates, about 5.5 nm diameter nanoholes of titania with a film thickness of 300 nm.

We used two different anodization methods for ATO templates.

i) Quick anodization with 1v% hydrofluoric acid (HF): First samples were electropholished by a mixture of 0.4 M hydrofluoric acid and 1 M nitric acid (\( HNO_3 \)) for 6 minutes. For the anodization, 1 v% of hydrofluoric acid was used as the electrolyte. Titanium foils were anodized for 1 hour under constant stirring. The anodization setup for ATO can be seen in Figure 2.4. Anodization of titanium in hydrofluoric acid resulted short tube length (<200nm), as shown in Figure 2.5(a) and (b).

ii) Long aspect ratio ATO tubes: Long aspect ratio tubes were acquired by using different electrolyte solution than HF. A mixture of 0.05 M potassium fluoride (KF) in 10% DI water + 90% Glycerol was used. Anodization was done under constant 60 V at room temperature for overnight. The anodization with potassium fluoride solution is a slow process but it leads longer ATO tubes, as seen in Figure 2.5(c) and (d).
Figure 2.4: The anodization setup for ATO, as electrolyte either HF or KF+Glycerol mixture was used. Calcium gluconate was kept under and around the setup for spill protection.
Figure 2.5: SEM images of ATO. (a) Top view (b) cross-sectional view, prepared by anodization in HF. (c) Top view and (d) cross-sectional view, prepared by KF-Glycol mixture.
Chapter 3

High-rate Assembly of Nanoelements in Nanotemplates

3.1 Introduction

Nanomaterials in the form of nanoparticles, nanowires and nanotubes are the main components in the burgeoning field of nanotechnology. Due to their unique physical and chemical properties, these nanoelements have the potential for use in a variety of applications such as memory devices\cite{20}, chemical sensors\cite{21}, biosensors\cite{22} in diagnostics\cite{23} and imaging\cite{24}. However, an important challenge in nanomanufacturing is the directed growth or assembly of nanoelements over macroscopic length scales. Different strategies have been explored to address the two dimensional (2D) and three dimensional (3D) assemblies of nanomaterials. Using capillary forces, spherical colloidal particles with sizes of the order of 150 nm have been assembled in microfabricated patterns of various shapes\cite{25}. Sub-50 nm colloids were assembled on a lithographically patterned surface by a mechanism known as capillary force assembly\cite{26}.
Another approach involves bias induced assembly. For example, CdS colloids have been assembled within the trenches created on block copolymers by application of bias voltage[27]. Very recently, a similar approach utilizing electric field assisted oriented assembly of CdS nanorods has also been demonstrated[28]. Biological elements in the form of DNA[29], bacteria[30] and viruses[31] have also been used as templates for assembly of nanoscale elements. All these techniques have limitations for assembly of nanoelements on large centimeter scales or for rapid assembly and utilize expensive and time consuming processes. Assembly of nanoparticles into macroscopic structures that have all critical dimensions in the nanometer length scale still remains a significant challenge.

In this chapter, we report a different kind of nanoassembly technique utilizing nanoporous templates. Nanoelements in solution are assembled inside the pores by means of an electrophoretic or dielectrophoretic process. These processes demonstrated directed assembly in truly 2D nanostructures. They also demonstrate a very simple technique for rapid and large-scale assembly of nanoelements.

### 3.2 Electrophoresis and Dielectrophoresis

Electrophoresis involves the motion of colloidal particles in solution in an applied electric field. In this work, we have demonstrated this technique for the assembly of nanobeads inside the nanoporous alumina template. In the case of colloidal particles, when such particles are in contact with a liquid, a thin layer or the so called electrical double layer with a nonzero net charge density is produced by rearrangement of local free ions in the solution.

The total force acting on a charged particle in solution is the sum of electrophoretic
and dielectrophoretic forces

\[ \vec{F} = \vec{F}_{EP} + \vec{F}_{DEP}. \]

(3.1)

The electrophoretic force acting on a charged particle is

\[ \vec{F}_{EP} = \Gamma \epsilon_m \zeta \vec{E} \]

(3.2)

where \( \Gamma \) is geometry factor, \( \epsilon_m \) is relative permittivity of the medium and \( \zeta \) is the zeta-potential of particle. For spherical particles, the relation between the velocity \( v \) of the particles and the electric field \( E \) is given by

\[ v = \mu_e E, \]

(3.3)

where, \( \mu_e \) is the electrophoretic mobility\[32\]. For a spherical particle in a liquid, electrophoretic mobility is calculated as

\[ \mu_e = \left( \frac{2 \epsilon \zeta}{3 \eta} \right) f_1(\kappa a), \]

(3.4)

where \( \epsilon \) is dielectric constant of the liquid, \( \zeta \) is zeta potential of the particle in the solution, \( \eta \) is viscosity of the liquid, and \( \kappa \) is Debye-Hückel parameter which depends only on temperature and the bulk electrolyte concentration. \( f_1(\kappa a) \) is monochromatically varying function in the range of \( 1.0(\kappa a = 0) - 1.5(\kappa a = \infty) \)[33]. The well-known Smoluchowski equation

\[ \mu_e = \frac{\epsilon \zeta}{\eta} \]

(3.5)

is the upper limit of this equation. Using Smoluchowski equation and equation (3.3), the speed of the beads during assembly was calculated approximately. It must be noted that other parameters such as high applied voltage, interparticle or particle-wall
interactions can also affect the electrophoresis[34]. However, the above calculations give a rough estimate for the required experimental parameters.

The dielectrophoretic force acting on a dielectric particle, on the other hand, can be calculated as

\[
\vec{F}_{DEP} = \Lambda \epsilon_p \text{Re}\{\kappa_f(\omega)\} |\vec{E}| |\nabla| |\vec{E}| \tag{3.6}
\]

where \(\Lambda\) depends on the geometry, \(\epsilon_p\) relative permittivity of particle and \(\kappa_f\) is Claussius-Mossotti factor depending both on geometry and frequency. If SWNT is considered as an ellipsoid, the electrophoretic force would not differ much from the spherical calculation except from the geometry factor, gamma. However, the dielectrophoretic force might change a lot depending on Claussius-Mossotti factor, \(\kappa_f\) [35]. \(\kappa_f\) for an ellipsoid particle with a cylindrical symmetry could be written as [36],

\[
\kappa_f = (\epsilon_p^* - \epsilon_m^*)/3((\epsilon_p^* - \epsilon_m^*)A + \epsilon_m^*) \tag{3.7}
\]

where \(\epsilon_p^*\) and \(\epsilon_m^*\) are the complex permittivity of particle and medium respectively and A is the depolarizing factor given by

\[
A = (1 - e^2)/2e^3(ln((1 + e)/(1 - e) - 2e)) \tag{3.8}
\]

e defined as

\[
e \equiv \sqrt{1 - (b/a)^2} \tag{3.9}
\]

where a and b are the major and minor axis of ellipsoid respectively.
3.3 Nanoparticle Assembly

3.3.1 Theoretical Modeling

Theoretical modeling has been used to predict the electrical field distribution within the nanoporous alumina array during the electrophoresis process. The effect of the thickness of the insulating alumina barrier layer at the bottom of the nanoholes, film thickness and type of electrolyte on the electrical field distribution was studied. Distribution of electric fields inside the nanopores due to the applied voltage was studied by means of the simulation program ANSYS (ANSYS, Inc., Canonsburg, PA).

A simplified model for porous alumina was assumed with 16 nanoholes, 250 units in thickness, with 10 units in barrier layer, and 80 units in diameter—conditions similar to the experimental parameters, the units used in simulations are arbitrary. The medium between the electrodes and inside the alumina holes is assumed to be water. The dielectric constants of alumina and that of the electrolyte were approximated to be around 9 and 80, respectively[37]. Similar to experimental conditions, 10 V DC bias was applied between the electrodes for modeling calculations.

The construction of the model is shown in Figure 3.1(a), illustrating the positive electrode inside the alumina layer with nanoholes. The color scale in the diagram indicates the variation in the magnitude of the electric field in arbitrary units. Figures 3.1(b) and (c) show the longitudinal and perpendicular cross-section images of electric field distribution. Thus, even in the presence of the insulating barrier layer, the strong electric field is directed towards the center of the nanoholes so that charged particles are attracted into the holes[38].

Ansys was also used to understand the effect of the dielectric constant of the liquid. The simulation showed that electric field distribution depends mutually on
Figure 3.1: Theoretical modeling for electric field distribution during electrophoretic assembly by ANSYS. (a) Configuration of positive electrode representing nanoholes, alumina nanoarrays, and aluminum electrode. (b) Longitudinal cross section of electric field distribution shows that electric field magnitude increases (illustrated by the brighter red arrows inside the nanoholes). (c) A perpendicular cross section shows that at the template boundary there is a considerable difference in the electric field magnitude between the holes (red arrows) and the surface (blue arrows). The length of the arrows is proportional to the magnitude of the vector and the color scale is in arbitrary units.
the dielectric constants of the template and liquid, as shown in Figure 3.2. In either, case everything except epsilon of liquid is constant.

![Figure 3.2: Computational simulation of the dependence of the field on the type of liquid. (a) Water, $\epsilon_{\text{liquid}} = 80$ and (b) Hexane, $\epsilon_{\text{liquid}} = 2$. In both cases, dielectric constant of the template is constant, $\epsilon_{\text{template}} = 9$.]

### 3.3.2 Parallel Plate Assembly

The first assembly setup used was a parallel plate configuration which yields a constant electric field between the electrodes, as shown in Figure 3.3. The positive electrode was connected to the aluminum layer under the 80 nm pore diameter template, while a graphite sheet was used as a counter electrode. The distance between the plates was kept at 1 cm and the applied voltage bias was 10 V, producing a constant electric field at 10 V/cm. 50 nm size polystyrene beads, with zeta potential of
-40 V, were assembled inside water. The entire setup was kept in sonication bath to eliminate clustering of the beads.

![Figure 3.3: Sketch of parallel plate assembly configuration. The arrows represent the velocity of the beads by the electrophoretic force.](image)

The results of bead assembly were promising as seen in the top view of the SEM images Figure 3.4(a). We can see that almost all of the holes were filled with one or more nanobeads. The darker ones which are harder to see are further down in the holes. In the cross-section view Figure 3.4(b), we clearly can see the beads at the bottom of the holes. There was more than 75% assembly over a cm$^2$ area, which is highly significant comparing the control experiments shown in Figure 3.5.

### 3.3.3 Pointed Tip Assembly

The second setup consisted of using a pointed electrode for assembly of 50 nm polystyrene latex beads inside nanoporous alumina templates. The nanobeads were mixed in ethanol (5µl/ml concentration) and the mixture was used as the electrolyte. Electrical contact was made to the conducting aluminum layer below the
Figure 3.4: SEM pictures of parallel plate assembly results. (a) top view (b) cross-sectional view.

Figure 3.5: SEM image of the control assembly without bias.

alumina template and was maintained at 10 V value with respect to the counter-electrode which was grounded. The potential difference between the electrodes is 10 V and the potential of the beads is -40 mV while the dielectric constant and the viscosities of the water are taken as 80.1 and 0.89 mPa s respectively [37]. The template was placed in the holder which allows electrical contact at the bottom, and the holder was filled with 50µl of electrolyte so that the alumina template was completely submerged. As counter-electrode, a Pt–Ir wire was brought at a distance of ∼ 50µm from template for a time duration of around 2 min. Schematic illustration of the experimental setup is shown in Figure 3.6.

At a DC bias of 10 V, the negatively charged polystyrene nanobeads are found to assemble inside the pores over 10µm diameter region around the counter-electrode
wire. This region corresponds to the region over which the bias is applied. Figure 3.7(a) and (b) are representative SEM images of nanobead assembly within nanoporous alumina template. The low resolution SEM shows that assembly only occurred in areas that are in close proximity to the counter-electrode and not in other areas on the template surface. The complete assembly was confirmed by the cross-section SEM image Figure 3.7(b) which shows 100% assembly: all of the holes are completely filled by nanobeads. Thus by varying the size and height of the nanohole, the number of beads assembled can be manipulated.

3.4 Single Wall Carbon Nanotube Assembly

After recent discoveries regarding their electronic properties like large current capacities as high as $10^9$ A cm$^{-2}$ [39] or mobility as high as 100 000 cm$^2$/Vs$^2$ [40], single wall carbon nanotubes (SWNT) have become one of the most popular structures in nanoscale electronics. They have tremendous importance especially as electrical
interconnects since current materials (e.g. copper wires) fail to meet the required resistivity for smaller diameters [41]. A key challenge in utilizing carbon nanotubes (CNT) in nanoscale electronics is to assemble them for large-scale device applications. Presently there are two main approaches for manufacturing CNT: growing or assembling them into the device. All the various methods for growing CNT require high temperature synthesis [42][43] and in most of them, nanotubes need a post process for cleaning, cutting and sorting to reach a narrow size distribution as well as for purification to eliminate the impurities which depends on the synthesis technique[44].

In contrast to methods for growing CNT, post-synthesis assembly methods permit arranging nanotubes in a desired position without the need for high temperatures. With assembly methods, we have the flexibility to choose and use any SWNT solution (e.g. commercially available) for desired applications. Many different assembly methods such as transfer printing[45], surface-functionalization[46], magnetic field assisted[47] and electric field assisted assembly [48][49][50][51]have been investigated.
so far. A key challenge in utilizing CNT in nanoscale electronics is the assembly of the CNT for large scale devices. For devices such as electronic memories, assembly of the nanoelements needs to be done over millimeter-square areas. In this paper we present a method to assemble SWNT into anodic aluminum oxide (AAO) nanotemplates utilizing electric field assisted assembly [52].

Nanoporous alumina templates consist of an array of vertically arranged nano-sized cylindrical holes and provide a useful platform for the development of a variety of novel nanofabrication techniques [53][54]. They also have been used in high temperature synthesis of ordered CNT arrays[55][56]. Here, we utilize AAO’s high-rate scalability over centimeter square areas to demonstrate a post-synthesis assembly method rather than growing SWNT to avoid high temperature synthesizing conditions. The assembly was performed on a silicon (Si) substrate, enabling seamless integration into today’s semiconductor electronics industry.

3.4.1 Dielectrophoretic Assembly

For successful assembly, it is necessary to avoid the charged nanotubes sticking on the positive electrode surface of AAO. For this reason an AC electric field is used together with DC field as seen in the assembly schematics Figure 3.8. The AC field does not contribute electrophoretic force hence does not cause the nanotubes to move directly towards alumina surface. However, it helps them to orient and to move into the holes by dielectrophoretic force. We used \(2.5\epsilon_0\) and \(105 \text{ Sm}^1\) for semiconducting nanotubes (s-SWNT) and \(-104 \epsilon_0\) and \(108 \text{ Sm}^1\) for metallic ones (m-SWNT) as the values of permittivity and conductivity respectively[48]. \(\epsilon\) and \(\sigma\) for ethanol is taken as \(25 \epsilon_0\) and \(6 \mu\text{Sm}^1\). By using these values Claussius-Mossotti factor was estimated as \(\kappa_f^{s-SWNT} = -0.3\) and \(\kappa_f^{s-SWNT} = -134\) for electric field parallel to the major axis of nanotubes under DC field or low frequencies (including 10 MHz which is used in
this experiment). These calculations show that actually metallic and semiconducting tubes are going toward the same direction but the force on metallic nanotubes is 400 times larger than the semiconducting ones.

![Diagram of CNT assembly](image)

**Figure 3.8:** Schematic of CNT assembly. Positive electrode is attached to Si substrate under alumina template while a Pt-Ir wire was used as counter electrode.

The SEM images after assembly show that SWNT are going into the holes. The SWNT also stay partly on the surface since the length of SWNT is much larger than array thickness. Although size of individual SWNT is too small to be visible under SEM; since they tend to form bundles, it is possible to see some of these bundles as seen at **Figure 3.9**.

### 3.4.2 I-V Measurements

Since SEM was not a good technique to investigate how successful was the assembly, the current passing between the top and bottom layer of AAO through assembled SWNT was also measured. The surface of alumina template was sputtered with 15 nm thick Au layer to make electric connection between all the SWNT on the surface. The I-V measurement was done using parameter analyzer with a probe station by attaching one electrode on Au layer on the surface of alumina and the other on Si surface at the bottom of the template. As control measurement I-V
Figure 3.9: SEM micrographs of assembled SWNT in anodic alumina array: (a) top view and (b) cross-sectional view. The top layer at this false colored picture is the sputtered Au for imaging purposes. The scale bars are 120 nm.

curve was measured using the same AAO templates on Si substrate covered by Au layer without the assembled SWNT. Schematics of the measurements can be seen at Figure 3.10. In the control experiment since there was no SWNT to provide electrical connection, no current was observed between top and bottom of the template (Figure 3.11(a)).

Figure 3.10: Schematic of I-V measurement of SWNT assembly and control.

On the other hand, I-V curve of assembled SWNT shows a Schottky diode type behavior (Figure 3.11(a)). The SWNT used for assembly were a mixture of semiconducting and metallic types. Although the calculations showed the force on metallic ones are two orders of magnitude greater than semiconducting ones, because of bundling of nanotubes, both types are assembling into the nanoholes. However the current passes preferentially on metallic nanotubes because they are the lower resist-
tant path and also there is no applied gate voltage. Hence, it is expected to have a Schottky barrier between Si and m-SWNT.

![Figure 3.11](image)

**Figure 3.11**: (a) I-V measurements curves for different connections: The blue (square) data set is the connection between SWNT-Si. The purple (circle) data set is the first control experiment with the absence of nanotube and the green (cross) data set is the second control experiment with SWNT suspension on AAO but without bias. No current is observed in the absence of either SWNT or the applied electrical field for the assembly. (b) Current density-voltage characteristics of Si-bulk Al and Si-SWNT connections. The current density of SWNT connection is calculated over the total measurement area.

I-V characteristics of a Schottky diode can be estimated by using the thermionic emission model[57]. The practical non-ideal diode is usually modeled as a series combination of a diode and a resistor, R. The forward current passing over the diode can be expressed as

\[
I = I_s \exp\left(\frac{qV_D}{nk_B T}\right)
\]

where \(I_s\) depends only internal characteristics and effective area of the diode and temperature; \(q\) is the electronic charge, \(n\) is the ideality factor of diode, \(k_B\) Boltzmann constant, \(T\) is the absolute temperature and \(V_D\) is the voltage across the diode, \(V_D = V - IR\). When the I-V curve of Au-SWNT-Si connection was fitted to thermionic emission model, the resistance was found as \(R^{Au-SWNT-Si} = 17 \pm 3K\Omega\).
The area available for I-V measurement is the area sputtered with Au on AAO surface for electrical connection, which was 0.015 cm$^2$ for the chip. Hence, we achieved 2 mA/cm$^2$ current density over assembled nanotubes per chip at 2 V bias, as seen in Figure 3.11(b), which is same as the current density observed over nanotubes grown in AAO by a high temperature synthesis method [58].

However, the actual current density is much larger than 2 mA/cm$^2$ since the electrical connection is through the SWNT which has much smaller diameter than the nanoholes. To have an approximation of the current density over the connection area, the effective area of assembly of the electrode setup was first estimated by low magnification SEM images of assembled beads. The SEM images showed that the radius of effective assembly site was 4 µm, which is smaller than the radius of the counter electrode (50 µm). The total effective assembly area for one chip was estimated as

$$\text{# of sites} \times (\text{circular area of assembly site}) = 50 \times \pi (4 \times 10^{-4})^2 \approx 2.5 \times 10^{-5} \text{cm}^2.$$  \hspace{1cm} (3.11)

From the SEM images of AAO, the average area of one nanohole is $1.6 \times 10^3 nm^2$. Several high-resolution SEM images, showing different cross-sections of the alumina template, were used to statistically determine the assembled nanotube per hole ratio, which was found to be $1.0 \pm 0.3$. Thus the number of assembled nanotubes is $1.6 \times 10^6$, by calculating the number of holes residing in the total effective assembly area. Since the total number of holes in the 0.32 cm$^2$ assembly area is approximately $2 \times 10^{10}$, the filling ratio of assembly area to total chip area can be estimated as 0.75/10000.

The total SWNT-Si connection area can be calculated by multiplying the number of assembled SWNT to the area of the one nanotube. Although the diameter of SWNT is known, their connection area cannot be calculated directly as they tend to form
bundles. We have statistically extracted the average diameter of assembled nanotubes (3±1 nm) from SEM images of the cross-sections of the alumina nanotemplate. Hence

\[ A_{connection} = 1.6 \times 10^6 \pi (1.5 \times 10^{-7})^2 \approx (8.4 \pm 0.6) \times 10^{-7} \text{cm}^2. \] (3.12)

Therefore, the current density reaches 360 A/cm\(^2\) at 2 V bias when the estimated area of connection between SWNT and Si is used instead of total chip area. Interestingly, the current density of the connection between bulk aluminum and silicon reaches 30 mA/cm\(^2\) at 2 V bias [shown in Figure 3.11(b)], which is \(\sim 4\) orders of magnitude lower than the estimated current density between SWNT and Si.
Chapter 4

Drug Release from Nanotemplates

4.1 Introduction

Controlled drug delivery applications have expanded rapidly with new advances in biomedical sciences as well as parallel developments in advanced materials and technologies [59, 60]. The aim of controlled drug delivery is to administer the necessary amount of drug safely and effectively to specific sites in the human body and to regulate the temporal drug profile for maximum therapeutic benefits. The idea of controlled drug delivery started around 1960s when Folkman et al [61] realized that a rabbit can fall asleep by circulating its blood inside a tube which had been exposed to an anesthetic gas. This was the first suggestion of a drug delivery implant [62].

Mechanically robust implants are being used in many different parts of the body for various applications in orthopedics, cardiovascular stents and defibrillators [63, 64]. However there are numerous problems to overcome such as reducing infections, enhancing implant bonding, preventing restenosis in cardiovascular stents, among others. A central strategy is to incorporate therapeutic agents that can enhance implants and overcome the key problems mentioned [65, 66, 67]. A popular approach is to incor-
porate polymer coatings that are loaded with the therapeutic agent [68, 69]. However in some cases, polymers are not the most suitable materials such as in cardiovascular stents where delamination of the polymer coating can lead to thrombosis [70, 71]. In those situations it is necessary to have a reservoir that does not degrade or erode. Non-eroding nanoporous oxide coatings offer an attractive alternative platform since they are non-erodible. In this chapter, the results of the release of a model drug, Doxorubucin (Dox), from different non-eroding nanoporous coatings will be presented.

4.2 Drug Loading to and Release from Nanotemplates

An important requirement for nanoporous coatings is that they must be able to load and release the drug, in addition to possessing biocompatibility, mechanical and chemical stability. In general, a substrate must have the ability to incorporate a drug, preserve it and deliver it gradually over the time to a specific target site [72]. For specific applications, some additional properties might also be necessary such as being adaptable to the implants, being biodegradable or being non-erodible. The holy grail of controlled drug delivery is to deliver the therapeutic dose of the drug to the site in need for the necessary amount of time. The dose of the drug and necessary release time vary depending on the application. However, since traditional drug delivery methods already cover the short time drug administration, sustained drug delivery has attracted more interest.

Drug loading for the nanoporous templates generally is performed through capillary action by either immersing the templates in the concentrated drug solution or dropping the solution slowly on the template surfaces [19, 73]. Different techniques have been used to increase and accelerate the intake of the drug, including surface
enhancement [74], sonication [75] or solution aids [76].

One of the unique features of these nanoporous coatings is the ability to precisely control the surface properties. By varying the pore size, distribution and density, drug loading and release can be altered. Surface charges of these pores can also be modified to hydrophobic or hydrophilic to accommodate variety of drug molecules. Additionally, through suitable surface modification the release kinetics of the drug molecules can be altered.

The pore size of the membrane is extremely important especially when it becomes comparable with the size of the molecule since the diffusion rate becomes pore size dependent—a phenomenon referred to as hindered or restricted diffusion. Diffusion through nanopores study has been pioneered by Pappenheimer [77] and Renkin [78], followed by different academic specialists from material scientist to physiologists [79, 80, 81, 82]. There are also experimental studies on elution from non-erodible nanoporous substrates: Urano and Fukuzaki [83] studied desorption kinetics of bovine serum albumin (BSA) from alumina particles. They explained the desorption kinetics with the first order kinetic model by Bourne and Jennings [84] as

\[- \frac{d\Gamma^f}{dt} = k^f \Gamma^f \text{ and } - \frac{d\Gamma^s}{dt} = k^s \Gamma^s\]  

where \(\Gamma^f\) and \(\Gamma^s\) are the amounts of faster and slower desorbing BSA respectively and \(k^s\) are the desorption rate constants. Kang et al [75] used porous alumina templates with different pore size and film thickness to demonstrate how pore diameter and film thickness affect release rate. They observed that the amount of released drug was even greater than the theoretical volume of the template which shows the surface of the template also was able to retain considerable amounts of drug. Another result from their experiment showed that there is a direct proportionality between the released
Drug amount and the pore size and surprisingly reverse proportionality with the film thickness.

Drug release measurement experiments depend on the type and properties of the drug used. For self-fluorescent or fluorescent-tagged drugs, such as Doxorubicin or fluorescein isothiocyanate-conjugated dextran, respectively, fluorometry is an easy way to measure the drug release [85]. UV spectrometry can be used to determine the amount of drug especially if the drug has a high UV absorbance [86]. Other release measurement methods include high performance liquid chromatography (HPLC) [75, 87] and micro-BCA assays to measure the protein content [88].

The most common method to prepare the release assays for the measurements is collecting aliquots from the release medium periodically and replacing it with fresh solution. When the amount of the drug is measured in the aliquots by any of the measurement techniques, the total drug amount in the release medium can be calculated. For non-destructive measurement techniques like fluorometry, in-situ release measurement is also possible. For an in-situ drug release measurement, the fluorescent signal can be directly measured from the release medium as long as the release experiments done in transparent containers and also the fluorometry setup designed properly, as will be explained later in Section 4.4. The experimental procedure of in-situ drug release measurements is summarized in Figure4.1.

4.3 Platforms for Elution

Three types of non-eroding and one type of polymeric platform were prepared for the study, namely anodic aluminum oxide pore sizes of 20 (AAO-20) and 200 nm (AAO-200), anodic titanium oxide (ATO) and a biodegradable polycaprolactone (PCL) as a control platform.
4.3.1 Nanoporous oxide platforms

AAO-20 platforms were prepared by two-step anodization of aluminum foil (Alfa Aesar) under 15 V constant voltage in 5 wt% oxalic acid solution as explained in Section 2.2.1. The second step anodization was carried out for 4 hours resulting 20 nm pore diameter and 2 $\mu$m array thickness, Figure 4.2(a).

AAO-200 platforms were also prepared by the same two-step anodization method but under 130 V and in 0.3 M phosphoric acid solution, as explained in Section 2.2.1. Following 4 hours of second step anodization, the platforms were immersed in 5% phosphoric acid for 80 minutes for pore widening. After the pore widening, the AAO
of 200 nm pore diameter and 10µm array thickness was achieved, Figure 4.2(b).

ATO platforms for this study were fabricated by Trifon Fitchorov as reported in literature[89] and Section 2.3.1. Titanium foil (Alfa Aesar) was anodized under 60 V constant voltage and in 0.05 M potassium fluoride in 10% DI water + 90% Glycerol solution. After 15 hours of anodization, nanotubes of 125 nm in diameter and 2.5µm in length were achieved, Figure 4.2(c).

After fabrication of the platforms, the templates were soaked in Doxorubucin (Adriamycin, 2 mg/mL) /PBS (500µL of 50µg mL⁻¹) for 24 hours. During loading, templates were shaken by a rocker constantly. PCL – Dox platforms were prepared by mixing dissolved Polycaprolactone (PCL) and Dox. First 0.4 g PCL was dissolved in 1 mL acetone to have a homogeneous solution. 20 v% 100µL Dox (2 mg mL⁻¹) was added to PCL solution and mixed thoroughly. The liquid solution was filled in a 500µL capacity micro-centrifuge tubes and dried overnight. The dried PCL has been removed from the centrifuge tubes and the resulting self standing PCL cones was used as a representative polymeric drug release platforms. The images of the platforms after loading are shown in Figure 4.3(a) and (b).

![Figure 4.3: Sample photographs of the drug release platforms: (a) Dox loaded AAO. (b) AAO with no drug loaded. (c) Dox loaded PCL cone. [The scale bars are 1 cm long.]](image)
4.3.2 Polymeric Platforms

First 0.4 g PCL was dissolved in 1 mL acetone to have a homogeneous solution. 20 v% 100 µL Dox (2 mg/mL) was added to PCL solution and mixed thoroughly. The liquid solution was filled in a 500 µL capacity micro-centrifuge tubes and dried overnight. The dried PCL has been removed from the centrifuge tubes and the resulting self standing PCL cones was used as a representative polymeric drug release platforms as seen in Figure 4.3(c).

4.4 In-situ Elution Detection

The templates were first washed with 1 mL DI water to remove the residues of Dox on the surface. All the release platforms, i.e. 4 replicas of AAO-20 and AAO-200, 3 replicas of ATO and 5 replicas of PCL, were placed in glass scintillation vials with 5 mL 1% PBS buffer solution as the release medium. The release studies were performed in-vitro, using PBS which is commonly used to simulate in-vivo conditions for drug release [90][91]. The elution kinetics is fundamentally the same in-vivo since the nanoporous platforms are not affected by the physiological conditions unlike polymeric or hydrogel counterparts. Besides, in case of small molecules like Dox, enzymes would not interfere with the drug and hence the elution kinetics is not altered by the presence of the biomolecules. In this study, the results prove that the nanoporous platforms can be used as non-eroding sustained release systems which can be utilized as coatings on currently available implants such as cardio-vascular stents, orthopedic / dental implants, fiducials or spacers.

A custom fluorometry setup Figure 4.4 was built to enable in-situ measurements i.e. the fluorescent intensity of Dox was measured directly inside the release medium. The laser beam at 532 nm wavelength was shone on the vial at predetermined time
intervals. The fluorescent emission of Dox at 590 nm was detected by the spectrometer (MS257 Oriel, CT). A cut-off filter at 550 nm was used to eliminate the signal from laser to reach the spectrometer. The acquisition time of the spectrometer was arranged to 0.5 s and to avoid bleaching of the Dox, the vials kept under the laser no longer than necessary. Between the measurements the vials were kept on a rocker to allow a constant mixing of the solution. The in-situ measurement method made it possible to achieve large number of readings without disturbing the release process. This design is very critical to understand the release kinetics since it allows collecting fast and frequent data over time.

4.4.1 Standard curve and calibration for time dependency of Dox

It is necessary to have the relation between the fluorescent signal and the amount of Dox to determine the exact amount of Doxorubucin (Dox). One of the methods to get this relation is to use a set of known concentrations of Dox and measure the

![In-situ custom made fluorescent measurement setup.](image)

**Figure 4.4:** In-situ custom made fluorescent measurement setup.
fluorescent intensity corresponding to the concentration of Dox. The curve obtained by this method is known as standard curve (STDC).

The general calculation of the mass in release medium is done by the linear fitting of STDC as

\[ I = A + Bm \]  \hspace{1cm} (4.2)

where \( I \) is the fluorescent intensity and \( m \) is the mass of Dox. However, the fluorescent intensity of a given concentration of Dox is not constant over the time, especially after few days since Dox is prone to bleaching due to light. For the experiments covering weeks of Doc measurements, to avoid the effect of time, STDC was calculated over the time during the experiments, Figure 4.5(a). As seen at Figure 4.5(b) and (c), the parameters \( A \) and \( B \) decays exponentially over the time. Hence, the modified STDC calculation can be done by using time dependent version of 4.2

\[ I = A(t) + B(t) \]  \hspace{1cm} (4.3)

where \( A(t) = 4 \times 10^4 + 9 \times 10^3 \exp(-t / (5 \times 10^3)) \) and \( B = 2 \times 10^4 + 4 \times 10^4 \exp(-t / (2 \times 10^4)) \). Origin software (OriginLab Corporation, MA) was used for the graphical analysis for all of the release calculations.

4.4.2 Mass measurements and calculations

The non-eroding platforms were washed by 1 mL DI water before placed in the measurement vials. Amount of Dox in the wash water (\( M_{\text{wash}} \)) was measured with the same fluorescent technique but the amount was under the detection limit of the spectroscope i.e. less than 100 nm.

Drug released at infinite time (\( M_\infty \)) was known for the polymeric platforms since
Figure 4.5: (a) Representative STDC fittings to 4.2 in different time points. The linear STDC equation changes due to decrease in the intensity over the time. Time dependent parameters (b) A and (c) B fit to an exponential decay.

Table 4.1: Dox amount in Tween solution ($M_T$) and at infinite time ($M_\infty$).

<table>
<thead>
<tr>
<th>Platform Type</th>
<th>$M_T$ ($\mu$g)</th>
<th>$M_\infty$ ($\mu$g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAO-20</td>
<td>20 ± 2</td>
<td>27 ± 1</td>
</tr>
<tr>
<td>AAO-200</td>
<td>18 ± 1</td>
<td>23 ± 3</td>
</tr>
<tr>
<td>ATO</td>
<td>8 ± 1</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>PCL</td>
<td>-</td>
<td>8 ± 0.5</td>
</tr>
</tbody>
</table>

A known amount of Dox was mixed in the PCL. For the non-eroding platforms, $M_\infty$ was estimated after the end of the measurements. The nanoporous platforms were cleaned in an emulsifier solution, Tween 80. Tween 80 increases the solubility of Dox, hence all the Dox from the platform was released into the solution. Amount of Dox in Tween solution, $M_T$, was measured and the added to the amount of Dox in the release medium, $m$. The summation is estimated as $M_\infty$, the values were summarized in Table 4.1.
4.4.3 Results

All the platforms show release profiles extending to order of weeks and were still eluting when the measurements were terminated. Figure 4.6 show a representative graph of percent drug release from different platforms. The percent release was calculated by comparing the drug released into the medium to the total drug released at infinite time, \( M_\infty \). After a rapid burst release which was similar for all the platform types, different sustained release kinetics was observed from nanoporous and polymeric ones.

![Figure 4.6: Representative curves for percent release of Dox from each platform.](image)

4.5 Elution I - Burst Effect

All the platforms show a considerable amount of drug release immediately after introduction to the release medium - this effect is known as the burst release. The time period for the burst release is relatively very short compared to the entire release
process and is typically about 100 minutes for the platforms investigated. Although burst release has been observed in many controlled release studies, but due to the short time range there is little detailed data in this region and it has been ignored in most of the mathematical models[92].

One of the advantages of the in-situ measurement setup used in these experiments is that it is possible to collect release data frequently, at minute intervals, which helps to understand the short term burst effect. The burst release data for the first 100 minutes fits to the same power law behavior for all non-eroding and polymeric platforms:

\[
\frac{m(t)}{M_\infty} = y_0 + at^{1/2}
\]  

(4.4)

where \(m(t)\) is the cumulative released drug, \(y_0\) and \(a\) are the fitting parameters Figure 4.7. The release rate of such a system is inversely proportional of square root of the time:

\[
\frac{dm(t)}{dt} = \kappa t^{-1/2}
\]  

(4.5)

where the rate constant \(\kappa = \frac{1}{2} M_\infty a\). The \(t^{1/2}\) dependence is characteristic of Fickian diffusion, considering that in this early stage, drug is diffusing into a semi-infinite medium with zero initial concentration [93].

4.6 Elution II - Slow Release

After the burst release phase, all the platforms start releasing the drug at a much slower rate. The non-eroding nanoporous coatings show an empirical logarithmic behavior, Figure 4.8:
Figure 4.7: A representative graph of initial release profile fitted to 4.4

\[
\frac{m(t)}{M_\infty} = \ln(b + ct)
\]

where \(b\) and \(c\) are fitting parameters. The parameters of this logarithmic behavior of each type of nanotemplates are summarized in Table 4.2.

Table 4.2: Fitting parameters of sustained release from non-eroding coatings to Equation 4.6. The values were averaged over the replicas of each type of platform.

<table>
<thead>
<tr>
<th>Type of nanoporous coating</th>
<th>b</th>
<th>c (min⁻¹) × 10⁻⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAO-20</td>
<td>1.2 ± 0.1</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>AAO-200</td>
<td>1.1 ± 0.02</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>ATO</td>
<td>1.4 ± 0.04</td>
<td>10 ± 1</td>
</tr>
</tbody>
</table>

The empirical constant \(c\) in the release equation depends on the effective surface area of the template which is quite magnified due to one of the advantages of nano-features of the platform viz. enhanced surface – volume ratio, Figure 4.9(a). This dependency suggests that the sustained release is not dominated mainly by the diffusion, but by the drug-surface interactions, viz. surface desorption. The release profile also suggests a density activated release model: the fewer the number of drug
molecules left on the surface, the faster the release rate is, Figure 4.9(b). Hence, the release profile is determined not only by the interaction between drug molecules and the surface, but also by the interaction between the drug molecules.

4.6.1 Model of density dependent desorption for non-eroding coatings:

The experimental results can be interpreted with a desorption model, which assumes that the time rate of change of the fractional surface coverage, \( f \), obeys the simple first order kinetic law

\[
\frac{df}{dt} = k(t) f^n
\]  

(4.7)
Figure 4.9: (a) Change of parameter c in release equation, Equation 4.6, as a function of effective template surface area. (b) A representative graph of the change in the number of particles over the surface which depends on the coverage itself. The line is the fitting of the data to surface desorption model, Equation 4.10.

where \(k\) is the desorbing rate of particles from the surface [94]. Since desorption is a thermally activated process, the rate constant obeys an Arrhenius law [95]

\[
k(t) = k \exp\left(\frac{-E_a(t)}{k_B T}\right).
\]  

(4.8)

The activation energy \(E_a\) and hence the desorption rate, can itself generally depend on surface coverage [96], reflecting the fact that this energy is determined both by the interaction of particles with the substrate and the interaction between particles. The latter becomes relevant for large surface coverage when the mean distance between particles becomes comparable to the particle radius. Since the probability for two particles to interact is proportional to \(f^2\), the activation energy can be written in the mean field limit as

\[
E_a = E_a^0 + \beta f^2
\]  

(4.9)

where \(E_a^0\) represents the interaction of the particle with the substrate and \(\beta f^2\) is the increase of the activation energy due to the pair wise particle interactions. This yields
Table 4.3: Fitting parameters of release data to surface desorption model, Equation 4.10.

<table>
<thead>
<tr>
<th></th>
<th>$\alpha (s^{-1}) \times 10^{-6}$</th>
<th>$\beta (J m^2) \times 10^{-47}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAO-20</td>
<td>$-6 \pm 1$</td>
<td>$-8 \pm 5$</td>
</tr>
<tr>
<td>AAO-200</td>
<td>$-7 \pm 1$</td>
<td>$-4 \pm 2$</td>
</tr>
<tr>
<td>ATO</td>
<td>$-17 \pm 0.6$</td>
<td>$-27 \pm 10$</td>
</tr>
</tbody>
</table>

The prediction

$$\frac{df}{dt} = -\alpha f \exp\left(-\frac{\beta f^2}{k_B T}\right)$$  \hspace{1cm} (4.10)

where we have defined as the vanishing-coverage-limit rate constant,

$$\alpha = \exp\left(-\frac{E_0}{k_B T}\right).$$  \hspace{1cm} (4.11)

The above theoretical prediction is seen to fit well for the data from all of the nanoplatforms. The absolute coverage rate decreases by declining coverage, Figure 4.9(b) shows a representative curve fitting. The fitting parameters for each non-eroding platform are summarized at Table 4.3.

4.6.2 Comparison with the polymeric platforms

We carried out drug release studies from PCL plugs as a comparison platform. PCL plugs showed same burst release behavior of $t^{1/2}$ dependency as the non-eroding ones. However, their profile was different than the non-eroding platforms for slow release, Figure 4.10. It is a power law similar to burst release but with a different exponent:

$$\frac{m(t)}{M_\infty} = kt^n$$  \hspace{1cm} (4.12)

where $k$ is the fitting parameter and the exponent $n = 0.31 \pm 0.01$, which is consistent with the elution kinetics of polymeric platforms in literature [97][98][99].
Figure 4.10: A representative slow release profile for non-eroding platforms fitted to Equation 4.12. Both axes are on log scale.
Chapter 5

Magnetic Nanoparticles in-Vitro: Superparamagnetism and Magnetic Hyperthermia

5.1 Introduction

Magnetic nanoparticles in forms of superparamagnetic iron oxide nanoparticles (SPION) have received increased attention due to their characteristic small size (~ 10 nm) and as the name suggests, due to their superparamagnetic properties. SPION exist mostly either as magnetite ($Fe_3O_4$) or maghemite ($\gamma - Fe_2O_3$). Since they are magnetic, they can be manipulated by an external magnetic field or field gradient. On top of this “action at a distance ”property, the penetrability of magnetic fields in human tissue makes these nanoparticles very attractive for biomedical applications. There is a significant interest in developing SPION for their application as MRI contrast agents as well as magnetic targeting and magnetic hyperthermia agents [100].
5.2 Superparamagnetism

Neel developed the superparamagnetism theory in 1949 [101]. He did not name the theory as superparamagnetism but as the theory for ferromagnetic fine particles. For ferromagnetic particles, the energy barrier for moment reversal ($\Delta E$) is much greater than thermal energy ($k_B T$). Below Curie temperature of a ferromagnet, all spins are coupled together and so cooperate to yield a large total moment. The reversal of this magnetization moment is possible by overcoming the energy barrier

$$\Delta E = KV$$  \hspace{1cm} (5.1)

between different directions of easy magnetization, where $K$ is the uniaxial anisotropy energy density and $V$ volume of the particle [102]. Decreasing particle size, decreases $KV$ until the thermal energy $k_B T$ can disrupt the bonding of the total moment to the particle. This moment is then free to move and respond to any applied magnetic field. Yet, an ensemble of these particles maintain distribution of magnetic orientations and behave like an ensemble of paramagnetic particles, obtain no hysteresis. An applied field would tend to align this giant moment but $k_B T$ would fight against as in paramagnetism and this phenomenon is called superparamagnetism.

Superparamagnetism is a time-dependent phenomenon due to the stochastic nature of thermal energy. The probability of overcoming the energy barrier in Equation 5.1 is proportional to the Boltzmann factor $exp(-KV/k_B T)$. A typical measurement with magnetometer takes around 10 - 100 seconds. If magnetization reverses faster than the measurement time the particle appears as superparamagnetic. The timescale for a successive energy jump, or the so called relaxation time is given by

$$\tau_N = \frac{1}{f_0} exp(-KV/k_B T)$$  \hspace{1cm} (5.2)
where $f_0$ is the characteristic frequency of order of $10^9$ Hz. Considering the measurement time, the transition between ferro-magnetism and superparamagnetism happens for $(KV/k_B T \approx 20)$. From this, one can calculate the critical temperature known as blocking temperature or $T_B$

$$T_B = KV/20k_B.$$  \hspace{1cm} (5.3)

Below $T_B$ the free movement of magnetic moment is blocked by anisotropy, above $T_B$ the thermal energy can disrupt this blockage and system appears superparamagnetic.

There is no precise qualitative description of superparamagnetic $M$ vs $H$ behavior. However, there are two main properties (i) no hysteresis above the blocking temperature and (ii) data of different temperatures superimpose onto a universal curve of $M$ vs $H/T$, Figure 5.1 [103].

![Magnetization data for SPION plotted versus H/T. The collapse of data to a single curve indicates superparamagnetism.](image)

**Figure 5.1:** Magnetization data for SPION plotted versus H/T. The collapse of data to a single curve indicates superparamagnetism.
5.2.1 Superparamagnetic Iron Oxide Nanoparticles

Magnetic nanoparticles ranging from the nanometer to micrometer scale have been used to improve drug targeting, vascular targeting, gene delivery, hyperthermia, and MRI [104, 105, 106, 107]. Superparamagnetic iron oxide nanoparticles (SPION) are the most widely used magnetic nanoparticles because of the biocompatibility of Fe. The main distinctive attribute of SPION is that at this size scale (~ 10 nm) they are superparamagnetic i.e. they generate a high magnetic moment in the presence of an external magnetic field [108]. The remarkable response of SPION to a magnetic flux density allows for the guidance and retention of significant concentrations of the therapeutic moieties at the desired site. Furthermore, the superparamagnetic property allows these particles to convert magnetic energy to heat which is the basis of magnetic hyperthermia.

The nanoparticles used for the studies in this thesis were either synthesized in the lab or commercially available ones bought from Chemicell (Berlin, Germany). Nanoparticle synthesis is performed by Dr Nagesha as explained in [109] using the thermal decomposition method developed by Hyeon et al. [110] with some slight modifications. Briefly, 1.28 g oleic acid was dissolved in 10 mL octyl ether, and then heated to about 100 °C. To this solution, 200 µL of Fe(CO)$_5$ was added. The mixture was refluxed at 280 °C for 1 h and then cooled down to room temperature. While heating, the color of the solution changed from pale-yellow/green to dark black. To this mixture 0.34 g of (CH$_3$)$_3$NO was added along with 500 µL of octyl ether to facilitate complete transfer of the salt. The mixture was then heated to 130 °C for 2 h under argon atmosphere. The temperature was slowly increased to reflux temperature of 280 C and maintained for an additional 1 h. The iron oxide nanoparticles were precipitated out from this solution using ethanol, separated from the supernatant by centrifugation, dried under argon, weighed, and re-dispersed in hexane to obtain a
1.5 mg/mL SPION solution. SPION obtained by this method were typically in the range of 4–6 nm as determined by transmission electron microscopy (TEM) Figure 5.2.

Figure 5.2: TEM images of hydrophobic $Fe_2O_3$ nanoparticles prepared by thermal decomposition method. Individual nanoparticles are seen with no clustering with average size less than 10 nm.

## 5.3 Packing Superparamagnetism

Plain SPION are not stable at normal physiological conditions and show a tendency to aggregate. They often need to be coated with different polymers in order to stabilize them in suspension. Attempts have been made to stabilize SPION by their incorporation into liposomes [111, 112], polymeric micelles [109, 113] as well as by their surface coating with dextran [114] and many other polymers. High accumulation of SPION in target organs is a usual requirement for better MRI contrast and/or AC-assisted hyperthermic cancer therapy. Still, even stabilized SPION when administered parenterally, distribute into tissues and organs non-specifically, with no specific accumulation into areas of interest. Packing SPION into polymeric structures often
brings a specific targeting method with it. We discuss two forms of encapsulation of SPION: in micelles and in liposomes. In both methods, specific targeting of SPION are enhanced by either anti-body conjugation (in case of micelles) or surface charge (in case of liposomes).

5.3.1 Magnetic Immuno-micelles

Polyethylene glycol - phosphatidylethanolamine (PEG–PE)- based polymeric micelles have been proven to be the delivery system of choice for various hydrophobic drugs/diagnostic agents, since they demonstrate a prolonged blood circulation and can be functionalized through PEG water-exposed termini with various target-specific ligands. It has been shown in literature that micelles prepared from PEG–PE conjugates can be efficiently loaded with various poor water-soluble cancer drugs and they accumulate into experimental tumors via the enhanced permeability and retention (EPR) effect [115]. In addition, these micelles can be decorated with various specific ligands attached to their outer surface. Thus, the anti-cancer nucleosome-specific monoclonal antibody 2C5 (mAb 2C5) can be attached to such micelles, which recognizes the surface of a broad variety of tumor cells (but not normal cells) via tumor cell surface-bound nucleosomes, making the resulting immunomicelles capable of specifically targeting a broad variety of tumors [116].

Magnetic immuno-micelles (MIM) packages were prepared by Dr. Sawant as explained in [109]. Briefly, MIM were prepared by lipid film rehydration method. 10 mg of PEG2000–PE in chloroform and 0.25 mg of SPION in hexane were mixed and the organic solvents were removed by rotary evaporator. Any residual solvents were removed using freeze-dryer overnight. The dry film was than rehydrated using 2 mL 10 mM HEPES-buffered saline (HBS) pH 7.4 by vortexing for 5 min followed by the bath sonication for 5 min to get SPION-loaded PEG–PE micelles. Since the transi-
tion temperature of PEG–DSPE is above 37 °C, we have performed the sonication at room temperature with cooling to keep the system below the transition temperature. The micelle size was determined by the dynamic light scattering (DLS) in ZetaPlus particle size analyzer (Brookhaven Instruments Corporation Holtsville, NY, USA) as 35 nm and confirmed via TEM as seen in Figure 5.3.

![TEM image of the encapsulation of multiple iron oxide nanoparticles in the core of the micelle.](image)

**Figure 5.3:** TEM image of the encapsulation of multiple iron oxide nanoparticles in the core of the micelle.

### 5.3.2 Magnetic Cationic Liposomes

Nanoplatforms in the form of liposomes have been employed as drug carrier systems for over 10 years. Since then, many liposomal drug delivery systems have been introduced to the market, the great majority of which have been anti-cancer drug carriers. Liposomal drug delivery systems have the advantages of large size range, the ability to guard encapsulated entities and change surface chemistry, extended plasma life-time, and highly efficient targeting capabilities using different surface coatings [117][118]. In addition, depending on their size they extravasate through fenestrated endothelium in tumors, thereby making them good candidates for targeted therapy [119].

Magnetic cationic liposomes (MCL) are an excellent example of nanocarriers,
demonstrating significant promise in the fields of formulation development, drug targeting, and anticancer therapy [112][120]. When MCL are employed, the improved delivery of drug payloads has been used to improve cancer therapy in preclinical models [121].

MCL were prepared by Aditi Jhaveri as explained in [121]. Briefly, the required amount of lipid (typically around 10 mol/ml) was dissolved in chloroform, and then transferred to a pyrex tube (or round bottom flask). The solvent in the tube was subsequently removed using a rotary evaporator. The temperature was held slightly above the phase transition temperature of the dominant lipid in mixture until a thin film was deposited on the inside wall of the tube. The lipid film was then allowed to freeze-dry for 4 h. The dried lipid film was hydrated with saline. The specific volume added to the dry film depended on experimental purpose. The newly formed preparation was warmed to a temperature slightly above the phase transition temperature of the dominant lipid of preparation, and vortexed intermittently until the lipid film was completely resuspended. To produce a homogeneous mixture of small unilamellar vesicles, liposome preparations were sonicated in a bath type sonicator (Laboratory Supplies Corporation, Hicksville, NY). Unincorporated MAG-C was separated from the MAG-C associated variety by centrifugation at 1000g for 15 min.

5.4 Magnetization Measurements

Magnetization measurements were done by using SQUID magnetometer by Quantum Design (San Diego, CA), more information on SQUID can be found in Appendix B. Since most of the samples for measurements were liquid, a liquid sample holder was necessary. For this purpose, the samples were filled in glass NMR tubes and sealed by fire using a torch. The complete sealing of the samples was necessary to avoid
contamination of SQUID chamber.

First set of measurements was done with SPION and SPION encapsulated in micelles (MIM). The concentration of SPION was kept same, 0.25 mg/mL for both SPION and MIM solutions. All magnetization and field values are in SI units (A/m) 
(For H, 1Oe = $10^3/4\pi$ A/m. For M, 1 emu/cm$^3$ = $10^3$ A/m).

For both of the solutions, 5 different measurements were taken, M-T at 8.0E3 A/m filed and M-H at 4 different temperature.

![M-T curve for (a) bare SPION and (b) SPION-micelle packages.](image)

**Figure 5.4:** M-T curve for (a) bare SPION and (b) SPION-micelle packages.

![M-H curves for SPION at (a) 5 K and (b) 90 K.](image)

**Figure 5.5:** M-H curves for SPION at (a) 5 K and (b) 90 K.

The blocking temperatures of the samples were determined from M-T graphs as
162 K for SPION, Figure 5.4(a) and 106 K for MIM (b). The reason for the differences in the blocking temperatures is the effect of clustering. Micelles prevented the SPION to stick each other.

![Graph Image]

**Figure 5.6:** M-H curves for SPION - micelle packages at (a) 5 K and (b) 150 K.

For M-H graphs, two different behaviors were observed. At low temperatures (below the blocking temperature), hysteresis with small coercivity is observed for both of the samples Figures 5.5(a) and 5.6(a). At high temperatures, (above the blocking temperature) no coercivity is observed, Figure Figures 5.5(b) and 5.6(b). This behavior is expected for superparamagnetic particles. However, M-H curves are not giving the same blocking temperatures as in M-T curves. For SPION, M-H curve at 150 K, with zero coercivity, obviously shows that the blocking temperature is below 150 K, it is not 162 K.

It is also observed that M-H curve can be fitted to Langevin function,

\[
L\left(\frac{H}{H_0}\right) = \coth\left(\frac{H}{H_0}\right) - \frac{1}{H/H_0}. \tag{5.4}
\]

where, \(H_0\) is \(k_BT/\mu[122]\). Table 5.1 shows the results of the analysis of M-T and M-H curves of the SQUID results.
Table 5.1: Comparing different properties of SPION and MIM at different temperatures.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Property</th>
<th>SPION</th>
<th>MIM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( T_B )</td>
<td>162 K</td>
<td>106 K</td>
</tr>
<tr>
<td>applied field</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.3E3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>applied temperature</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 K</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( H_S )</td>
<td>2.3E5</td>
<td>1.9E5</td>
<td></td>
</tr>
<tr>
<td>( H_0 )</td>
<td>5.0E4</td>
<td>2.0E4</td>
<td></td>
</tr>
<tr>
<td>( M_S )</td>
<td>2.2E4</td>
<td>6.0E4</td>
<td></td>
</tr>
<tr>
<td>coercivity</td>
<td>6.7E4</td>
<td>7.2E4</td>
<td></td>
</tr>
<tr>
<td>applied temperature</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>90 K</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( H_S )</td>
<td>9.9E4</td>
<td>1.2E5</td>
<td></td>
</tr>
<tr>
<td>( H_0 )</td>
<td>1.4E4</td>
<td>1.7E4</td>
<td></td>
</tr>
<tr>
<td>( M_S )</td>
<td>2.6E4</td>
<td>8.2E4</td>
<td></td>
</tr>
<tr>
<td>coercivity</td>
<td>8.0E3</td>
<td>3.4E4</td>
<td></td>
</tr>
<tr>
<td>applied temperature</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150 K</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( H_S )</td>
<td>2.4E4</td>
<td>1.1E5</td>
<td></td>
</tr>
<tr>
<td>( H_0 )</td>
<td>1.0E4</td>
<td>1.4E4</td>
<td></td>
</tr>
<tr>
<td>( M_S )</td>
<td>7.0E4</td>
<td>8.9E4</td>
<td></td>
</tr>
<tr>
<td>coercivity</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>applied temperature</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>260 K</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( H_S )</td>
<td>1.2E5</td>
<td>1.0E5</td>
<td></td>
</tr>
<tr>
<td>( H_0 )</td>
<td>1.2E4</td>
<td>1.0E4</td>
<td></td>
</tr>
<tr>
<td>( M_S )</td>
<td>2.7E4</td>
<td>8.1E4</td>
<td></td>
</tr>
<tr>
<td>coercivity</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

As a control experiment, commercially available bulk iron-oxide, not in nano-size, was also measured in SQUID, Figure 5.7. As expected, M-T curve of bulk iron-oxide was completely different. In M-H curve, even at 260 K, coercivity is observed which shows that the sample is ferromagnetic at that temperature. It is also observed that the magnetization of bulk material is much higher than the nano-size particles for every case.

Another set of measurements was done with SPION encapsulated in liposomes. 100 \( \mu L \) of MCL solutions at different concentrations, 0, 2.5, 5 and 10 mg/mL, were measured. They show no coercivity at room temperature, Figure 5.8, showing they sustained their superparamagnetic property at this temperature.
Figure 5.7: M-H curve for micron size iron oxide, even at 260 K it shows coercivity.

5.5 NMR Spectroscopy of SPION

One of the most common applications for SPION is that they behave as excellent MRI contrast agents. Their superparamagnetic property affects the $T_2$ relaxation time of the proton and this effect can be visible through MR image. Nuclear magnetic resonance (NMR) studies were carried out to characterize relaxation properties of SPION.

NMR is a phenomenon which occurs when the nuclei of certain atoms are immersed in a static magnetic field and exposed to a second oscillating magnetic field. Some nuclei experience this phenomenon, and others do not, dependent upon whether they possess a property called spin. In NMR, a magnetic field $B$, along the Z direction is used to align the nuclear spin of unpaired protons, typically in the nuclei of H atom.
5.5.1 Principles of NMR

When the subject body is under a high magnetic field ($\vec{B}_0$) like the magnet of NMR, the magnetic dipole moments of the hydrogen nuclei align in the direction of the field with a slight preference to the parallel direction, Figure 5.9(a). Hence, there is a net magnetization in the direction of $\vec{B}_0$, known as longitudinal magnetization or $M_z$. Due to the intrinsic angular momentum of the hydrogen nuclei, protons not only align but also undergo precession, Figure 5.9(b). The characteristic speed of the precession of the nuclei, Larmor frequency, is proportional to the applied magnetic field and given by the Larmor equation

$$\omega_0 = \gamma_0 \cdot B_0.$$  (5.5)
where $\omega_0$ is the Larmor frequency in megahertz, $\gamma_0$ is the gyromagnetic ratio (42.58 MHz/T for hydrogen nucleus) and $B_0$ is the strength of the magnetic field in Tesla [123].

Figure 5.9: (a) Magnetic dipole moments align with the applied magnetic field. (b) Magnetic dipole moments precess under applied magnetic field with the frequency called Larmor frequency.

A radio frequency (RF) pulse at the same Larmor frequency can excite the nuclei under the magnetic field causing the longitudinal magnetization to become perpendicular to the direction of $\vec{B}_0$, given that it is applied long enough. The plane perpendicular to the $\vec{B}_0$ is called as transverse (xy-) plane and the magnetization that lies on this plane is called transverse magnetization or $M_{xy}$. The transverse magnetization precess about z-axis with all protons in phase and induce the MR signal at Larmor frequency.

When the RF pulse is turned off, the system seeks its lowest energy by spins going back to the lowest energy state and by spins getting out of phase [124]. Hence, MR signal rapidly fades due to two simultaneous and independent processes i.e. spin-lattice interaction causing $T_1$ longitudinal relaxation and spin-spin interaction causing $T_2$ transverse relaxation, Figure 5.10.
Figure 5.10: Relaxation of excited spins through (a) spin-lattice interaction ($T_1$) and (b) spin-spin interaction ($T_2$).

$T_1$ Relaxation

As transverse magnetization decays, longitudinal magnetization recovers slowly, Figure 5.10(a), this process is known as longitudinal or $T_1$ relaxation because it refers to the time it takes for the spins to realign the longitudinal axis ($z$-axis). The spins turn to ground state by dissipating the energy obtained from RF pulse back to the surrounding lattice. The time constant of this relaxation, $T_1$, depends on the strength of the applied field $\vec{B}_0$ and internal motion of the molecules. The equation governing $T_1$ as a function of the time $t$ after its displacement is:

$$M_z = M_0(1 - \exp(-\frac{t}{T_1})).$$

(5.6)

$T_1$ can be defined as the time required to change the $z$ component of magnetization by a factor of e.
Magnetization on the z axis produces no signal in the detector. Only when magnetization tipped to x-y plane by a pulse is a signal produced. Recovery rates are measured by inverting the equilibrium magnetization with a 180° pulse, and watching the recovery to equilibrium. The 180° pulse tips the magnetization from +z to -z direction. Because z-magnetization does not produce signal, the recovery is monitored in a series of spectra, in which the z-magnetization at various times after the 180° are monitored by applying additional 90° pulse \[125\]. A sample measurement is shown in Figures 5.11.

![Figure 5.11: Monitoring z-magnetization recovery after 180° pulse. The sample used for this graph is the group 1A (Table 5.2).](image)

The z-magnetization values are recorded for different time intervals and the data is fitted to Equation 5.6 as seen in Figure 5.12.

**T₂ Relaxation**

As mentioned before, after the applied RF pulse all spins precess about the z-axis in phase. While relaxing, spins get out of phase by two phenomena namely, field inhomogeneities and interaction between spins. Magnetic field inhomogeneities over the sample volume is an instrumental imperfection and the reason of the magnetic field optimization, or *shimming*, for each sample.

The second reason for magnetic field differences is the spin-spin interactions in the sample, which represent natural relaxation process. All the nuclei produce small
Figure 5.12: Curve fitting of the data collected after 180° pulse. The sample used for this graph is from group 1 in Table 5.2.

magnetic fields ($\triangle B$) during the precession as seen in Figure 5.13. The field acting on proton number 1 is

$$B_1 = B_0 + \triangle B.$$  \hspace{1cm} (5.7)

where $B_0$ is the applied external field and $\triangle B$ is the effect of proton number 2. Similarly, the field on proton number 2 will be

$$B_1 = B_0 - \triangle B.$$  \hspace{1cm} (5.8)

The differences on the field affecting on the protons will cause slightly different precessing frequencies and lead to dephasing. When all the spins are out of phase with each other their vectorial sum will be zero, Figure 5.10(b). The spin-spin interaction effect depends on the proximity of the spins to each other, meaning dephasing will occur at different time scales for different tissue types.

The time constant which describes the return to equilibrium of the transverse
magnetization, $M_{xy}$, is called the spin-spin relaxation time, $T_2$

$$M_{xy} = M_{xy}^0 \exp\left(\frac{-t}{T_2}\right)$$  \hspace{1cm} (5.9)

The $T_2$ decay is observed through this exponential decay is called as free induction decay (FID) which is a plot of signal intensity versus time.

The combined relaxation time, for both field inhomogeneities and spin-spin interactions, is given by $T_2^*$

$$\frac{1}{T_2^*} = \frac{1}{T_2} + \frac{1}{T_2^\dagger}$$  \hspace{1cm} (5.10)

where $T_2$ is the relaxation time from the genuine relaxation process, $T_2^\dagger$ is from the field inhomogeneities [126].

Fourier transformation of the time domain exponential decay, FID, gives a Lorentzian in frequency space with a full width half maximum (FWHM)

$$\Delta \omega = \frac{1}{\pi T_2}.$$  \hspace{1cm} (5.11)

For samples with long $T_2$, bigger time constants in Equation 5.9, accordingly have very narrow line width and vice versa. The examples of measurements through time
and frequency domains are given in Figures 5.14 and 5.15.

### 5.5.2 NMR Experiments

All the measurements were done by Varian INOVA 500 MHz NMR spectrometer and with the help of Dr Roger Kautz. First set of experiments were done with plain micelles. Five different micelle formulations were prepared, micelles with SPION (MIM), MIM with unspecific anti-body (IgG), MIM with specific anti-body (2C5) and for control plain micelles and micelles with 2C5 anti-body. Each group had two different concentrations of micelles, 1.0 and 2.0 mg/mL.

<table>
<thead>
<tr>
<th></th>
<th>$T_1$ (s)</th>
<th></th>
<th>$T_2$ (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>formulations</td>
<td>cells</td>
<td>formulations</td>
</tr>
<tr>
<td>micelle concentration</td>
<td>1 mg/mL</td>
<td>2 mg/mL</td>
<td>1 mg/mL</td>
</tr>
<tr>
<td>plain micelles</td>
<td>3.2 ± 0.1</td>
<td>3.1 ± 0.1</td>
<td>3.1 ± 0.1</td>
</tr>
<tr>
<td>2C5 - micelles</td>
<td>3.2 ± 0.1</td>
<td>3.2 ± 0.1</td>
<td>3.1 ± 0.1</td>
</tr>
<tr>
<td>2C5 - SPION - micelles</td>
<td>3.0 ± 0.1</td>
<td>2.5 ± 0.1</td>
<td>3.1 ± 0.1</td>
</tr>
<tr>
<td>SPION - micelles</td>
<td>2.9 ± 0.1</td>
<td>2.3 ± 0.1</td>
<td>3.1 ± 0.1</td>
</tr>
<tr>
<td>IgG - SPION - micelles</td>
<td>3.0 ± 0.1</td>
<td>2.6 ± 0.1</td>
<td>3.2 ± 0.1</td>
</tr>
<tr>
<td>only cells</td>
<td></td>
<td>3.2 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>

These formulations were incubated for 2 hours with human breast carcinoma MCF-7 (American Type Culture Collection, Manassas, VA, USA) cells. After the incubation, the cells were washed using three 1 mL washes of Hanks Balanced Salt Solution (HBSS). The cells were then digested using trypsin and collected in HBSS.
Figure 5.14: Determination of $T_2$ relaxation time for a long $T_2$ sample. (a) Measurement in frequency domain is not possible due to very narrow peak. (b) FID curve in time domain, the time point where envelope of the decay meets 1/e is the relaxation time. The sample used for this graph is from group 2 in Table 5.2.
Figure 5.15: Determination of $T_2$ relaxation time for a short $T_2$ sample. (a) Measurement in time domain is not possible due to short relaxation time. (b) FWHM value of Lorentzian curve provides the relaxation time. The sample used for this graph is from group 4 in Table 5.2.
Triplicates of each sub-group prepared for the measurements, the micelle formulations and cell work was done by Dr Sawant. $T_1$ and $T_2$ relaxation times for the formulations and cell samples were measured at room temperature. Results summarized in Table 5.2 and Figure 5.16.

![Graph showing $T_2$ values for different formulations and cell samples.](image1)

**Figure 5.16:** $T_2$ values of NMR micelle measurements.

![Graph showing $R_2$ values for cells-MIM, normalized by protein amount.](image2)

**Figure 5.17:** $R_2$ values of cells-MIM, normalized by protein amount.

The results show definite difference in $T_2$ values between groups with and without SPION in formulations. However, in cells the difference between the groups drop by
more than 10 folds due to high concentration of proteins and other molecules inside
the cells hence less water protons to give signal. Due to the same reason we can see
the difference in $T_2$ values in formulation depending on the presence of anti-bodies.
The relaxation rate $R_2$, inverse of the relaxation time, of the samples were calculated
and normalized with the protein content as seen in Figure 5.17. In Table 5.3, we
see the effect of specific anti-body on cell attachment through percent difference in
relaxation rate.

**Table 5.3**: Normalized relaxation rate values for micelle study.

<table>
<thead>
<tr>
<th>micelle concentration</th>
<th>$R_2$ (1/s) 1 mg/mL</th>
<th>$R_2$ (1/s) 2 mg/mL</th>
<th>% difference in $R_2$ a</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPION - micelles</td>
<td>4.30 ± 0.02</td>
<td>5.20 ± 0.05</td>
<td>55%</td>
</tr>
<tr>
<td>IgG - SPION - micelles</td>
<td>4.30 ± 0.01</td>
<td>6.00 ± 0.02</td>
<td>55%</td>
</tr>
<tr>
<td>2C5 - SPION - micelles</td>
<td>5.60 ± 0.01</td>
<td>7.10 ± 0.02</td>
<td>66%</td>
</tr>
<tr>
<td>plain cells</td>
<td>1.90 ± 0.01</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

a compared to plain cells

The second set of NMR spectroscopy experiments were done with magnetic cationic
liposomes (MCL). MCL with 10mg/mL SPION loading was diluted to achieve five
different SPION concentrations. Relaxation rates of each dilution measured as ex-
plained in Section 5.5.1. The results are given in Table 5.4.

**Table 5.4**: Relaxation time and rate values for MCL study.

<table>
<thead>
<tr>
<th>SPION (mM)</th>
<th>$T_1$ (s)</th>
<th>$R_1$ (1/s)</th>
<th>$T_2$ (s)</th>
<th>$R_2$ (1/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>43</td>
<td>0.13 ± 0.01</td>
<td>7.5 ± 0.006</td>
<td>1.80E-4 ± 0.02E-4</td>
<td>5561 ± 1</td>
</tr>
<tr>
<td>22</td>
<td>0.19 ± 0.01</td>
<td>5.2 ± 0.003</td>
<td>3.25E-4 ± 0.10E-4</td>
<td>3079 ± 2</td>
</tr>
<tr>
<td>4.3</td>
<td>0.77 ± 0.02</td>
<td>1.3 ± 0.001</td>
<td>9.65E-4 ± 0.30E-4</td>
<td>1037 ± 1</td>
</tr>
<tr>
<td>0.43</td>
<td>1.54 ± 0.04</td>
<td>0.6 ± 0.001</td>
<td>3.03E-3 ± 0.11E-3</td>
<td>330 ± 1</td>
</tr>
<tr>
<td>0</td>
<td>3.10 ± 0.03</td>
<td>0.3 ± 0.001</td>
<td>2.6 ± 0.1</td>
<td>0.385 ± 0.001</td>
</tr>
</tbody>
</table>

The quality of a contrast agent is defined by its relaxivity which shows the effect
on proton relaxation versus contrast agent concentration and is represented by $r_1$
and \( r_2 \) for \( T_1, T_2 \) relaxations respectively. The relaxivity values for MCL (10mg/mL loading) was then calculated as \( r_1 = 0.17 \text{ mM}^{-1}\text{s}^{-1} \) and \( r_2 = 125 \text{ mM}^{-1}\text{s}^{-1} \). The high \( r_2 \) relaxivity of MCL proves it as an excellent \( T_2 \) contrast enhancement agent.

\[ \] 5.6 Magnetic Hyperthermia

The feasibility of treating cancer by induced hyperthermia has led to the development of many different methods designed to heat up tumor cells, magnetic hyperthermia is one of such methods. In general, the procedure involves introducing magnetic particles throughout the target tissue, and then applying an AC magnetic field of sufficient strength and frequency to cause the particles to heat. This heat conducts into the immediately surrounding diseased tissue and the cancer is destroyed after applying long enough. However, there is an important drawback in hyperthermia field that is keeping the temperature under control. Most of the cancerous tumors are surrounded by healthy tissues and coincidental heating of these healthy tissues is unacceptable. Magnetic particle hyperthermia is appealing because it offers a way to ensure only the intended target tissue is heated.

Physical basis behind magnetic hyperthermia can be summarized as conversion of electromagnetic energy into heat. For small field changes and ignoring effect of magnetic particles on each other, the magnetization response to an AC field can be described as

\[ P_{SPION} = \mu_0 \pi f \chi'' H^2 \]  

(5.12)

where \( P_{SPION} \) is the heat generated by unit volume of SPION, \( \mu_0 \) is permeability of free space, \( f \) is the frequency of the applied field, \( \chi'' \) is the imaginary part of complex susceptibility and \( H \) is the field amplitude [100].

The frequency and strength of applied magnetic field is limited by physiological
responses to high frequency magnetic fields [127]. These physiological responses include stimulation of peripheral and skeletal muscles, possible cardiac stimulation or non-specific inductive heating of healthy tissue. The safe region for humans is accepted to be between 0.05-1.2 MHz for the frequency and 0-15 kA/m for the field amplitude.

![Figure 5.18](image)

**Figure 5.18:** Temperature increase due to hyperthermia, different concentration of MCL giving different temperature curves.

The quality of hyperthermia agents are determined by the specific absorption rate (SAR). Specific absorption is defined by the fraction of the incremental energy ($dE$) absorbed by incremental mass ($dm$). The time derivative of specific absorption gives SAR [128]

$$\text{SAR} = \frac{d}{dt} \left( \frac{dE}{dm} \right).$$

(5.13)

$dE$ can be calculated by

$$dE = m_m c_m dT$$

(5.14)

where $m_m$ and $c_m$ refers to the mass and specific heat of the medium. When we use
Equation 5.14, the Equation 5.13 becomes

$$\text{SAR} = m_m c_m \frac{d}{dt} \left( \frac{dT}{dm} \right). \quad (5.15)$$

SAR value of MCL was measured through hyperthermia curves in Figure 5.18 by calculating of the slope of the curves while they are linear at the beginning, Table 5.5, there is a finite value for only liposomes due to heat induction by the hyperthermia coil. The SAR value, now, can be calculated by taking slope of $dt/dm$ vs MCL mass and using specific heat of water ($4.2 \text{ J} \text{C}^{-1} \text{g}^{-1}$) can be found as $\sim 60 \text{ W} \text{g}^{-1}$.

Table 5.5: The slope of hyperthermia curves for different concentration of MCL

<table>
<thead>
<tr>
<th>MCL (mg)</th>
<th>$dT/dt$ (C/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.008</td>
</tr>
<tr>
<td>2.5</td>
<td>0.128</td>
</tr>
<tr>
<td>5</td>
<td>0.086</td>
</tr>
<tr>
<td>10</td>
<td>0.126</td>
</tr>
<tr>
<td>20</td>
<td>0.321</td>
</tr>
</tbody>
</table>
Chapter 6

Magnetic Nanoparticles in-Vivo: MRI Contrast Agents and Magnetic Targeting

6.1 Introduction

In the previous chapter, we explained properties of superparamagnetic iron oxide nanoparticles (SPION) and encapsulating them into different entities like micelles or liposomes. Our approach in this chapter involves combining such nanostructures into a versatile multi functional theranostic nanoplatform for enhanced drug delivery and monitoring of cancer treatment. The nanoplatform combines the advantages of drug delivery using liposomes, active targeting via magnetic guidance and monitoring of treatment using non-invasive imaging techniques. It has been shown that cationic liposomes preferentially target tumor vessels compared to vessels in normal tissues; however, the interaction of cationic liposomes with tumor vessels is heterogeneous [129]. To target tumor vessels more uniformly we seek to combine the electrostatic
properties of cationic liposomes with the strength of external magnetic guidance by incorporating SPION into long circulating PEGylated cationic liposomes to produce magnetic cationic liposomes (MCL), which have an extraordinarily high affinity for the tumor vascular supply following intravenous administration.

Although the general distribution of MCL within the tumor following intravenous and intratumoral injection can be predicted on the basis of their unique formulation characteristics, few imaging techniques can accurately determine the exact location and functional effects of magnetic nanosystems within a developing tumor mass. For this reason, we have introduced a highly sensitive approach to observe the functional distribution of MCL using a combination of MRI contrast enhancement and biodistribution studies in vivo.

Previously the feasibility of SPION incorporated, drug loaded cationic liposomes for magnetic targeting of tumor vasculature has been reported [130]. In this chapter, we report the efficacy of MCL as MRI probes for the monitoring of the magnetic tumor targeting.

6.2 Magnetic Resonance Imaging

MRI is a powerful, noninvasive technique which can provide real time soft tissue information with high resolution [131]. The power of MRI is further enhanced by many different types of contrast mechanisms in endogenous tissues like biochemical, density or diffusion contrast. Different image contrast techniques can be used to evaluate anatomy of soft tissues as well as their physiological function [132]. The image quality of MRI can be further improved by utilizing contrast agents which alter the proton relaxation rates that enhance image contrast [133][134]. The loading of SPION in magneto-liposomes allows them to be utilized as excellent MRI contrast
agents. They induce a significant reduction in \( T_2 \) and \( T_2^* \) relaxation times which leads to negative contrast on MR images; thus the presence of MCL in the body can be monitored easily.

MR imaging for this study was performed on a 7 T preclinical MRI system (BioSpec 70/20USR, Bruker BioSpin Corp., Billerica, MA) at the Center for Translational Neuro-Imaging at Northeastern University. A whole body quadrature coil was used for reception and emission. All animals were scanned before and after the administration of MCL.

### 6.2.1 MRI Image contrast

Three main intrinsic features of the tissue play a role in the construction of MR image: (i) proton density, (ii) the time \( T_1 \) and (iii) the time \( T_2 \). (For more information on relaxation times, see Chapter 5.5.1.) The proton density determines the maximum signal that can be achieved from the tissue. The more water molecules in the material, the more signal is obtained. On the other hand, \( T_1 \) and \( T_2 \) affect signal intensity indirectly. All the three parameters are the property of the material and cannot be changed. However, it is possible to get different contrast images from the same materials. These intrinsic features can be exploited by manipulating other parameters and that requires different image weighting for different tissues or purposes.

#### \( T_1 \) weighting

The same slice will be excited and recorded many times for successive MR imaging. The time difference between two consecutive RF pulse is known as repetition time or \( TR \) for short. For a given TR, the image signal strongly depends on the \( T_1 \) relaxation time [135]. In the case of long TR, more of the excited spins are recovered and contribute to \( M_z \). So for the next pulse, there is more longitudinal magnetization to
excite resulting stronger MR signal. In the case of short TR, there are two different outcomes.

While the tissues with short $T_1$ will recover in this short time, the tissues with long $T_1$ can undergo only little relaxation and lose the signal strength in overall process. Hence, with short TR, tissues with short $T_1$ appear bright (more signal) whereas tissues with long $T_1$ appear dark (signal lost). This dependency on $T_1$ is known as $T_1$ weighting and thus the scans with short TR produce heavily $T_1$ weighted images. Figure 6.1 explains this the TR dependence of z-magnetization.

![Figure 6.1: Interaction of TR with z-magnetization. For tissues with different $T_1$ values like in this graph, long TR results very little difference between the signal originating from these tissues. On the other hand, short TR results large signal difference which supply plenty of $T_1$ information making the image $T_1$ weighted.](image)

$T_2$ weighting

After exciting the tissue, the MR signal is not received immediately but after a delay. The time between the excitation and acquisition is called *echo time* or $TE$ for short. Tissues with different $T_2$ values can produce altered signal intensities depending on $TE$. In the case of long $TE$, this dependence of the image on $T_2$ is known as $T_2$ weighting. Figure 6.2 explains this the TE dependence of transverse magnetization.
Figure 6.2: Interaction of TE with transverse magnetization. TE indicates the time span between excitation and measurement. For tissues with different $T_2$ values like in this graph, short TE results very little difference between the signal originating these tissues. On the other hand, long TE results large signal difference which supply plenty of $T_2$ information making the image $T_2$ weighted.

The power of MRI lies behind the flexibility of the image contrast by changing the extrinsic parameters. By manipulating TR and TE values same sample can reveal different information. However, choosing the right TR or TE is not always an easy task[136]. This phenomena can be explained better with a sample illustration, Figure 6.3.

**Contrast agents**

In cases that manipulation of external parameters, like TR or TE, are not enough for a clear contrast there is a need to manipulate the intrinsic parameters. Both $T_1$ and $T_2$ times can be manipulated, shorten, by introduction of contrast agents. The structures with shortened $T_1$ appears brighter on the $T_1$ weighted images and this effect is referred as positive contrast. The structures with shortened $T_2$ on the other hand, appears darker on the $T_2$ weighted images, this effect also known as the negative contrast. The contrast agents shortening $T_1$ known as $T_1$ contrast agents and the ones shortening $T_2$ known as $T_2$ contrast agents. The contrast agents affect the relaxation
Figure 6.3: In this illustration of TE effect on image information, both healthy tissue and tumor has long $T_1$ values so the contrast must be weighted over $T_2$ to differentiate them. In this example, while the images with $TE_1$ or $TE_3$ will supply enough contrast between tumor and healthy tissue, in the image with $TE_3$ the tumor is indistinguishable.

times as shown in

$$\frac{1}{T_{1,2}} = \frac{1}{T_{1,2}^0} + r_{1,2}C. \quad (6.1)$$

where $T_{1,2}^0$ is the relaxation time before introduction of the agent, $r_{1,2}$ and $C$ are the relaxivity and the concentration of the agent.

$T_1$ contrast agents are generally paramagnetic and great majority of them are Gadolinium compounds and they work via dipolar mechanism. There are two ways that a paramagnetic agent works, first the inner-sphere effect where the bound water is relaxed efficiently by rapid chemical exchange with the agent. The water not contained in this sphere can be relaxed by the agent and this effect known as outer-
sphere relaxation. Outer-sphere relaxation is more complex and does not involve bonding or chemical exchange. It is the result of rotational or translational diffusion of water. Simply put, the more water can approach and interact the agent the greater the effect of the contrast agent[137].

$T_2$ contrast agents are generally superparamagnetic. Due to their high magnetization under applied field, they create local magnetic field gradients and reduce $T_2$ relaxation time of nearby H nuclei due to spin dephasing, section 5.5.1. Their effect also is referred as *susceptibility effect* and it is field dependent.

There are certain criteria for a compound to be used as contrast agents [138] and can be summarized as

1. They should alter the relaxation times necessary to reach a contrast. They should also be efficient in enhancing the contrast at low concentration in order to minimize the dose.

2. They should have some tissue / organ specificity which results higher concentration of accumulation in specific part of the body. And they should stay where they introduced long enough for imaging.

3. They should have non or low toxicity in the concentration required for contrast.

4. They should be stable for a reasonable amount of time while they are stored.

### 6.2.2 Efficacy of MCL on MRI contrast

The efficacy of MRI contrast is normally evaluated in terms of the values of relaxivities, $r_1$ and $r_2$. The higher the ratio $r_2/r_1$ is the better efficiency of a $T_2$ contrast agent. In the case of MCL formulation used in this study, the relaxivity ratio is quite
high due to the superior SPION loading, as the higher the internal SPION concentration the better the contrast obtained [139]. The lipid bilayer provides a protective barrier that alters the interaction of water molecules with the contrast agent and increases local concentration, both of which alter relaxation rates. The modulation of water transport restricts $T_1$ relaxation which is confirmed by our low $r_1$ value. The high susceptibility and high local concentration of SPION modify the local magnetic field altering $T_2$ relaxation. These two independent mechanisms both contribute to an enhanced relaxation rate enhancement that establishes the efficacy of MCL as an exceptional MRI contrast agent.

The result of high SPION payload (10 mg/mL) shows itself in saturation magnetization, $M_S = 2.1 \times 10^4 \, A/m$, and relaxivity measurements, $r_1 = 0.17 \, mM^{-1} \, s^{-1}$ and $r_2 = 120 \, mM^{-1} \, s^{-1}$. The relaxivity ratio $r_2/r_1 = 700$ reveals excellent contrast capabilities and this effect was immediately evident through negative contrast on $T_2$-weighted MR images, Figures 6.9 and 6.12. In healthy animal study, Figures 6.5 SI in $T_2$-weighted images decreased 70% in the spleen and up to 90% in the liver one hour after administration, demonstrating a strong contrast due to MCL.

### 6.2.3 MRI Phantom Studies

To confirm the effect of MCL on $T_2$ relaxation time, different concentrations of MCL solution were imaged with MRI. MCL solutions were filled in capillary tubes and fixed around a 50 mL centrifuge tube which was used as a skeleton for the capillaries. Gradient-echo sequence (Table 6.2) was used for scanning the capillaries. $T_2^*$ calculations were performed on a voxel by voxel basis by using linear regression analysis on the voxel signal intensities. The signal intensities were measured in each of the successive axial images obtained by the MR sequence. Numeric fitting and all relevant quantitative MR image analysis for the construction of the $T_2^*$ values was performed.
using Bruker user interface ParaVision software. The resulting image and $T_2^*$ values are given in Figure 6.4 and Table 6.1.

![Image of MR images](image)

**Figure 6.4**: Axial view MR images of different concentrations of MCL with two controls, namely a: only liposome and m: only PBS solution. The concentrations and $T_2^*$ values of this image are given in Table 6.1.

<table>
<thead>
<tr>
<th>concentration (µg/mL)</th>
<th>$T_2^*$ (ms)</th>
<th>concentration (mg/mL)</th>
<th>$T_2^*$ (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>Liposomes only</td>
<td>83</td>
<td>g</td>
</tr>
<tr>
<td>b</td>
<td>0.1</td>
<td>84</td>
<td>h</td>
</tr>
<tr>
<td>c</td>
<td>0.5</td>
<td>50</td>
<td>i</td>
</tr>
<tr>
<td>d</td>
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<td>40</td>
<td>j</td>
</tr>
<tr>
<td>e</td>
<td>5</td>
<td>28</td>
<td>k</td>
</tr>
<tr>
<td>f</td>
<td>10</td>
<td>17</td>
<td>l</td>
</tr>
</tbody>
</table>

$\dagger$ $T_2^*$ value is too low to measure.

Table 6.1: $T_2^*$ values of MRI phantom study.

MRI phantom results showed that $T_2^*$ values of MCL solutions were distinguishable at as low as 0.5µg/mL concentration. These results are in line with the high relaxivity values of MCL obtained by NMR.
6.3 In-vivo Studies

6.3.1 Blood Pool

First a healthy animal model, 10 week old SCID mice, was used for optimizing the MR sequence for contrast imaging. Animals were imaged before and immediately after a tail vein injection of 100 µL of a 5 mg/mL of MCL. This initial study showed where magnetic cationic liposomes (MCL) accumulate and whether or not the MR method was successful in monitoring MCL. With the help of pre and post images (Figure 6.5) we decided using three different imaging sequence, one for $T_1$ weighted one for $T_2$ weighted and another for $T_2^*$ mapping. Details of the chosen MR sequences are given in Table 6.2.

The mice were initially anesthetized with a cocktail mixture of ketamine/xylazine and kept under exposure of isofluorine during the imaging. After anesthetization, a catheter was placed into the tail vain of the animals. An injector with 100 µL MCL was attached to the catheter with a long tube ready for administration. When the mice were placed in MRI, the injector was secured outside so that administration can be done without moving the animals.

<table>
<thead>
<tr>
<th>sequence</th>
<th>TR(msec)/TE(msec)</th>
<th>flip angle</th>
<th>Slice (1 mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_2$</td>
<td>Turbo-RARE</td>
<td>4200/12</td>
<td>-</td>
</tr>
<tr>
<td>$T_1$</td>
<td>RARE spin-echo</td>
<td>1738/10</td>
<td>-</td>
</tr>
<tr>
<td>$T_2^*$</td>
<td>gradient-echo</td>
<td>722.7/4.9</td>
<td>30</td>
</tr>
<tr>
<td>$T_2^*$ map$^1$</td>
<td>gradient-echo$^2$</td>
<td>2763/12.6</td>
<td>90</td>
</tr>
</tbody>
</table>

6.3.2 Tumor Model

Animal experiments were conducted at Northeastern University and animal protocols were approved by The Division of Laboratory Animal Medicine (DLAM) and
the Northeastern University-Institutional Animal Care and Use Committee (NU-IACUC). We used 8-10 week old male SCID mice for MRI and biodistribution studies. Metastatic melanoma B16-F10 cells (ATCC, Manassas, VA) were injected to the right flank or dorsal region of the mice, depending on the study and then allowed to grow until the tumor reached appropriate size, typically $250 - 300 \text{ mm}^3$. The mice were initially anesthetized with cocktail mixture of ketamine/xylazine prior to administration of MCL (100 $\mu$L), and were exposed to a required amount of isofluorine continuously for the duration of the imaging acquisition process. Two different administration
method was used, intravenously (IV), by tail injection and intratumoral (IT) by injection directly in the tumor mass. The experimental procedure is summarized in Figure 6.6.

6.3.3 MR Signal Analysis

MR images were processed using MATLAB and MIVA (Medical Image Visualization and Analysis Software) to select regions of interest. Quantitative analysis of tumors was performed by carefully choosing regions of interest (ROI) in the bulk and calculating average signal intensities (SI) from individual voxels. The average SI of adjacent muscle ROI were also chosen as a reference and the tumor/muscle ratio (TMR) was calculated for all animals. This ratio was used to obtain the contrast enhancement before and after injection. In the case for magnetically targeted tumors, the area directly adjacent to where the magnet was placed was treated separately since inhomogeneous magnetic force targets mostly this area. This area was referred to as the magnet targeted area and quantified separately from the rest of the tumors. In addition to TMR, two animals from each group were used to calculate $T_2^*$ values.
before and after injection. These values were obtained choosing small square ROI (< 3mm^2) in desired areas of the $T_2^*$ maps obtained and calculating their averages. The $T_2^*$ values obtained from $T_2^*$ maps, as explained in section 6.2.3, can be used to estimate local concentration of MCL. Change of relaxation rates after the administration of contrast agents is directly proportional to the concentration of the agent, given by the equation

$$R_{1,2} = R_{1,2}^{\text{int}} + r_{1,2} \cdot C$$  \hspace{1cm} (6.2)

where $R_{1,2}$ is the relaxation rate ($1/T_{1,2}$), is the relaxation rate before the addition of the contrast agent, $r_{1,2}$ is relaxivity and $C$ is concentration of the agent.

6.4 Magnetic Targeting

Magnetic targeting was achieved using a $1cm^2$, 0.4 T magnet attached over the tumor area using surgical tape. The magnet was left in place only during first hour post injection. Time line of the injection, magnet application and imaging is summarized in Figure 6.7. Tumor bearing mice were divided into four subgroups according to MCL injection site and magnet application. Eight mice were chosen to be injected in the tail vein; four of such mice had magnet applied for one hour, while four others used as control, namely IV injection with applied magnet (IV-M) and IV injection non-magnet (IV-NM) groups. Correspondingly eight mice were chosen to be injected intratumourally; four of such mice had magnet applied for one hour while the other four used as control, namely IT injection with applied magnet (IT-M) and IT injection non-magnet (IT-NM) groups. The definition of the animal groups is summarized in Table 6.3.
Figure 6.7: Time line for the course of study.

Table 6.3: Definition of animal groups.

<table>
<thead>
<tr>
<th>Group Name</th>
<th>number of mice</th>
<th>MCL administration</th>
<th>Magnetic Guidance</th>
<th>MRI</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV-NM</td>
<td>4</td>
<td>IV</td>
<td>no magnet</td>
<td>0 hours 24 hours</td>
</tr>
<tr>
<td>IV-M</td>
<td>4</td>
<td>IV</td>
<td>with magnet</td>
<td>0 hours 24 hours</td>
</tr>
<tr>
<td>IT-NM</td>
<td>4</td>
<td>IT</td>
<td>no magnet</td>
<td>0 hours 24 hours</td>
</tr>
<tr>
<td>IT-M</td>
<td>4</td>
<td>IT</td>
<td>with magnet</td>
<td>0 hours 24 hours</td>
</tr>
</tbody>
</table>

6.4.1 Magnetophoretic Guidance

The force of the applied magnet field on the MCL for the magnetic guidance can be calculated by

\[
\overrightarrow{F}_m = \frac{1}{2} \mu_0 V_L \nabla (\overrightarrow{M} \cdot \overrightarrow{H})
\]  

(6.3)

where \( \mu_0 \) magnetic permeability, \( V_L \) is volume of a liposomes, \( \overrightarrow{M} \) is volumetric magnetization[140].

The equation can be simplified for constant magnetic field gradient and low concentration of magnetic particles i.e. external field is not affected by the presence of magnetic particles, hence written as[141]

\[
\overrightarrow{F}_m = V_L M_z \frac{\partial B_z}{\partial z} \hat{z}.
\]  

(6.4)
Magnetization (M) of MCL reaches 83% of the saturation magnetization \( M_S = 2.1 \times 10^4 \text{ A/m} \) around 0.145 T magnetic field which is the field value 0.5 cm away from the magnet, Figure 6.8. Thus, magnetophoretic force on one MCL 0.5 cm away from the magnet can be estimated as \( 3 \times 10^{-15} \text{ N} \), this is the estimated force due to applied magnet which is responsible for magnetic targeting. The magnetophoretic velocity of MCL \( (u_m) \) due to this force can be calculated by balancing hydrodynamic drag force, Equation 6.5, with the magnetic force [142], resulting \( u_m = 0.5 \mu\text{m/s} \).

\[
\vec{F}_d = -3\pi\eta D \vec{v}.
\] (6.5)

Considering diameter of the blood vessels, this speed is fast enough to guide liposomes to vessel walls from where they can extravasate to tumor mass. And the result of the magnetophoretic guidance is self-evident by the contrast difference in MR images between the magnet and non magnet groups.

**Figure 6.8:** (a) Change in the magnetic field of the magnet used in the studies. (b) SQUID results of normalized magnetization (M) by saturation magnetization \( (M_S) \) as a function of applied magnetic field (B).
6.4.2 IV administration

For the IV injection groups, the difference in tumor accumulation of MCL between the groups with or without applied field is quite clear in the MR images, Figure 6.9. With magnetic guidance, there is a remarkable signal decrease in the tumor, indicating greater MCL accumulation with magnet than the no magnet group. Diffusion through tumor bulk with magnetic guidance was more efficient than the group without. The uniform darkening of the target area illustrates the effect of the external magnet where a more homogeneous accumulation is achieved as compared to non magnet group.

![Figure 6.9: \(T_2\) images from IV group without (NM) and with (M) applied magnet. Top and bottom rows show images of same mouse before and 24 hours after the administration of MCL. The darkening of the tumors indicates the diffusion of MCL from blood through tumors. Accumulation in the tumor part where magnet localized on top (shown with dashed rectangle), is quite distinguishable than the rest of the tumor.]

The magnet was applied during the first hour of injection while imaging was done 24 hours after the injection. Hence, the images demonstrate the ability to not only target MCL towards the tumor, but to retain such accumulation. One animal was chosen to be imaged after 48 hours following injection to further investigate MCL
retention. The results illustrate significant accumulation and retention even after 48 hours post injection Figure 6.10.

Figure 6.10: $T_2$ images from IV group with magnet before, 24 and 48 hours after the administration. External magnet was fixed on the tumor during first hour after injection. The images were obtained without further administration or magnetic guidance.

$T_2^*$ maps shown in Figure 6.11 reflect a spatial distribution of $T_2^*$ values before injection and after injection. In animal shown from group IV-M significant decrease in $T_2^*$ values in the bulk of the tumor is evident where $T_2^*$ values went from approximately 60 ms before injection to as far down as 30 ms after injection. In the animal shown from group IV-NM heterogeneous accumulation around tumor bulk is observed throughout. $R_2^*$ ($1/T_2^*$) values showed 2-fold increase for tumor comparing magnet and non magnet group, Table 6.4, demonstrating twice as much accumulation of MCL with magnetic targeting.

Table 6.4: Change in $R_2^*$ relaxation rate and MCL accumulation ratio for different organs. Accumulation ratio was derived by using Equation 6.2.

<table>
<thead>
<tr>
<th></th>
<th>$\Delta R_2$ ($s^{-1}$)</th>
<th>Accumulation Ratio</th>
<th>$\Delta R_2$ ($s^{-1}$)</th>
<th>Accumulation Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IV-NM</td>
<td>IV-M</td>
<td>(M/NM)</td>
<td>IT-NM</td>
</tr>
<tr>
<td>Tumor</td>
<td>0.003 ± 0.003</td>
<td>0.006 ± 0.005</td>
<td>2</td>
<td>0.1 ± 0.2</td>
</tr>
<tr>
<td>Liver</td>
<td>0.1 ± 0.2</td>
<td>0.1 ± 0.1</td>
<td>1</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>Spleen</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.013 ± 0.004</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.009 ± 0.003</td>
<td>0.007 ± 0.006</td>
<td>1</td>
<td>0.011 ± 0.004</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.001 ± 0.004</td>
<td>0.001 ± 0.004</td>
<td>1</td>
<td>0.014 ± 0.002</td>
</tr>
</tbody>
</table>

SI analysis for IV injection group showed almost 3-fold enhancement in the contrast for the magnetic guidance group comparing non-magnet group. The values of
Figure 6.11: Representative $T_2^*$ maps of tumors from IV group before and 24 hours after the injection; without and with magnetic guidance. Accumulation of MCL is evident through the color change of the tumors between pre and after images: From red (high $T_2^*$) to yellow (low $T_2^*$). Magnet placement was shown with dashed lines.

SI analysis are given in Table 6.5.

Table 6.5: Average percent signal intensity decrease in different organs for different groups. The signal intensity for all groups was normalized by the signal intensity for muscle.

<table>
<thead>
<tr>
<th></th>
<th>IV-NM</th>
<th>IV-M</th>
<th>IT-NM</th>
<th>IT-M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor</td>
<td>20 ± 5</td>
<td>57 ± 12</td>
<td>91 ± 7</td>
<td>87 ± 2</td>
</tr>
</tbody>
</table>

6.4.3 IT administration

In both IT groups, the effect of magnetic guidance was much harder to visualize through MRI due to the blooming effect which amplifies the signal decrease because of affected protons even at distant sites [143]. The drastic decrease in relaxation values hinders the ability to distinguish retention at such high concentrations of SPION. However great retention is evident in both magnet and non-magnet groups. This can be appreciated in Figure 6.12, where tumor signal intensities disappear post-injection for both groups, IT-NM and IT-M. The decrease of $T_2^*$ values of IT group for tumor
cannot be calculated exactly since high concentration of MCL caused drastic decrease in $T_2^*$ that went below the capabilities of the instrument. It is still possible to see the effects of applied magnet indirectly from $R_2^*$ values of the rest of the body, Table 6.4. SI decrease was also too high for both magnet and non-magnet groups, Figure 6.12.

Figure 6.12: Representative $T_2^*$ maps of tumors from IT group without (NM) and with (M) applied magnet. Top and bottom rows show images of same mouse before and 24 hours after the administration of MCL. Distortions in and around the tumors (inside dashed borders) are due to the high concentration of MCL in the area which indicates that MCL are locked in the tumors even after 24 hours.
Chapter 7

Conclusions

In this thesis, we investigated two types of nanoplatforms, nanoporous oxide coatings and superparamagnetic nanoparticles. We discussed their fabrication, properties and some applications in different fields.

We demonstrated the feasibility of high-rate 3D assembly of nanoelements, in forms of polymeric nanobeads and SWNT, inside the nanoholes in porous alumina using electric field. This work demonstrated that reliable approaches to nanoassembly are feasible and forms the basis for a variety of other nanoassembly challenges. For example, drug-loaded polymeric nanoparticles could be assembled with these nanoporous arrays and used as drug delivery platform.

This electric field assisted assembly method has many advantages such as mild condition assembly, post produced or commercially available SWNT utilization, high-rate and large scale assembly as well as integration of SWNT into existing Si platforms. It is also possible to attach AAO to Si after anodization in order to avoid exposure to acidic environments [144]. Using nanoscale features in the templates, we achieved large scale assembly of SWNT order of $10^6$ elements over centimeter square areas. The strength of the connection established through these assembled SWNT is sim-
ilar to the connection through in-grown nanotubes on Si. This assembly technique can potentially be used in many SWNT applications such as in CNT interconnects with the integration with Si microelectronics, in field emission displays or electronic memory devices.

Another application of these nanotemplates we investigated is sustained drug release. We demonstrated the results of the release of a model drug, doxorubicin (Dox), from different non-eroding nanoporous coatings. All the platforms show release profiles extending to order of weeks and were still eluting when the measurements were terminated. The percent release was calculated by comparing the drug released into the medium and the total drug released at infinite time, \( M_\infty \). We showed that bio-compatible, non-eroding nanoporous coatings can be fabricated that are suitable for drug releasing implants for a variety of therapeutic situations. Nanoporous platforms were loaded with Dox and the release profile was observed by in-situ fluorometry. After a rapid burst release which was similar for all the platform types, different sustained release kinetics was observed from nanoporous platforms. An activated surface density dependent desorption was in effect for nanoporous templates.

The studies on the inorganic nanoporous coatings or devices discussed here show that these systems can be used effectively for sustained release applications. They meet the requirements of drug loading and sustained release profiles extending to several weeks, and they also are compatible with the current implant technologies. Future challenges that need to be met are to achieve better control on the release profile and more in vivo studies to evaluate their efficacy. One of the main future aims in drug delivery is to achieve smart release systems that can be activated by the doctor or patient to release drug.

Superparamagnetic iron oxide nanoparticles (SPION) were another type of nanoplat-
somes they can be used as excellent contrast agents for MRI. PEGPE-based polymeric micelles offer a convenient carrier system for suspending SPION in aqueous medium. The formulations were stable with no apparent aggregation compared to that observed in the case of plain SPION. SPION-loaded PEGPE micelles provided dramatically improved $T_2$ relaxation times. These SPION-micelles can be easily modified with anti-cancer nucleosome-specific mAb 2C5 with the preservation of the antibody specific activity to make them cancer cell-specific. mAb 2C5- SPION-immuno-micelles specifically recognize and bind cancer cells in vitro, bring higher quantity of SPION to these cells, and have a potential to serve as an MRI contrast agent with improved $T_2$ for better tumor imaging. In addition to this, the specific accumulation of mAb 2C5-SPION-immuno-micelles into the cancer cells makes these micelles good candidates to be used in conjunction with AC-field assisted hyperthermic cancer therapy.

We also showed that liposomes can be a good candidate as SPION carrier, known as magnetic cationic liposomes (MCL). We demonstrated that they can be guided into the tumor site efficiently by an external magnet, and that the accumulation can be monitored non-invasively through MRI. The quantitative MR analysis and biodistribution studies showed up to 2-fold greater accumulation of administered MCL into tumor mass by magnetic targeting. MCL are long circulating, and are retained for therapeutically significant durations when accumulated in the tumor. The MCL nanoplatfrom is quite versatile, enabling incorporation of a variety of cargo elements such as drugs, biologics, optical and radio imaging agents, and can be conjugated with targeting moieties. These results clearly show the efficacy of MCL as MRI contrast agents, prove the use of magnetic guidance. Future work in this project will be to demonstrate the therapeutic applications of MCL by means of magnetic hyperthermia on top of being imaging and guidance agents.
Appendix A

Protocol for AAO Templates

Pre-treatment Steps

1. Cut the aluminum sheet for pieces we want to make templates.

2. Clean them with acetone.

3. Put the dry samples into the oven at maximum temperature for overnight annealing.

4. Put a large beaker on the hot plate filled with water. Put the electrolyte beaker in that and heat them. When the temperature of acid soln. closes to 70C it is ready for electropolishing. Electropolishing must take at most 10s under 20V constant voltage. Then wash the electrodes with water twice and dry them with air. Dump the waste water from the first washing into the waste bottle.

First Anodization

1. Turn on the cooler and arrange it to 2.8C (for oxalic acid, 1.3C for sulfuric acid) wait till the actual temperature of electrolyte is close to that.
2. Place the electrodes into the acid.

3. Arrange the voltage desired for the template. 50V for oxalic acid, 20V for sulfuric acid.

4. First anodization should be at least 4 hours long to have somewhat ordered structures. Try to keep current readings to make sure everything going normal.

5. After anodization wash the electrodes twice with water and dry with air. Dump the waste water from the first washing into the waste bottle.

**Second Anodization**

1. To clean the alumina layer completely, soak the templates into hot (around 60°C) chromic-phosphoric acid soln. for overnight.

2. After removal of alumina wash the electrodes thrice with water and dry with air. Dump the whole waste water into the special waste bottle.

3. To start the second anodization, arrange the temperature and the voltage just like in the first anodization.

4. The time of second anodization determines the thickness of the alumina layer. For oxalic acid, 10 minutes gives 1micron alumina layer.

5. After anodization washed the electrodes twice with water and dry with air. Dump the waste water from the first washing into the waste bottle.

**Pore Widening**

1. Put the templates in the pore widening soln. The time determines the widening, for oxalic acid templates; 80 minutes remove 55nm in diameter and 500nm in
the thickness.

2. After pore widening wash the templates with water twice and dry with air. Dump the waste water from the first washing into the waste bottle.

**Solutions**

1. Electropolishing Solution: Sulfuric acid, phosphoric acid mixture H2SO4:H3PO4:H2O; 4:4:2. Be very careful since it is really concentrated acid and becoming much more dangerous when it is hot.

2. Electrolyte Solution: Sulfuric acid: 20% sulfuric acid. 80ml water 20ml H2SO4. Oxalic acid: 5 wt% oxalic acid solution.

3. Chromic-Phosphoric Acid Solution: 170ml water, 5ml phosphoric acid, 25ml chromic acid. Be very careful with chromic acid since it is carcinogen. Do not dump anything related to it into the sink; everything needs to be in a special waste bottle for it.

4. Pore Widening Solution: 5% phosphoric acid.
Appendix B

SQUID Magnetometer

Superconducting Quantum Interference Device (SQUID) uses the properties of electron-pair wave coherence and Josephson Junctions to detect very small magnetic fields. A Josephson junction is made up of two superconductors, separated by an insulating layer so thin that electrons can pass through. SQUID is usually made of either a lead alloy (with 10% gold or indium) and/or niobium, often consisting of the tunnel barrier sandwiched between a base electrode of niobium and the top electrode of lead alloy. Figure B.1(a) shows the basic diagram of SQUID.

Figure B.1: (a) Basic circuit diagram of SQUID. (b) A superconducting ring in SQUID.

A SQUID consists of tiny loops of superconductors employing Josephson junctions to achieve superposition. The central element of a SQUID is a ring of superconducting...
material with one or more weak links. Figure B.1(b) shows one of such rings, with weak-links at points W and X whose critical current, $i_c$, is much less than the critical current of the main ring. With a thin enough insulating layer, the phase of the electron wave-function in one superconductor maintains a fixed relationship with the phase of the wave-function in another superconductor. This linking up of phase is called phase coherence. One of the characteristics of a Josephson junction is that as the temperature is lowered, superconducting current flows through it even in the absence of voltage between the electrodes.

Sample in SQUID is moved with a linear stepper motor through coils which are linked to the input coil of a SQUID through a superconducting flux transformer. The Figure B.2 show the sample space and the sample holder.

In these experiments we used the SQUID magnetometer running on DC mode. A direct current (DC) SQUID, consists of two Josephson junctions employed in parallel so that electrons tunneling through the junctions demonstrate quantum interference, dependent upon the strength of the magnetic field within a loop. DC SQUID demonstrates resistance in response to even tiny variations in a magnetic field, which is the capacity that enables detection of such minute changes.
Figure B.2: Sample space and fiber optic sample holder.
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