Application of Ultrasound Imaging for Noninvasive Characterization of Phase Inverting Implants

by

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09 March 2012

*We also certify that written approval has been obtained for any proprietary material contained therein.
To my wife, brother, and mother
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Mw</td>
<td>molecular weight</td>
</tr>
<tr>
<td>GI</td>
<td>gastrointestinal</td>
</tr>
<tr>
<td>T_g</td>
<td>glass transition temperature</td>
</tr>
<tr>
<td>PLA</td>
<td>polylactic acid</td>
</tr>
<tr>
<td>PGA</td>
<td>polyglycolic acid</td>
</tr>
<tr>
<td>PLGA</td>
<td>poly(lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>PCPP:SA</td>
<td>poly(bis[p-carboxyphenoxy]) propane:sebacic acid</td>
</tr>
<tr>
<td>PEO</td>
<td>polyethylene oxide</td>
</tr>
<tr>
<td>PPO</td>
<td>polypropylene oxide</td>
</tr>
<tr>
<td>PVP</td>
<td>polyvinylpyrrolidone</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>PG</td>
<td>Propylene Glycol</td>
</tr>
<tr>
<td>NMP</td>
<td>N-methyl-2-pyrrolidone</td>
</tr>
<tr>
<td>PVA</td>
<td>polyvinyl alcohol</td>
</tr>
<tr>
<td>ISFIs</td>
<td>In situ forming implants</td>
</tr>
<tr>
<td>FPI</td>
<td>fast phase inverting</td>
</tr>
<tr>
<td>SPI</td>
<td>slow phase inverting</td>
</tr>
<tr>
<td>EPR</td>
<td>electron paramagnetic resonance</td>
</tr>
<tr>
<td>ESR</td>
<td>electron spin resonance</td>
</tr>
<tr>
<td>CW-EPR</td>
<td>continuous-wave EPR</td>
</tr>
<tr>
<td>AFC</td>
<td>automatic frequency control</td>
</tr>
<tr>
<td>AMC</td>
<td>automatic matching control</td>
</tr>
<tr>
<td>CCD</td>
<td>Charge-coupled device</td>
</tr>
<tr>
<td>LODEPR</td>
<td>longitudinally detected EPR</td>
</tr>
<tr>
<td>MDCT</td>
<td>multi-detector computed tomography</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>DSA</td>
<td>digital subtraction angiography</td>
</tr>
<tr>
<td>PET</td>
<td>positron emission tomography</td>
</tr>
<tr>
<td>DWI</td>
<td>Diffusion-weighted MRI</td>
</tr>
<tr>
<td>ADC</td>
<td>apparent diffusion coefficient</td>
</tr>
<tr>
<td>Gd-albumin</td>
<td>gadolinium conjugated to albumin</td>
</tr>
<tr>
<td>Gd-DTPA</td>
<td>Gd-diethylenetriamine pentaacetic acid</td>
</tr>
<tr>
<td>FDG</td>
<td>fluorodeoxyglucose ((^{18}\text{F}))</td>
</tr>
<tr>
<td>GV</td>
<td>gray-scale value</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscope</td>
</tr>
<tr>
<td>US</td>
<td>ultrasound</td>
</tr>
<tr>
<td>RF</td>
<td>radiofrequency</td>
</tr>
<tr>
<td>RFA</td>
<td>radiofrequency ablation</td>
</tr>
<tr>
<td>DGI</td>
<td>dark ground imaging</td>
</tr>
<tr>
<td>FMT</td>
<td>fluorescence molecular tomography</td>
</tr>
<tr>
<td>Vd</td>
<td>distribution volume</td>
</tr>
</tbody>
</table>
Da
daltons
kDa
kilodaltons
m
meter
Cm
centimeter
mm
millimeter
µm
micrometer
g
gram
kg
kilogram
mg
milligram
µg
microgram
l
liter
ml
milliliter
µl
microliter
°C
degree centigrade
RPM
revolutions per minute
s
second
min
minute
h
hour
day
t
transarterial chemoembolization
CED
convection enhanced delivery
NCT
neutron capture therapy
RCT
randomized controlled trials
DEB
drug eluting beads
WHO
World Health Organization
RECIST
Response Evaluation Criteria in Solid Tumors
EASL
European Association for Study of Liver
NIH
National Institutes of Health
FDA
Federal Drug Administration
IACUC
Institutional Animal Care and Use Committee
C
drug concentration
D
diffusivity
HCC
hepatocellular carcinoma
hGH
human growth hormone
MTX
methotrexate
Dox
doxorubicin
BCNU
carmustine
PTX
paclitaxel
B
boron
BSA
bovine serum albumin
PBS
phosphate buffered saline
NaOH
sodium hydroxide
HCl
hydrogen chloride
THF
tetrahydrofuran
DiI
1,1'-dioctadecyl-3,3',3'-tetramethyl-indocarbocyanine perchlorate
BBB
blood brain barrier

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>Ex/Em</td>
<td>excitation/emission</td>
</tr>
<tr>
<td>GPC</td>
<td>gel permeation chromatography</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>3-D</td>
<td>3 dimensional</td>
</tr>
<tr>
<td>4-D</td>
<td>4 dimensional (X,Y,Z,time)</td>
</tr>
<tr>
<td>SC</td>
<td>subcutaneous</td>
</tr>
<tr>
<td>ETP</td>
<td>experimental time point</td>
</tr>
<tr>
<td>DTP</td>
<td>discrete time point</td>
</tr>
<tr>
<td>CTP</td>
<td>continuous time point</td>
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I. Abstract

Cancer is a global disease that affects millions of people annually. Primary treatment relies on the surgical resection of the lesion followed by adjuvant chemotherapy regimens. As a result of the systemic delivery of these drugs, healthy tissue is also targeted, resulting in dose limiting toxicity. One method for targeting the delivery of drugs to the tumor and limiting the action of these agents on healthy tissues is through the placement of drug eluting depots within the tumor, which can reduce systemic exposure of the delivered agent while elevating the local concentrations. \textit{In situ} forming implants provide an ideal means by which these depots can be physically targeted to the tissue. These injectable implants are liquid solutions outside of the body, but solidify into solid implants upon contact with an aqueous environment through a process known as phase inversion.

Currently, use of \textit{in situ} forming implants is limited due to a poor understanding of how the environment of the injection site affects overall implant behavior. This thesis
outlines the development of a novel noninvasive technique for characterizing the behavior of these implants \textit{in situ} with ultrasound imaging. A correlation was developed that can be used to approximate the burst release of drug from these implants as a function of the phase inversion data obtained through image analysis. Additionally, implant formulation parameters were modified so that the effect of cargo properties on implant behavior could be evaluated. Tunable swelling, phase inversion, degradation, erosion, and release were obtained by altering the Mw of the polymer used in the initial implant formulation. Finally the effect of the injection site on implant behavior was evaluated using diagnostic ultrasound. These studies identified that the stiffness of the injection site has a direct effect on the drug release profile, and the polymers that undergo a greater degree of swelling ultimately release more drug \textit{in vivo}. The techniques developed in this thesis may be used to further elucidate the underlying parameters that control phase inversion and drug release \textit{in vivo}, and may lead to the development of optimal implant formulations that can provide improved treatment efficacy.
II.1. Rationale

Cancer is the number one cause of death worldwide [1, 2], affecting millions of people regardless of age, gender, or race. Defined by the National Institute of Health as an “uncontrolled growth of abnormal cells in the body”, cancer will be the cause of death of an estimated 577,190 Americans this year alone [1]. The size and location of the cancerous lesion as well as the age of the patient, often times determine both the choice of therapeutic approach and the effectiveness of the selected treatment regimen. In ideal circumstances, the tumor is surgically resected, and adjuvant regimens of chemotherapy are given to prevent recurrence. Among patients who are diagnosed with cancer, at least 75% will receive some form of chemotherapy as part of their treatment [3, 4].

For some types of cancer, such as hepatocellular carcinoma (HCC), a disease for which 4 out of 5 patients are ineligible for surgical intervention, chemotherapy is the only available therapeutic option [5-7]. These treatment regimens can significantly affect the quality of life of the patient undergoing the therapy due to the side effects of the prescribed drugs such as myelosuppresion, alopecia, and nausea. These side effects are caused because the systemically administered chemotherapeutic drugs target healthy, mitotically active tissues (such as the bone marrow or lining of the gastro intestinal tract) in addition to the cancer cells. Due to the detrimental effects of systemic chemotherapy on healthy tissues, the dosages that the patient can safely receive are limited, and can reduce treatment efficacy [8-10]. Among those patients with HCC who receive chemotherapy, only 1 out of 4 show even a partial response to the drugs [5-7].

In addition to systemic toxicity issues, patient compliance can also be a particular problem for those taking multiple medications with complex dosage schedules as well as
for patients whose drugs make them feel weak or sick. It has been reported that as many as 25% of patients do not take their medicine as prescribed, this issue of patient compliance is estimated to result in over $100 billion additional costs to the already staggering cost of the health care system [11].

Intratumoral delivery of chemotherapeutic agents provides a means of circumventing the intrinsic limitations of systemic delivery. The local delivery system can elevate drug levels while reducing systemic involvement since the controlled release system is physically placed directly at the site of action [8, 12]. Furthermore, the use of implantable controlled release systems removes issues of patient compliance with treatment. *In situ* forming implants (ISFI) provide a unique avenue by which local delivery can be achieved, while improving patient compliance, through the use of an injectable polymer solution loaded with drug. One major limitation of these implants is that the *in situ* behavior has not been well characterized, with most studies destructively evaluating the *in vitro* behavior of these systems, which has limited the number of *in vivo* applications. The engineering objective for this thesis is to design a system that can be administered with image guidance, evaluated *in situ* noninvasively, with a tunable release profile. Ultimately, this system will incorporate chemotherapeutic drugs and used to provide primary or adjuvant therapy directly at the lesion, maximizing the treatment dose, while minimizing side effects.

**II.2. Specific Aims**

In order to achieve this objective, the work has been divided into four specific aims:
Aim 1: **Development a method by which formation rate, release rate, swelling, and rate of degradation of the drug depot can be monitored noninvasively.** In this aim, ultrasound was utilized to monitor formation rate, release rate, swelling noninvasively. Characterization of implants was performed *in situ* both *in vitro* and *in vivo*. The effect of cargo properties on ultrasound characterization was also evaluated, and image data used to determine if there is a correlation between phase inversion and drug release so that release could be approximated noninvasively.

**Aim 2: Characterization of the role that drug properties play on implant behavior in situ.** Drug matrix interactions were evaluated by altering the properties of drugs that were loaded into the implants. Fluorescein, Doxorubicin, 1,1-Dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate (DiI), and bovine serum albumin (BSA) were added to the polymer solution and phase inversion were characterized using ultrasound. Additionally, the burst release and phase inversion were correlated to provide a noninvasive metric by which drug release can be approximated.

**Aim 3: Formulation and characterization of an ISFI system with tunable release.** The matrix properties were altered by using molar ratios of different molecular weight (Mw) poly(D,L-lactic-co-glycolic acid) in the polymer solution formulation. These ratios were used to alter the swelling, release, degradation, erosion, and phase inversion properties, allowing for tunable drug release. Ultrasound was again used to characterize the system, and blended polymers were evaluated *in vivo*.

**Aim 4: Evaluation of the effect that the injection site has on implant behavior.** In this aim, DHD/K12/TRb cells were used to create a subcutaneous tumor model in BDIX rats. Implants were then injected into four different environments: the subcutaneous
space, necrotic tumor, non-necrotic solid tumor, as well as ablated tumor. The phase inversion, swelling, and drug release were evaluated using techniques developed in aim 1 and 2 in these environments, and the effect of injection site was characterized relative to the in vitro release profile.

The work in these aims has led to the development of a noninvasive means by which implant behavior can be characterized in situ. Additionally, these studies have demonstrated that factors beyond polymer concentration, solvent type, drug type, and bath side components alter the release behavior of implants in vivo. The insight gained has provided techniques by which more complex release systems can be developed that overcome the intrinsic limitation of diffusion distance from traditional implantable devices.

II.3. Thesis Overview

This thesis outlines the development of a noninvasive characterization technique that uses diagnostic ultrasound for the in situ characterization of ISFIs. This system was then used to determine both the formulation parameters and the environmental factors that alter implant behavior. Background information is provided in Chapters 1 and 2. In Chapter 1 factors that play a role in altering implant behavior are discussed while current techniques and systems used for image guided drug delivery are evaluated in Chapter 2.

In Chapters 3 to 6, the development of a noninvasive technique for the characterization of ISFIs using ultrasound is described. Chapter 3 describes the methods by which ultrasound was used to characterize phase inversion and swelling behavior of this implant system. Chapter 4 demonstrates that ultrasound characterization can be
carried out regardless of the implant payload, describes the role of drug properties on implant behavior, as well as describes the relationship between the phase inversion and drug release. Chapter 5 is used to demonstrate how implant properties (such as swelling, erosion, degradation, release, and phase inversion) can be tailored, as well as a method for characterizing implant behavior in four dimensions (X, Y, Z, and time). Chapter 6 highlights the effect that the injection site has on ISFI behavior. Finally, Chapter 7 provides an overview of the key results found in each of the studies followed by work that will continue after completion of this thesis.

II.4. References

Chapter 1: Phase Inverting Polymer Systems in Drug Delivery and Medicine

Modified with permission from Polymer Phase Behavior 2011, Chapter 4, Pp 171–203; Copyright © 2011 Nova Science Publishers and Engineering Polymer Systems for Improved Drug Delivery 2012, Chapter 11

Luis Solorio, Loran D. Solorio, Sarah Gleason, Alex M. Olear, and Agata A. Exner
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Chapter 1: Introduction

Drugs can be administered in a variety of ways, from swallowing a pill, receiving an intravenous injection, applying a dermal patch, to surgically implantiing a controlled drug delivery device [1, 2]. While the vast majority of drugs are given orally, factors such as rapid metabolism, poor oral bioavailability, narrow therapeutic index, and nonspecific targeting can render oral delivery methods undesirable or impossible [1, 2]. The development of more sophisticated parenteral methods of drug delivery has become critical as treatment options advance and become more complex. One example that highlights the immediate need for more advanced delivery techniques is the administration of drugs for the treatment of cancer.

For many chemotherapeutic treatments, highly toxic drugs must be ingested, traveling through the gastrointestinal (GI) tract before entering the circulatory system. Once the drug is absorbed into the circulatory system (which is where the journey begins in the case of intravenous injection, which bypasses the GI phase), it can be metabolized, eliminated, or passed into the interstitial space of both diseased and healthy tissues. If the drug reaches the cancerous lesion, it must then diffuse against pressure gradients induced by poor lymphatic drainage in order to reach the target, a single cancer cell. Since many chemotherapeutic drugs act on cancer cells by targeting their propensity for proliferation, systemic exposure to these toxic drugs affects healthy, mitotically active tissues. Examples include hair follicles, bone marrow and the lining of the GI tract, which results in hair loss, nausea, anemia, and infection due to immune suppression. More advanced methodologies of drug administration could enable local rather than systemic
chemotherapeutic drug delivery, potentially limiting the adverse effects on healthy tissues.

1.1. Polymeric Implants for Controlled Release Applications

Polymer implants have been developed as a method of protecting drugs from harsh physiological conditions as well as providing a means for localizing a therapeutic agent to a target area. In these implants, a drug is encapsulated in an implantable polymer matrix of fixed geometry (which can be degradable or nondegradable) then surgically inserted directly into the site of action. These implants can be placed subcutaneously or directly into the diseased tissue [3-5]. When these implants are placed directly into the diseased tissue, high local levels of drug can be delivered without systemic involvement, ultimately reducing side effects and protecting drug activity. While the drug release profile of both degradable and non-degradable implants are dependent on the properties of the encapsulating polymer matrix, one major benefit of the degradable systems is that there is no need for surgical removal, since the implants degrade over time [4, 6-9]. While degradation results in more complicated implant design, these systems can provide additional, more flexible avenues by which dissolution of drug can be modulated.

While a polymer cannot be approved by the FDA, there are five polymers commonly found in FDA approved devices, and have demonstrated an appropriate host tissue response for their designated applications (Figure 1.1). The polyetherester polydioxanone has been approved for use as suture clips and bone pins marketed as OrthoSorb [10]. The slow degrading semicrystalline poly(caprolactone) had been used for the controlled release of contraceptives and as a suture marketed as Capronor and
monocryl [10, 11]. Poly(PCPP-SA anhydride) has been used in the field of drug delivery in order to treat residual tumor cells after surgical resection and are marketed as the Gliadel Wafer ® [8, 10, 12]. Finally, poly(glycolic acid) (PGA), poly(lactic acid) (PLA), and their copolymer (PLGA) have been used as degradable sutures, bone pins, and drug delivery vehicles marketed as the Leupron depot, Atrigel, Eligard, Atridox, Dexon, Vicryl [10, 13-18].

1.1.1. Pre-formed Implants and Their Fabrication

The only commercially available implant used for intratumoral delivery of chemotherapeutic agents is the Gliadel wafer, which can be classified as a pre-formed drug eluting depot. Pre-formed implants are fabricated into the desired geometry outside of the body, and then physically placed into the desired location using minimally invasive surgical techniques. By creating an implant with a pre-formed geometry, the release profile can be more readily controlled.

![Polymers commonly found in FDA approved devices](image-url)

**Figure 1.1.** Polymers commonly found in FDA approved devices
Fabrication of these implants can be achieved using a number of techniques including: compression molding, melt casting, solvent casting, and extrusion [4, 19, 20]. Implants fabricated using compression molding are made by first mixing the polymer and drug in a piston shaped mold, then applying elevated pressure at 5-10°C above Tg to create disk or rod shaped implants [20-22]. The low fabrication temperature is beneficial for maintaining activity of reactive drugs, but since the drug may not be homogenously distributed, there is can be a large variability in drug release [20]. Implants formed using melt molding require that the polymer is elevated above Tm, resulting in a viscous solution in which drug can be homogenously mixed and subsequently solidified in a mold of the desired geometry [20]. The fabricated implant has a reproducible release profile, but the elevated temperature can result in a loss of therapeutic activity if the drug is sensitive to temperature especially when proteins are used as therapeutics [20]. Solvent casting is performed by dissolving the polymer in a volatile solvent, with drug if the drug is soluble. If the drug is non-soluble then the powdered drug is homogenously distributed into the polymer solution. The solution can then be added to a mold of the desired geometry, and maintained at a lowered temperature so that the solvent will evaporate off slowly [20]. Solvent casting provides a technique with which to fabricate implants loaded with heat sensitive drugs, but this technique has a number of disadvantages. The phase inversion dynamics intrinsic to solvent evaporation can lead to a porous microstructure, resulting in implants with poor mechanical properties, as well as have the potential to deactivate the therapeutic agent [20]. Additionally, because of the amount of time required for the solvent to evaporate, the suspended drug may not stay homogenously distributed in the matrix [20]. While the ability to create an implant with
a fixed geometry provides an avenue by which the release of drug can be controlled, these implants are limited for a number of reasons. First, implant fabrication can often times be time consuming and tedious process. The characteristics of the drug and additives will dictate which fabrication technique can be used, and may result in added cost to the device. Finally, depending on the process used to make the implant, the drug may be poorly distributed throughout the matrix, which can alter the release profile.

1.1.2. Factors that Affect Drug Release

Drug release is not only affected by the geometry of the implant, but the rate of dissolution can be affected by factors such as the ratio of polymer relative to other components in the implant, how the drug is loaded into the implant, the crystallinity of the polymer, the crystallinity of the drug, as well as implant microstructure [20, 23-31]. Percolation theory provides an ideal means by which the effect of drug loading can be evaluated on implant behavior. In percolation theory the matrix is envisioned as a lattice of interconnected points, and the introduction of drug removes a point from the lattice. Initially, the reduction of points in the lattice does not overly disrupt the lattice stability, but as the number of points in the lattice continue to disappear so does the stability of the system. The critical point at which the polymer no longer forms a connected network, is referred to as the percolation threshold [32-34]. In addition to the percolation threshold, polymer crystallinity can play a role as to where the drug partitions within the system [24, 28]. Typically drug diffuses through the implant pores, therefore high interconnectivity leads to elevated release.
Additional factors that control the release of the drug from the polymer include the size of the drug diffusing through the polymer network, the affinity of the drug with the polymer, as well as the tortuosity of the porous network [8]. The diffusivity of degrading implant systems change as a function of polymer degradation. Simple models of release for bulk eroding systems assume that polymer degradation follows first-order kinetics, leading to an exponential increase in diffusion with respect to time [30].

\[
\frac{d[\text{COOH}]}{dt} = k[\text{COOH}][\text{H}_2\text{O}][\text{Ester}] \tag{1}
\]

\[
D_{\text{eff}} = D_0 e^{kt} \tag{2}
\]

\[
\frac{\partial c}{\partial t} = D_{\text{eff}} \frac{\partial^2 c_d}{\partial x^2} \tag{3}
\]

In eq. (1) and (2), [COOH] refers to the concentration of carboxylic acid, [H$_2$O] refers to the concentration of water, and [Ester] refers to the total concentration of degradable esters, \(k\) is the first-order degradation kinetic constant of the polymer, \(D_{\text{eff}}\) is the effective diffusivity as a function of the degradation kinetics, \(D_0\) is the initial diffusivity at time zero before degradation begins, and \(t\) is time [30]. The first-order degradation kinetics can be determined from the changes in polymer Mw over time, and makes the simplifying assumption that there is a near constant concentration of both the hydrolysable esters and water [35-37]. The effective diffusivity coefficient can then be used with the general models of diffusion. For the system evaluated in eq. (3), convection, generation, and metabolism were assumed to be negligible in eq. (3), and the
implant was assumed to be a flat sheet. The result of degradation is that there is an increase in release over time as the polymer diffusivity changes (Figure 1.2). For surface eroding systems, release of drug is typically proportional to the rate of polymer erosion [20].

![Figure 1.2. Cumulative release over time from a degrading and nondegrading polymer implant.](image)

Dual release can be achieved through a process known as dip coating, where the pre-formed implants are dipped into a polymer solution containing a water soluble component such as NaCl or poly(ethylene oxide) and drug in order to form a thin outer membrane [6, 9, 21]. Once implanted into the tissue, the aqueous environment leads to the dissolution of the soluble component and an initial burst release from the porous outer membrane [6, 21]. Ultimately it is the properties of the drug and the desired application that will dictate the fabrication technique used to make the implant.

For *in vivo* systems, evaluating drug release is more complex due to the presence of cells and microvascularization in the tissue space [4, 21, 38, 39]. Evaluation of release from these systems requires multidimensional analysis of the implant and surrounding tissue, with careful consideration of the boundary conditions between the implant and...
tissue interface. For this system, the rate of drug distribution within the surrounding tissue involves investigating the diffusion of drug within the tissue space from the implant, the elimination of the drug by the vasculature, and the metabolism of the drug by the cells [4, 21, 38, 39].

\[
\frac{\partial C}{\partial t} = D\nabla^2 C - v\nabla C - kC
\] (4)

Where \( k \) is the rate of drug metabolism, the \( v \) accounts for the velocity of the surrounding vasculature, and \( D \) is the diffusivity of drug from the implant in the surrounding tissue [4]. A result of diffusion based release, is that the metabolic activity of the cells and the removal of drug as a function of the microvasculature limits the distribution distance of the drug within the tissue.

1.1.3. Strategies to Improve Drug Penetration

While drug eluting implants can deliver very high local drug concentrations to a tumor site without avoiding systemic toxicity, relatively few of these devices have been translated for clinical use due to poor drug penetration into tissue. Typically therapeutic tissue drug concentrations are only achieved a few millimeters away from the implant boundary [40]. Penetration and movement of drug into tissue is dependent on two tissue specific factors drug diffusivity, \( D \), and drug elimination, \( \gamma \), and can be described by the following first order transport equation:

\[
\frac{\partial C(r,t)}{\partial t} = D\nabla^2 C(r, t) - \gamma C(r, t)
\] (5)
where $C(r,t)$ is the tissue drug concentration, $t$ the time, and $\nabla$ the gradient operator. Therefore to improve drug penetration from the implant boundary either the tissue permeability must be increased, by increasing $D$, or the tissue drug elimination must be reduced, by decreasing $\gamma$. One strategy to both increase tumor drug permeability and reduce drug elimination is to induce tissue necrosis. Cell membranes in necrotic tissue are usually destroyed and greater drug diffusivity has been shown to occur in these types of tissues [40]. Additionally, dead cells are metabolically inactive, and tissue vasculature is often destroyed with necrosis thereby eliminating the major routes of drug elimination from the tissue. Both ablative tumor treatments as well as chemotherapy induced tumor necrosis have been shown to increase drug penetration[40, 41].

In addition to inducing tumor necrosis several other strategies have also been developed to improve drug penetration in tumors. One such strategy is to bind the drug molecule to a large Mw carrier that is not easily eliminated from the tissue. A study by Dang et al. demonstrated that methotrexate (MTX) which was covalently bonded to dextran was able to retain its cytotoxic activity while reducing tissue drug elimination [42]. Even though, the diffusivity of the larger Mw MTX-dextran molecule was lower, the reduction in tissue elimination was more than able to offset that factor and significant gains in tissue penetration were achieved [42]. Yet though this is a promising strategy to increase tumor drug penetration, it can only be used for chemotherapeutic agents that act on surface receptors such as MTX which acts on cell surface folate receptors. Many commonly used chemotherapeutic agents such as cisplatin, BCNU, PTX, and doxorubicin work on intracellular targets which would become inaccessible if they are attached to a dextran carrier. Finally if tissue specific elimination and drug diffusivity
properties are known, a study by Weinberg et al. has shown that a mathematical modeling approach to place multiple implants within a tumor volume can be used to achieve complete penetration [43]. Using this type of method, a patient specific treatment approach can be designed by reconstructing 3-D CT or MRI data of a tumor and using mathematical modeling to determine the number and location of implants to be placed in a tumor to achieve therapeutic levels throughout the volume [43]. However, while this method shows great promise, accurate tissue specific diffusivity and elimination parameters may be difficult to obtain due to tumor tissue and anatomical variability. One solution to help overcome this barrier is the development of imaging contrast tagged drug agents, whose tissue distributions can be non-invasively monitored to establish tissue specific drug transport parameters.

1.1.4. Polymer Degradation and Erosion

As previously stated, polymer degradation can play a significant role in altering the release profile of a controlled release devise. The combination of polymer degradation and erosion lead to the loss of implant mass over time. Polymer degradation refers to the process by which the repeating structural units of the polymer chain are cleaved resulting in a reduction in molecular weight (Figure 1.3).
This can be facilitated by a number of factors [8, 25, 33]. For example, enzymes can catalyze the polymer degradation process which is sometimes referred to as biodegradation. Additionally polymer degradation can be initiated by external factors such as the presence of UV light, mechanical perturbation, as well as γ-radiation which is used to sterilize the polymers for clinical applications [25]. For drug delivery purposes, the most common method of degradation occurs through hydrolysis, which is the cleavage of the polymer backbone due the interactions of the polymer with water.

After the polymer begins to degrade, the loss of the oligomers and monomers to the surrounding environment resulting in the reduction of polymer mass is known as erosion [8, 10, 25, 26, 33]. When erosion is enhanced by physiological processes, the
process is referred to as bioerosion [8]. Since erosion is simply the loss of mass over time, erosion does not always require degradative processes in order to occur. In the case of a matrix containing water-soluble components, erosion can occur simply through dissolution of the matrix [8], such as a cookie crumbling in milk or a bar of soap reducing in size with use. Erosion is a process that can occur solely on the surface of the implant through a process known as surface erosion (as in the case of the soap), or throughout the bulk of the polymer, which is then referred to as bulk erosion, as in the cookie example (Figure 1.4).

![Figure 1.4. Schematic of bulk erosion and surface erosion](image)

The primary factor that determines whether the polymer will be surface eroding or bulk eroding is the reaction kinetics of degradation. If the rate of polymer degradation is faster than the rate of water diffusion into the polymer, then the polymer will be surface eroding. If the rate of water diffusion is faster than the reaction kinetics, then the polymer will be bulk eroding [44].

It is the presence of hydrolysable bonds in the polymer backbone that allows for tunable degradation and erosion profiles; therefore the choice of chemical bond is a critical factor in controlling the rate of degradation and the method by which the polymer
will erode. The three most common chemical bonds found in controlled release devices listed in order of reactivity are: anhydrides, esters, and amides [25, 45]. While both esters and amides require either acidic or basic conditions in order for hydrolysis to occur, the reactivity of anhydrides is high enough that hydrolysis occurs under neutral conditions, only requiring the presence of moisture in the air to initiate degradation [45, 46]. The reaction begins when the oxygen of water acts as a nucleophile, and attacks one of the anhydride carbonyls, which leads to the elimination of one carboxylic acid and the formation of a second (Figure 1.5).

![Anhydride reaction with water, arrows represent the movement of electrons](image)

Since esters are less reactive than anhydrides, an acidic or alkaline environment is needed in order for the hydrolysis of esters. When the hydrolysis is acid-catalyzed, the reaction is a reversible process, requiring the presence of excess water or removal of degradation byproduct to drive hydrolysis [45, 46]. The two stage reaction begins when free hydrogen associates with the carbonyl, which allows water to act as a nucleophile, forming a tetrahedral intermediate that facilitates the alcohol to act as a leaving group (Figure 1.6) [46].
When the reaction occurs in an alkaline environment, free hydroxyl ions act as a nucleophile. The nucleophilic attack leads to the formation of a tetrahedral intermediate causing the elimination of an alcohol (Figure 1.7). Like acid-catalyzed hydrolysis, this reaction leads to the formation of a carboxylic acid and an alcohol, but it is not an equilibrium reaction and will continue until the polymer has completely degraded.

The hydrolysis of amides is similar to esters; and can occur in either alkaline or acidic environments, but because the bond is less reactive than an ester linkage, stronger conditions are required in order for hydrolysis to occur [45, 46]. Both ester hydrolysis and amide hydrolysis leads to the formation of a carboxylic acid, but unlike ester hydrolysis the eliminated group from amide hydrolysis is an amine (Figure 1.8).
While the chemical bond used to form the polymer backbone is an important factor in controlling the rate of polymer degradation, the groups adjacent to the reactive bond can also be important factors in controlling the reaction rate. For example, the methyl group found on poly(lactic acid), leads to a slower degradation rate than what is observed in poly(glycolic acid) due to the presence of the hydrophobic moiety, which sterically hinders the nucleophilic attack of water [25, 47, 48]. Since the erosion profile of a polymer is sensitive to both the rate of degradation as well as the rate of water uptake, parameters that decrease the influx of water can be used to alter the erosion profile of the implant. Therefore the polymer hydrophobicity is an important parameter in controlling whether the polymer will be a surface or bulk eroding polymer.

Crystallinity is a measure of the polymer ability to form a structured array, which occurs as a result of the chain regularity, and is typically reported as a percentage of crystallinity [49]. Due to the close packing inherent in crystalline domains, the free volume for diffusion is lower in the crystalline regions, which can decrease diffusion coefficients by several orders of magnitude and play a role in altering the rate of polymer degradation.
degradation [30, 37]. Due to the random nature of polymers, there will always be amorphous domains such that a polymer can never be purely crystalline [10]. Crystalline domains can be present initially, or can form as a result of oligomer formation caused by polymer degradation, leading to latent crystallization [25, 28, 30].

In addition to polymer crystallinity, the glass transition temperature (T_g) has been shown to play a role in altering the free volume available for diffusion. The T_g is the temperature at which the polymer transitions from a glassy state into a rubbery state. When a polymer is above the glass transition temperature, the free volume is higher which results in an increased diffusivity [30]. Even the physical dimensions of the polymer can be used to transition a system from bulk eroding to surface eroding [44]. For systems that have low water absorption and rapid degradation kinetics, the implants would have to be extremely small in order to behave as a bulk eroding system. For example a poly(anhydride) disk would need to be smaller than 75 µm thick in order to behave as a bulk eroding system [44]. Conversely, polymer disk that rapidly absorb water with slow degradation kinetics would have to be significantly thicker to behave as a surface eroding system. Poly(α-hydroxy-ester) would need to be at least 7.4 cm thick to surface erode, while a poly(amide) which is even less reactive would need to be at least 13.4 m thick [44].

1.1.5. Biocompatibility of Degradable Polymers

With the insertion of any material into the body, understanding how the host and implant will interact is imperative. Biocompatibility describes the ability of an implanted material to maintain performance without initiating a negative host response [10, 50, 51].
Oftentimes biocompatibility is evaluated based upon the inflammatory and healing responses of the body to a particular implant [50]. The tissue response continuum, as explained by Anderson et al., organizes the host response into a sequence of events characterized by the cell types which are present. Phase I, or the acute and chronic inflammatory phase occurs over the course of the first two weeks, and is initiated by the implantation or direct injection of the device. During phase I, neutrophils, monocytes, and lymphocytes will be present with monocytes becoming the dominant cell type within days of implantation [50]. Phase II is characterized by an excess of monocytes and macrophages, leading to the development of a fibrous capsule around the implant. The duration of the second phase is determined by the rate of polymer degradation, and can take as little as 50 days or more than 400 days [50, 52-55]. Phase III is dominated by the degradation of polymer and an increase of fibrous tissue filling the void left behind by the eroding polymer [50]. Histological evaluation of the tissue response has proven useful in categorizing the effect of introducing an implant to the local environment.

One major concern when using a degradable device is the toxicity of the degradation byproducts. In the case of polylactic acid (PLA), polyglycolic acid (PGA) and their copolymer poly (lactic-co-glycolic acid) (PLGA), the polymer was designed to degrade into natural metabolites. The hydrolytic degradation of these polyesters results in the formation of lactic and/or glycolic acid, based upon the original polymer used [51, 56, 57]. In healthy tissues with high clearance, these intermediates are metabolized by the body into carbon dioxide and water and show no adverse effects upon introduction of the material to the body [58]. However, in situations where the clearance is low, which may occur as a result of a diseased state or be a function of injection site with low
metabolism and overall clearance, the effect of elevated levels of acidic byproducts must be considered. Therefore, the clearance rate of these compounds is an important parameter in implant design.

1.1.5. Clinical Utilized Implants

One of the most commercially successful pre-formed depots for local drug delivery is the Gliadel® wafer, used to treat malignant glioma as an adjunct to surgical resection and radiation. Gliadel® wafers, composed of poly(bis[p-carboxyphenoxy]) propane:sebacic acid (PCPP:SA), degrade by surface erosion to locally release carmustine. When placed directly into voids left after tumor resection, the wafers release carmustine into the adjacent tissue space [4, 9, 59-64], treating residual tumor cells along the periphery of the resection zone. These implants have shown modest success in both extending the patient survival time (by as much as two months), as well as increasing the patient’s chances for survival (by as much as 25%) [64].

While some drugs may require local delivery to provide the most effective treatment, other situations may necessitate a sustained, system-wide drug administration. Percutaneous placement of pre-formed drug eluting implants has provided a means by which constant plasma levels of drug can be achieved. In the treatment of prostate cancer, one form of therapy was daily injections of leuprolide acetate, a potent gonadotropin hormone-releasing hormone agonist, which reduces circulating levels of androgens. As an alternative to daily injections, the Leupron® Depot was developed and launched in 1989 [15, 29, 65, 66]. The Leupron® Depot is a polymer microsphere formulation injected subcutaneously to provide sustained delivery of leuprolide acetate for up to 4
months [29]. With this treatment, patient compliance improved due to a single quarterly injection replacing inconvenient and potentially painful daily injections [9, 61, 65, 67-75]. The Leupron® Depot has since been used as a treatment for a number of other hormonal disorders as well, including endometriosis and precocious puberty[15].

Despite the benefits of drug encapsulation in polymers, both pre-formed implants and injectable microsphere suspensions have disadvantages. For example, one major drawback of the Leupron® Depot is the complicated and expensive multistep process necessary for microsphere fabrication. Additionally, if a complication arises due to the treatment, removal of the microspheres is a difficult process [29, 66]. Preformed implants such as the Gliadel® Wafer have a more favorable manufacturing cost, since a large number of these implants are formed through compression molding and extrusion techniques, but they have limited application because they require surgical placement and simple modifications in formulation to customize the system can be difficult to implement [9, 61].

1.2. In situ Forming Implants

An alternative class of drug eluting implants was first described by Dunn et al. in a series of patents that described a system utilizing biocompatible, biodegradable polymers dissolved in a biocompatible solvent that could be mixed with an active therapeutic agent [76, 77]. This system provided a means by which many of the complications involved in drug eluting depots could be circumvented [13, 15, 29, 66, 71, 75-77]. Upon injection of the polymer solution into the body via a syringe, a solid drug eluting depot forms in situ through a process known as phase inversion [9].
Phase inversion refers to the process by which a polymer precipitate is formed through the immersion of the polymer implant solution into a bath that is miscible with the implant solution solvent but acts as a nonsolvent for the polymer phase [78, 79]. Once the implant solution is in contact with the nonsolvent bath, mass transfer of solvent and nonsolvent occurs, resulting in an unstable ternary system [79, 80]. With continued mass transfer of solvent and nonsolvent, stability of the system is restored. The mass transfer results in liquid-liquid demixing that facilitates precipitation of the polymer from the solvent/nonsolvent milieu [74, 79-83]. While the use of phase-inverting polymer systems is a well-established area of research for the fabrication of asymmetric and symmetric membranes, the use of phase inversion to design drug eluting polymer depots was not established until 1990 [13, 15, 29, 65, 66, 68, 71, 74, 76, 79, 80].

Phase-inverting drug depot systems have a number of advantages over traditional preformed implants. *In situ* forming implants (ISFIs) provide a means by which a drug eluting depot can be delivered in a minimally invasive manner directly to the site of action through injection of the polymer solution. The placement of the implant at the site of action ultimately reduces systemic exposure to therapeutic agents while maintaining elevated local levels of the drug [9, 61]. In addition to local delivery of therapeutic agents, these implants can be injected subcutaneously to achieve sustained delivery of therapeutic levels of quickly eliminated hormones or growth factors for the treatment of diseases such as diabetes and prostate cancer [9, 13, 15, 16, 29, 61, 65-67, 69, 73, 74, 84-96]. A large body of literature outlines many of the parameters affecting drug release rates from these phase-inverting implants.
1.2.1. Factors Affecting In Vitro Release and Phase Inversion

The phase inversion dynamics within in situ forming implant systems are governed by a number of factors including solvent type, polymer composition, and various additives. Within these systems, the drug release kinetics are related to both the phase inversion behavior and the resulting implant morphology. This section is subdivided into descriptions of fast and slow phase inverting systems, co-solvent mixtures, polymer types, and various additives.

1.2.1.1. Fast Phase Inverting Systems

Implants that form via phase inversion can be classified into two morphological classes: fast phase inverting (FPI) and slow phase inverting (SPI) systems. FPI systems occur when a polymer is dissolved in a strong, highly water miscible solvent such as NMP (Table 1.3), and the resultant implant morphology has a vast network of interconnected pores and macrovoids (Figure 1.9) [24, 68, 69, 71, 74, 97, 98]. These FPI implant formulations often have much lower viscosities than the SPI systems, and begin to precipitate upon contact with the aqueous environment. For ternary systems (consisting of solvent, polymer, and nonsolvent phases) the internal pore formation is hypothesized to be a result of changes in nonsolvent diffusivity [79].

For FPI systems, instantaneous demixing occurs when the polymer solution comes into contact with the bath-side (nonsolvent). This instantaneous demixing process happens as a consequence of the high miscibility between the solvent and nonsolvent and results in the formation of a thin, dense polymer shell [79]. Once formed, the polymer
shell acts as a diffusional barrier, causing the development of opposing concentration gradients of nonsolvent and solvent within the implant [79].

If one were to consider a spherical implant, the shell would exist at the surface exposed to the nonsolvent, with the nonsolvent concentration highest near the polymer shell. The solvent would have a concentration gradient in the opposite direction, with the solvent concentration highest at the center of the implant (Figure 1.10). The polymer precipitation at the implant surface is instantaneous, and polymer precipitation is delayed towards the center, again as a result of the opposing concentration gradients.

Just inside the interfacial polymer shell, droplets consisting predominantly of a mixture of solvent and nonsolvent form. These droplets are referred to as polymer-lean domains [79]. The

Figure 1.9. SEM micrographs of drug eluting depots made using 3 different solvents (A) NMP, (B) triacetin, and (C) ethyl benzoate. “Journal of Controlled Release, 62, K.J. Brodbeck, J.R. DesNoyer, A.J. McHugh, Phase inversion dynamics of PLGA solutions related to drug delivery Part II. The role of solution thermodynamics and bath-side mass transfer, 333-344, Copyright (1999), with permission from Elsevier.”

Figure 1.10. A schematic illustration summarizing the effects of opposing concentration gradients on macrovoid formation, with a corresponding SEM image highlighting these features.
polymer-lean domains are stabilized if diffusional flow of nonsolvent into the polymer phase is sufficiently large, so that polymer precipitation occurs and droplet expansion is arrested [79]. Since the concentration of solvent is sufficiently high towards the implant interior, the nucleated polymer-lean droplets begin to expand and aggregate as a result of insufficient nonsolvent exchange from the droplets into the surrounding polymer solution, ultimately forming macrovoids [79]. Since the development of macrovoids results from a transition from instantaneous demixing to delayed demixing, implants that have a sufficiently thin cross-section will not form macrovoids, but simply form a dense polymer shell [79, 80, 99]. One hallmark trait of FPI systems is the initial burst release of hydrophilic drugs followed by a plateau, due to the porous networks that are formed within these implants [68, 71, 74, 90].

Therefore, the phase inversion dynamics directly affect the implant morphology, and consequently the drug release behavior. The initial burst of drug characteristic of FPI systems has been hypothesized by McHugh et al. to occur as a result of drug dissolution into the polymer-lean phase. As a result of the lower viscosity and improved diffusion of drug through these polymer-lean phase regions, a period of burst release occurs until the drug is depleted from the porous network. After the drug has been depleted from the

Figure 1.11. A schematic illustration of the effects of the interconnected porous network on the release of drug through spherical implants composed of different solvents (A) NMP, (B) Triacetin, and (C) ethyl benzoate. The arrow thickness indicates the rate of diffusion through the matrix.
porous network, cumulative drug release is significantly reduced. Prolonged release is a function of polymer degradation as well as diffusion of drug through the polymer-dense regions (Figure 1.11) [68, 71, 74, 100]. The following table is a list of various solvents typically used in ISFIs and their solubility characteristics.

Table 1.1. Solvent Characteristics

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Miscibility in water (mg/mL)</th>
<th>Phase Inversion System</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Methyl Pyrollidone</td>
<td>150[^101]</td>
<td>Fast Phase Inversion</td>
</tr>
<tr>
<td>Dimethyl Sulfoxide</td>
<td>&gt;95[^101]</td>
<td>Fast Phase Inversion</td>
</tr>
<tr>
<td>Triacetin</td>
<td>77.8[^102]</td>
<td>Intermediate Phase Inversion</td>
</tr>
<tr>
<td>Ethyl Benzoate</td>
<td>Insoluble[^103]</td>
<td>Slow Phase Inversion</td>
</tr>
<tr>
<td>Benzyl Benzoate</td>
<td>3.4 in hot water[^105]</td>
<td>Slow Phase Inversion</td>
</tr>
<tr>
<td>Benzoic Acid</td>
<td>40[^103]</td>
<td>Slow Phase Inversion</td>
</tr>
<tr>
<td>PEG-400</td>
<td>Soluble</td>
<td>Fast Phase Inversion</td>
</tr>
</tbody>
</table>

1.2.1.2. Slow Phase-Inverting Systems

In contrast to FPI systems, SPI implant systems incorporate a solvent with a weak affinity for the nonsolvent, such as ethyl benzoate which is poorly miscible in water (Table 1.1). Often times these water-immiscible solvents are also weak solvents for the polymer used to make the implants, which results in a high viscosity solution [24, 65, 74, 97]. When amorphous polymers are used, delayed demixing occurs without a period of instantaneous demixing due to the low solvent miscibility in SPI systems. As a result of the slow precipitation, the composition throughout the implant is maintained as a constant, and no macrovoid formation occurs [24, 68, 74, 79, 83, 104]. Due to the absence of an internal porous network, drug is released from the implant via diffusion through the viscous polymer phase (Figure 1.11) [68, 71, 74, 100]. One advantage of
these SPI systems is that they have a nearly zero-order release, with almost no initial burst. However, these implants are more difficult to inject than FPI implants due to their relatively high viscosities, and thin film precipitation requires several hours or even days. Due to the morphological differences that arise during polymer precipitation, a high correlation exists between the phase inversion and drug release [68, 71, 72, 74, 105, 106].

A unique feature of SPI systems is that the drug release from these implants can be significantly enhanced by increasing the crystallinity of the polymer used [24]. For these systems, the presence of water can induce polymer crystallization as early as four days after implant formation [24]. As water continues to diffuse into the implant, a solid-liquid separation leads to the development of a semi-crystalline matrix, which excludes the drug into the polymer-lean domains. The resultant morphology of SPI systems utilizing crystalline polymers is a highly interconnected network of small pores (substantially smaller than the macrovoids seen in FPI systems). As in FPI systems, the development of a porous network leads to a substantial burst in drug release [24, 68, 71]. Unlike the burst release seen with FPI systems, burst release from SPI implants made with crystalline polymers begins four to five days after implant formation and continues for about one week. After this burst period, there is little change in the structure, and most of the drug has been depleted indicating that very little drug is trapped within the polymer dense regions [24]. The burst can be modified adding varying concentrations of an amorphous polymer, which will result in prolonged drug release [24]. Thermo-reversible crystallization can also occur for amorphous polymers dissolved in benzyl benzoate [107]. Crystallization begins to occur as soon as 6 h after precipitation during polymer storage at 25°C, and can be reversed by heating the sample to 65°C. If the
samples are not properly heated before use, there is a significant increase in drug release from the implants.

The composition of the injection environment plays a significant role in determining the drug release performance of an ISFI implant, this is especially true for SPI. In general, as the polymer solution becomes less miscible with water, the bath-side composition becomes increasingly more important to the drug release behavior [68]. The effects of bath-side additives such as triglycerides and organic salts on SPI systems have been studied, as both are found in the subcutaneous environment where these depots would likely be injected. The bath-side addition of triglycerides and salts both result in a significant increase in overall protein release rate, but these effects are governed by different phenomena. The first case is demonstrated by adding triacetin, a short-chain triglyceride, to the bath-side of implant systems using the weakly miscible solvent ethyl benzoate [68]. In this system, triacetin added to the bath-side solution is relatively more hydrophilic than the polymer-solvent depot. As triacetin diffuses into the implant, the overall implant viscosity is decreased and its capacity for water absorption is increased. As a result, the liquid demixing process is accelerated, triggering an increase in protein release rate. In the case of adding salt to the bath-side solution, no changes are observed in the liquid demixing rate [68]. Instead, surface erosion of the polymer depot occurs, and the increased protein release is attributed to polymer surface degradation. As a whole, these studies demonstrate that the specific composition of the aqueous implant environment is very important in SPI systems, and one must be aware that components entering the system may influence the drug release behavior.
1.2.1.3 Effects of Cosolvent Composition

While the classical systems described above demonstrate the effect of a pure solvent on drug release, phase inversion, and implant morphology, systems which combine solvents have been developed as a means for achieving intermediate release profiles [70, 71, 85, 86, 89, 92, 108-114]. The combination of NMP and triacetin reduces the affinity of the polymer depot for water, and subsequently reduces both the rate of phase inversion and drug release [71, 89]. Morphological analysis shows that the addition of 10wt% of triacetin into the implants causes a transition from a vastly porous network with a large number of macrovoids, to a more spongy structure, with additional increases in triacetin leading to a more dense structure [71]. Additional studies have shown that the introduction of solvents with low water miscibility has resulted in a reduction in burst release from the FPI implants [89, 109].

In some instances a limited burst is desired, and SPI systems can be modified by the addition of water miscible solvents [86, 113, 114]. Both fast and slow phase inverting systems have benefits and drawbacks, but because multiple parameters can be modified in order to produce the desired drug release profile, the application often dictates which implant formulation is used. Disorders that required near constant drug plasma levels, such as maintenance of basal insulin levels for the treatment of diabetes or the treatment of Turner syndrome by delivery of human growth hormone (hGH), may require the use of a SPI system to achieve optimal therapeutic results [68, 69, 92, 93, 105, 115]. In the case of diseases that require a high initial dose of drug followed by a small maintenance dose, such as the treatment of hormone imbalances, where the target organ needs to receive a saturation of signal in order to induce down regulation of the receptors, or
cancer chemotherapy where one wants as much drug as possible to be taken up by the cells, so that drug resistance can be avoided, FPIs are a better choice [15, 29, 66, 105, 115-117].

1.2.1.4. Polymer Type

PLGA is among the most common polymer used for ISFIs, but implants have also been formulated using a number of different polymers including PCL, PLA, Poly(ethylene carbonate), sucrose acetate isobutyrate, and fluoroalkyl-ended poly(ethylene glycol) [24, 65, 75, 91, 93, 94, 108, 118-121]. The chemical composition of a polymer (including but not limited to the Mw, functional groups, and degree of hydrophobicity) affects how the polymer will interact with other components in the ISFI system. Therefore the choice of polymer provides another tool by which researchers can tune the rate of phase inversion and drug release in order to fit their system demands. For example, implants formed using hydrophobic polymers have been demonstrated to release less drug than implants formed from their hydrophilic counterparts [14, 119, 122].

The effects of polymer Mw on phase inversion varies based on a number of factors. Intuitively, the rate of phase inversion would appear to be inversely related to the critical water concentration of the polymer solution. Due to the lower water affinity, one may assume that the formation of implants with small Mw would occur more slowly than implants made from polymers with larger Mw. However, the order of phase inversion is reversed. It has been demonstrated that the phase inversion of polymer films formed from polyethersulfone occurred more slowly with an increase in Mw [71, 82]. This trend has also been observed in our laboratory, with implants made from smaller Mw PLGA phase
invert faster than implants made from larger Mw PLGA (Figure 1.12). This change may be attributed to many factors including a sufficient increase in opposing concentration gradients due to the formation of the dense polymer shell, a change in the hydrophobicity resulting in a decrease in water absorption, and changes in diffusivity throughout the ISFI [71, 82, 123].

**Figure 1.12.** Representative ultrasound images of 15kDa (A), 29kDa (B), and 53kDa (C) implants formed in PBS illustrating the polymer precipitation of the implants over time. The 15kDa implants have already undergone phase inversion, and the central black region is result of macrovoid formation. (B) illustrates the gradual precipitation of the polymer before macrovoid formation occurs after 120 h. (C) illustrates delayed demixing after shell formation.

In addition to varying the polymer Mw, another method of modifying system characteristics is through changing the polymer concentration. Increasing the polymer concentration has been shown to decrease overall drug release as well as the rate of phase inversion in ISFIs [29, 67, 71, 79, 82, 86, 89, 90, 108, 122, 124, 125]. In FPI systems, the use of a more concentrated polymer solution influences the implant morphology, changing the microstructure from a highly interconnected porous network into a less porous spongy structure [71, 79, 81, 82]. The change in implant behavior can attributed to
a number of factors including lower water uptake, thicker skin formation, increased hydrophobicity of the polymer solution, as well as decreased diffusivity [71].

### 1.2.1.5. Polymer Formulation Additives

Changes in either the solvent or polymer used for ISFI fabrication provide a means by which the drug release and phase inversion profiles of ISFIs can be manipulated [68, 71, 72, 89, 105, 106, 113, 115, 118]. Due to the high viscosity of SPI implant solutions, injection is difficult or impossible without preheating the polymer solution [98]. Thus, while the ideal delivery profile may be achievable utilizing SPI systems, their use in a clinical setting is sometimes impractical. Therefore, methods by which the phase inversion and drug release rate of FPI systems can be altered through the use of additives, is an ongoing area of research [71, 74, 96, 98, 113, 115, 126]. Since burst release is often times an undesirable effect of the phase inversion process, additives have most often been used to reduce the burst release of drug from implants [24, 74, 115, 116].

The use of Pluronic as an additive (which is an amphiphilic triblock copolymer of polyethylene oxide (PEO) and polypropylene oxide (PPO), shown to increase a cell’s sensitivity to cancer drugs and heat [19, 73, 127]), provides a means by which the drug release can be reduced [98, 115]. Despite the reduction in drug release, the rate of phase inversion and water absorption of implants formulated with Pluronic were shown to increase due to the presence of the hydrophilic PEO blocks [98]. While morphological changes may occur within the ISFI (depending on the choice of Pluronic used), the changes do not elicit the transition into a more dense spongy microstructure [98, 115].
Instead the reduction in drug release is hypothesized to occur as a result of the orientation of the Pluronic molecule in the polymer matrix [98]. Since the PPO block is hydrophobic, it is reasonable to assume that the PPO block would readily associate with the polymer, while the PEO block would extend into the polymer-lean pores [98]. If the concentration is raised significantly, the Pluronic begins to fill the polymer-lean domains, resulting in a diffusivity barrier for the drug [98]. While increased Pluronic concentrations have been shown to decrease drug release from implants, this effect can be lost if the concentration of the additive exceeds the percolation threshold leading to increased diffusivity through the implant [115]. Other additives for reducing the rate of drug release include ethyl heptanoate and glycerol [116].

While the majority of additives studied are used to reduce drug release, some additives have the opposite effect on release [71, 128]. The use of hydrophilic polymers (such as polyvinylpyrrolidone (PVP)) has been shown to eliminate macrovoids if a large enough mass is included in the polymer solution [71, 78, 79]. However, when only a small mass of PVP is added to the polymer solution, an increase in the rate of polymer gelation and drug release occurs without increasing the rate of water absorption [71]. Another additive that can be used to increase release from FPI systems include the use of aliphatic esters which have been shown to increase the water absorption, solvent release, and enhance polymer degradation [128]. A table of additives and their effects is provided below.
Table 1.2. Additives

<table>
<thead>
<tr>
<th>Additive</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pluronic P85</td>
<td>Reduced Burst</td>
</tr>
<tr>
<td>Pluronic L101</td>
<td>Reduced Burst and overall release</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Reduced Burst</td>
</tr>
<tr>
<td>Ethyl Heptanoate</td>
<td>Reduced Burst</td>
</tr>
</tbody>
</table>

1.2.2. Solvent Safety

Primary concerns for the *in vivo* use of phase sensitive ISFIs center around the issue of solvent safety. Some of the most commonly used solvents for ISFIs include DMSO, NMP, ethyl benzoate, and low Mw PEO [72, 81, 106, 129]. These solvents have been used historically to aid in solubilizing drugs, and their toxicological profiles have been well-studied [130]. While any solvent can induce an adverse effect when the concentration reaches an unsuitably high level, studies of these solubilizing agents have focused on: systemic toxicity (Table 1.4) [130], effects on renal and liver function [131], teratogenic effects [132], as well as eye sensitivity [133].

Poly(ethylene glycol) 400 was shown to induce signs of renal toxicity when the gavage dosing approached 2.5 ml/kg/day, five days a week for 13 weeks in Fischer-344 rats, but this toxicity was reversible and slight [134]. The effects of the dietary administration of NMP to beagle dogs over the course of 13 weeks were evaluated [133]. No signs of toxicity or behavioral changes were observed, but because the acute oral LD$_{50}$ has been reported to be 4.2 g/kg in rats, the agent is considered moderately toxic for ingestion [132, 133]. The acute myotoxic potential of NMP, DMSO, and 2-pyrrolidine was evaluated by measuring the cumulative creatine kinase efflux from isolated rodent muscles exposed to implants formulated using the listed solvents for 2 h. NMP and
DMSO both had a high myotoxic potential, leading to the belief that they may not be safe for use in applications involving the intramuscular delivery of drugs [135].

However, when ISFIs formulated using poly(caprolactone) dissolved in either NMP or DMSO were injected both subcutaneously and intramuscularly into the backs of 10 rhesus monkeys, the ISFIs yielded similar results to other preformed biodegradable implants. In this study, each monkey received two IM and two SC implants, and after 28 days a histological analysis was performed. The histology was rated using a 0-4 scale. Control tissues, which contained no implant, were given a score of 0. Conversely if severe cellular infiltrate and tissue necrosis were observed, the sample was given a 4 [75]. In this study the highest histological rating given was 1.5, based upon the presence of neutrophils, lymphocytes, macrophages, and a small number of foreign body giant cells, similar to what is observed with preformed implants [75]. These results indicated, that there was a relatively small impact of DMSO and NMP as a toxin in the quantity used for a phase inverting polymer systems, both subcutaneously and intramuscularly [75].

Similar results were also observed with implants using a combination of PLGA and poly(ethylene glycol) 500 dimethylether (PEG500DME) [129]. After 48 days in a rabbit model granular tissue was discovered at the injection site in all animals involved in the study, histopathological examination of the injection site showed no evidence of irritation and inflammation [129]. Implants using PLA dissolved in triacetin followed a typical wound healing response, and met the International Organization for Standardization regulations of a biocompatible implant. Current commercial applications of ISFIs provide a safe, platform for the controlled release of therapeutic agents, using
solvent volumes well below the LD<sub>50</sub> level [13, 16, 76]. Common solvents, their LD<sub>50</sub>, and potential side effects are listed in Table 1.3 below.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt;*</th>
<th>Side Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>6.2 g/kg [136]</td>
<td>Local, teratogenic and systemic toxic effects [137]</td>
</tr>
<tr>
<td>N-methyl-2-pyrrolidone</td>
<td>4.2 g/kg [138]</td>
<td>Local, teratogenic and mutagenic toxic effects [137]</td>
</tr>
<tr>
<td>Dimethyl Sulfoxide</td>
<td>14.5 g/kg [139]</td>
<td>Local, teratogenic, mutagenic and systemic toxic effects [137]</td>
</tr>
<tr>
<td>Triacetin</td>
<td>3 g/kg [140]</td>
<td>Data Not Available</td>
</tr>
<tr>
<td>Polyethylene Glycol</td>
<td>30 g/kg [141]</td>
<td>Local and systemic toxic effects [137]</td>
</tr>
<tr>
<td>Propylene Glycol (PG)</td>
<td>22 g/kg [142]</td>
<td>Local, mutagenic and systemic toxic effects [137]</td>
</tr>
</tbody>
</table>

*All values are for oral administration for a rat

1.2.3. In Vivo Release

Early in vivo ISFI studies focused predominately on evaluating drug release through analysis of serum plasma levels which screened for released drug, proteins, or specific drug efficacy markers such as testosterone [15, 16, 29, 66, 67, 69, 73, 85, 86, 88, 92, 143, 144]. While the release data obtained from these studies cannot be directly compared to that obtained from in vitro release studies (due to metabolism, adsorption, and drug clearance occurring in the body), the effect of implant formulation on release could still be evaluated [105]. In a series of studies performed in both rat and dog models, Dunn et al. measured testosterone levels as an efficacy marker for leuprolide acetate release from ISFIs. Testosterone can be used to determine leuprolide acetate efficacy, because continuous delivery of leuprolide acetate causes a reduction in circulating sex hormones due to pituitary desensitization [15, 29].
FPI systems are optimal for this application due to their characteristic burst release, which can rapidly increase pituitary signaling to desensitize the gland more rapidly. The role of polymer Mw, polymer concentration, polymer hydrophobicity, and drug loading were evaluated for FPI implants consisting of NMP and 75:25 PLGA [15, 29]. From these studies, it was shown that changes in polymer concentration and drug loading had a negligible effect in reducing serum testosterone levels, while changes in polymer Mw and hydrophobicity were more significant factors for controlling drug release in these in vivo FPI systems. The average percentage of residual leuprolide acetate followed the expected trend of increased drug concentration with increases in polymer concentration, loading drug concentration, and polymer Mw (Figure 1.13). Additionally, changing the polymer hydrophobicity resulted in a prolonged lag time before desirable levels of testosterone were achieved, taking 35 days compared to 14 days for the more hydrophilic counterpart [29].

![Figure 1.13. Bar graph generated using the percent of residual drug content data from: International Journal of Pharmaceutics 194 (2000) 181–191, with implants explanted 105 days after implant formation in male Sprague–Dawley rats.](image-url)
Another common formulation parameter that has been varied in order to evaluate its effects on \textit{in vivo} release is the organic solvent used to dissolve the polymer [69]. The sustained release of human growth hormone (hGH) from 50:50 PLGA was evaluated using polymer formulations comprised of solvents with increasing nonsolvent miscibility [69]. Serum hGH levels were monitored after subcutaneous injection of implants made with NMP, triacetin, ethyl benzoate, or benzyl benzoate. The solvent affinity for water played a significant role in altering the release profile of implants. It was shown that ISFIs made with benzyl benzoate maintained a relatively flat serum hGH profile when compared with the other solvent systems, indicating a more constant rate of growth factor release, which is indicative of a SPI system [69].

1.3. Conclusion

ISFIs are an exciting area of study that can be used for the continuous release of a therapeutic agent as well as local targeted delivery of drug to a diseased tissue. Since these implants are liquid solutions outside of the body, and do not precipitate to form drug-eluting depots until after exposure to an aqueous environment, they provide a means to circumvent the intrinsic limitations of pre-formed implant systems. Additionally, the simple manufacturing process results in a cost efficient system for the noninvasive administration of a variety of drugs and proteins. While the chief concern for ISFI technology has focused on solvent biocompatibility, a large number of studies have been performed to establish the safety of these implants. Advances in medical imaging have provided unique insight into the behavior of ISFIs \textit{in vivo}, demonstrating that the environment in which the implant is injected plays a significant role in the drug release
and phase inversion behavior. The insight into ISFI dynamics in vivo may lead to methods for the minimally invasive treatment of non-resectable tumors, or to the design of systems that can postpone or eliminate the long-term complications of diabetes. Improvements in the understanding of these implant systems may one day provide a treatment option for clinicians to treat an incalculable number of diseases.

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Chapter 2: Advances in Image Guided Intratumoral Drug Delivery Techniques

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* R. Patel and L. Solorio contributed equally to this work
2.1. Introduction

Modern imaging techniques no longer focus solely on generating anatomical images, but instead are capable of performing a wide array of qualitative and quantitative tasks ranging from monitoring physiological changes that occur in diseased tissues to assessing function at a molecular level. These advances have not only helped diagnostic medicine, but have resulted in a new paradigm in drug delivery. The ability to guide or place devices in the body in a minimally invasive manner, noninvasively determine concentration and distribution of active agents, and monitor treatment efficacy, sometimes performing all with a single imaging modality, are paramount in providing feedback to both clinicians and researchers (Figure 2.1).

Figure 2.1. Graphical representation of the various components that comprised the field of image guided drug delivery.

The advancements in medical imaging techniques have been particularly valuable in the field of interventional oncology, where a variety of imaging modalities are used to noninvasively evaluate the efficacy of therapeutic treatments through changes in tumor necrosis and vascularization [1-8]. Medical imaging has also led to the improvement of
minimally invasive treatment options such as transarterial chemoembolization (TACE) and convection enhanced delivery (CED). While the concepts of image guided drug delivery can extend into the systemic delivery of contrast enhanced particles and therapeutics, the focus of this chapter will be on the local administration of therapeutics to solid tumors for interventional drug delivery, using image guidance for treatment planning and evaluation of treatment efficacy. This chapter will highlight advances in TACE, methods for improving drug penetration within a tissue, as well as CED.

2.2. Transarterial Chemoembolization (TACE)

The availability of high speed real time imaging modalities in combination with minimally invasive surgical techniques has led to the development of several local direct drug infusion protocols. One such minimally invasive image guided local drug delivery procedure that has been commonly clinically employed is transarterial chemoembolization (TACE). During this procedure a catheter is threaded into the local blood supply of a tumor under image guidance, a chemotherapeutic drug cocktail is administered directly to the tumor, and an embolic agent is released to block blood flow. By selectively occluding the tumor blood supply, washout of the chemotherapeutic agent is prevented and higher drug dosages can be maintained within the tumor volume.

TACE is most often employed for treatment of hepatocellular carcinoma (HCC) in the liver; however it has also been used for cholangiocarcinoma [9, 10] and liver metastases [11, 12]. Normal liver parenchyma is supplied by collateral portal venous and hepatic arterial circulation and receives two-thirds of its blood supply from the portal vein and one-third from hepatic arterial blood flow [13]. Meanwhile, hypervascular HCC
tumors often receive much of their blood supply from the hepatic artery [13]. Therefore embolotherapy blockage of peripheral hepatic arterial blood flow can initiate ischemic tumor necrosis, while leaving normal liver parenchyma intact as it is still supplied by collateral portal circulation [13, 14]. TACE-treated HCC tumors which have greater portal involvement have a worse prognosis and have a greater chance for tumor recurrence than TACE treated tumors with only hepatic arterial involvement [15]. While surgical resection is still the gold standard and preferred treatment option for HCC, this option is often not available due to commonly occurring concurrent cirrhotic liver disease, portal hypertension, or poor hepatic reserve function, which are all associated with significant perioperative morbidity and mortality [14, 16, 17]. Consequently, minimally invasive treatments such as TACE are often the only treatment options available for patients with HCC. Several randomized controlled trials (RCT) have shown that TACE is a good therapeutic option for improving both patient survival and providing palliative therapy [18-21]. A recent cohort study by Miraglia et al. has shown that TACE is effective in achieving complete control of tumor growth in a majority of patients with a single HCC tumor less than 6 cm in diameter with complete tumor necrosis seen in 50-69% of cases [14]. On the other hand the authors found that complete necrosis of HCC tumors greater than 6 cm was only achieved in 13% of patients [14].

2.2.1 Preprocedural imaging

Before TACE treatment can be undertaken, it is necessary to conduct baseline diagnostic imaging to determine tumor extent, as well as rule out any procedural contraindications such as portal-hepatic arterial shunts or portal vein thrombosis.
Therefore pre-procedural evaluation using cross-sectional imaging modalities such as biphasic multi-detector computed tomography (MDCT) or magnetic resonance imaging (MRI) is required (Figure 2.2A). In addition, digital subtraction angiography (DSA) of the celiac trunk must be conducted before injection of chemotherapeutic or embolic agents during the TACE procedure to determine the course of the tumor feeding arteries (Figure 2.2B) [22]. Determining the correct tumor feeding vessels is essential for selective positioning of the catheter during TACE to reduce collateral damage to normal liver parenchyma. Therefore two imaging stations, one cross-sectional imaging modality

**Figure 2.2.** DSA image of the celiac trunk shows a HCC tumor and its corresponding feeding vessel branches of the hepatic artery (A). Contrast enhanced CT shows a hypodense tumor region in the right liver lobe (B). After TACE treatment, iodized lipiodol is shown to accumulate in the tumor mass with planar x-ray imaging (C) and in non-contrast CT (D). All tumor regions are encircled by the dashed line. Images were provided courtesy of Dr. Huimin Liang, Huanzhong University of Science and Technology.
such as MDCT or MRI to provide soft tissue information, and one real time fluoroscopy or DSA station to provide guidance for catheter placement, are necessary. Since transferring patients from one site to the other is inconvenient, hybrid CT/DSA [23] or MRI/DSA [24] suites have been employed over the past decade.

More recently, with the availability of digital flat-detector angiographic systems, a new class of C-arm CT imaging devices that can provide both conventional DSA and CT-like soft tissue images have been developed. C-arm CT data is obtained by rotating the flat-detector around a patient and obtaining multiplanar reconstruction images. Concurrent injection of contrast agent into the hepatic artery provides hemodynamic information. These C-arm CT devices have improved convenience due to a single machine providing all required information as well as greater sensitivity in detecting small hypervascular HCC lesions (<2 cm) than MDCT [25, 26]. In comparison to conventional DSA, C-arm CT has been shown to have significantly higher sensitivity, specificity, and accuracy in finding tumor feeding arteries according to a study by Iwazawa et al [22]. However one limitation of these systems is the limited field of view with a study by Meyer et al. showing that two-thirds of their cases had incomplete liver coverage using a medium sized flat detector [25]. Therefore pre-procedural planning using MDCT is still recommended, but C-arm CT can be used exclusively during TACE treatment once a narrower tumor field of view has been established [22, 25].

2.2.2 Embolic Agents

A variety of embolic agents including polyvinyl alcohol (PVA) particles, gelatin sponges, autologous clots, steel coils, and starch microspheres have been used to occlude
vessels during TACE. PVA particles and steel coils are permanent embolic agents while gelatin sponges only temporarily occlude vessels. With the latter, re-vascularization occurs after several weeks. Due to their proven safety, gelatin sponges are the most widely used embolic agent [27]. In addition to the aforementioned agents which perform the sole function of vascular occlusion, several other notable agents are multifunctional and act as occlusion agents and drug delivery vehicles. Of these, the most important is lipiodol, an iodinated ethyl ester of the fatty acids in poppy seed oil. Lipiodol selectively accumulates in tumor tissue due to lack of Kupffer cells and can remain there for several weeks to a year after injection [28]. However in normal liver parenchyma, lipiodol collects in portal venules where it is phagocytized by Kupffer cells and quickly cleared [28]. Lipiodol acts as a drug delivery vehicle due to its ability to carry and localize chemotherapeutic agents inside a tumor volume. To incorporate delivery capability, chemotherapeutic drugs, most commonly doxorubicin, 5-fluoruracil, or cisplatin, are mixed with lipiodol until an emulsification is created. A study by Nakamura et al. has shown when the ratio of lipiodol to drug solution is correctly adjusted, approximately 3:1 (lipiodol/doxorubicin) for their studies, drug is slowly released from the emulsion, and pharmacokinetic outcomes are better than direct chemotherapeutic infusion [29]. It has also been shown that subsequent Gelfoam ® embolization can also slow down release of drug from the lipiodol emulsion and prevents washout, resulting in increased drug concentration in the tumor volume [30]. As an embolic agent lipiodol occludes microvessels and can reach portal veins around the tumor periphery through arterioportal communication routes [29]. Since tumors that have portal involvement are the most resistant to TACE, the ability of lipiodol to reach and accumulate in portal circulation
surrounding a tumor can greatly strengthen anti-tumor effects [31]. Finally since lipiodol is iodinized, it also acts as a CT contrast agent and its tumor accumulation can be examined post-operatively using CT [32].

Recently another type of combination drug delivery-embolic agents, injection of controlled release chemotherapeutic drug eluting beads (DEB), has been developed and is currently undergoing clinical trials. Currently there are two types of drug eluting microsphere based systems available for TACE, DC Bead microspheres (Biocompatibles, UK) and Quadrasphere micropsheres (Biosphere Medical). The DC beads are comprised of a polyvinyl alcohol polymer hydrogel with a sulfonic acid additive and can be polymerized into different sized microspheres from 100-900 µm[33]. These beads can be loaded with a variety of chemotherapeutic agents with the two most common being doxorubicin and irinotecan and have been shown to be effective in improving patient survival[34, 35]. The Quadrasphere microspheres are the more recently developed of the two DEB systems and are comprised of hydrophilic, acrylic copolymer that can absorb up to 64 times their dry state volume[36]. These particles have a dry volume of 50-200 µm, which can expand to 200-800 µm[36]. While much less studied than PVA particles, recent studies have shown that Quadrasphere particles loaded with cisplatin or doxorubicin are promising for use with TACE therapy[37, 38].

In comparison to traditional TACE with direct infusion or as an emulsion with lipiodol, the half-life of doxorubicin is greatly increased with PVA DEB and has been shown to range from 150-1730 h depending on bead size [39]. Therefore the antitumor effect of DEB after TACE is prolonged, and tumor necrosis can take place up to 40 days post treatment [39]. Recent clinical studies have shown that DEB particles are more
effective in improving survival in patients with unresectable HCC tumors than traditional TACE embolic agents, and many centers have shifted to using DEB particle based TACE [39]. With the development selective micro catheters to isolate tumor feeding arteries and improvements in embolic agents the safety of this type of treatment has greatly increased. Recent trials have shown that severe complications, such as pulmonary embolism, are extremely rare with current treatment protocols. One study, found that out of 1348 patients treated with a total 2012 TACE procedures there was only one case of pulmonary embolism, three cases of liver abscess, and a very few other severe complications from TACE therapies[40].

2.2.3 Peri-operative Imaging and Treatment Efficacy

After TACE treatment, proper follow up and assessment of treatment efficacy is vital. However traditional measures of tumor response to therapy such as the World Health Organization (WHO) and the Response Evaluation Criteria in Solid Tumors (RECIST) do not correlate well with response to local percutaneous therapies such as TACE [18, 21]. Tumors treated with TACE may have necrosis, edema, or hemorrhage which can have variable effects on tumor size, the primary criteria used for WHO and RECIST. For TACE, the European Association for Study of Liver (EASL) criteria which estimates the reduction in viable tumor volume through loss of tumor enhancement in imaging is the preferred method of measuring tumor response to therapy [18, 41]. Yet although, loss of tumor enhancement does correlate well with tumor necrosis, the assessment of enhancement with CT can be difficult if the tumor is infused with lipiodol, which radio-opaque (Figure 2.2D). Evaluation of tumor enhancement with MRI or
contrast enhanced ultrasound however is not affected by iodized oil and has been shown to correlate well to tumor necrosis when using the EASL criteria [39, 42]. While lipiodol obscures CT assessment of tumor enhancement, tumor uptake of lipiodol using non-contrast CT may itself also serve as marker for tumor necrosis [32, 43, 44](Figure 2.2C+D). Tumors which show homogenous accumulation of lipiodol are strongly correlated with necrosis on histopathology, while incomplete or spotty uptake of lipiodol is suggestive of viable tumor [44].

In addition to quantifying tumor enhancement to assess treatment, functional imaging using CT, positron emission tomography (PET), and MRI can also be used to evaluate postoperative tumor necrosis. Diffusion-weighted MRI (DWI) measures the apparent diffusion coefficient (ADC), which detects the Brownian motion of water molecules in tissue. Water mobility in viable tumors is reduced due to the presence of intact cell membranes and results in a low ADC value. On the other hand, increased water diffusion and a high ADC value is correlated with tumor necrosis [42]. PET measures tumor viability by detecting radioactive decay of a glucose analog, fluorodeoxyglucose (\(^{18}\)F) (FDG), to measure tumor metabolism. However while PET is sensitive and has been used to measure tumor response to TACE therapy [45], it has poor spatial resolution and soft tissue contrast. Newer combined PET/CT imaging workstations may be more suitable for this application since they combine the high spatial resolution of CT and exquisite sensitivity of PET. CT perfusion imaging can also be used as an alternative to assess tumor response to therapy by measuring perfusion parameters pre- and post-treatment [46]. A study by Chen et al., demonstrated that tumors undergoing successful treatment had significantly decreased hepatic arterial
perfusion, hepatic arterial fracture, and hepatic blood volume perfection CT parameters post-TACE treatment compared to pre-TACE, while the same perfusion parameters were insignificantly different for viable tumor groups [47].

2.3. Convection Enhanced Delivery

Treatment of neuro-oncologic conditions has proven to be extremely difficult, due in part to the physiological and anatomical protection provided by the blood brain barrier (BBB). The BBB protects the central nervous system (CNS) by limiting the passage of molecules from the circulating blood into the cerebrospinal fluid and interstitial fluid in the parenchyma, preventing the transport of many potentially potent therapeutic agents to the disease site [48]. In order to circumvent this naturally existing barrier, several researchers have focused on developing ways to disrupt the BBB. One such technique is opening the BBB by shrinking the endothelial cells of the BBB with an osmotically active solution resulting in an increased permeability of inter-endothelial tight junctions [49, 50]. This effect is largely reversed after 10 minutes [50]. Another technique for BBB disruption includes the use of focused ultrasound with ultrasound contrast agents under MRI guidance [3, 51, 52]. This technique has shown that the BBB can be disrupted for as long as 4 hours and allow molecules larger than 100 kDa to pass into the CNS [3, 51, 52]. While these techniques have shown some promise in the field of neuro-oncology [51, 53], they are limited to treatment regions near vascular beds [54]. Additionally, other diffusion based treatments such as polymeric devices and drug pumping catheters can be surgically placed in a resected tumor bed [55, 56], but the spatial distribution is limited by the diffusion distance of the drug (millimeters).
Therefore additional surgeries may be necessary if an increased dosage is required [54, 57]. While all of these technologies provide a means by which drugs can be administered to the CNS, they are all limited by the low, non-homogenous, distribution volumes that are the inherent short comings of diffusion based delivery systems [54, 57-59].

![Figure 2.3. Graphical representation of CED, with an ideal delivery volume (A) and CED if reflux has occurred (B).](image)

To improve CNS drug penetration beyond diffusional limitations convective drug delivery methods were developed. Convective enhanced delivery (CED) is a direct intracerebral means of drug delivery performed by sterotactically placing a cannula into the targeted region and maintaining a continuous pressure gradient to convectively deliver therapeutic agents through the extracellular space to a large tissue volume. CED has been shown to be a promising means of delivering drugs to treat a wide variety of illnesses from Parkinson’s disease (PD) to malignant gliomas [55, 60-62]. In CED, because the distribution volume (Vd) is generated by the convective movement of the infusate, the drug is more homogenously distributed than diffusion based systems, and the molecular weight (Mw) of the drug plays a less significant role in the mass transport (Figure 2.3). Initially, this technique was limited by drug reflux along the cannula due in
part to improper infusion rates and poor cannula designs (Figure 2.3B). Ultimately reflux results in a poor drug distribution within the tissue and at times can even lead to the leakage of drug from the target region into the CSF, potentially leading to systemic toxicities [54].

As imaging techniques have increased in sophistication, the insight gained has led to advances in not only cannula design, but in the role that the tissue properties play in the drug distribution volume [54, 58, 63, 64]. The use of gadolinium conjugated forms of albumin (Gd-albumin) has been especially instrumental in developing real time imaging techniques of CED in the brain stem [60, 65-67]. An interesting aspect in these studies was that the spatial distribution of larger Mw drugs could be correlated with the Vd of Gd-albumin (72 kDa) [67]. In a study evaluating the distribution of drug in rat and primate models, there was a 4.8% mean difference in the Vd between drug agent, $^{125}$I-interleukin 13 bound to Pseudomonas, and Gd-albumin, which correlated to less than a 200 µm difference in the diameters of the Vd occupied by the surrogate tracer and the transported drug [67]. More recently, Gd-albumin was used to determine the effects of tissue properties on the spatial distribution of infused drug in the hypothalamus and hippocampus in rat models using high resolution MRI (11.1 Tesla) [60]. When CED was used to deliver Gd-albumin to the hypothalamus, it was shown that regions with dense cell layers such as the dentate gyrus served as convective barriers for mass transport appearing as hypointense regions within the hypothalamus. It was hypothesized that due to the decreased hydraulic conductance in these dense cell layers, the drug would flow around these structures into regions of higher conductivity resulting in elevated
concentrations in the less densely packed regions of the hypothalamus, highlighting the
effect of neuroanatomy on the spatial distribution of drug within a region [60].

While Gd-albumin works well as a surrogate tracer for the real-time imaging of
drugs with larger Mw, under conditions of low flow rates (less than 0.5 µl/min) or with
infusion volumes greater than 2 ml, the distribution of smaller Mw drugs is more
accurately determined using a Gd-diethylenetriamine pentaacetic acid (Gd-DTPA, Mw
of 938 Da) tracer due to the importance of diffusion in the mass transport of the drug.
This surrogate tracer system has been successfully used in the treatment of Gaucher
disease in human patients [66, 68, 69]. While the use of surrogate tracers facilitate the
real-time evaluation of drug dispersion and flow parameters that ensure proper drug
dispersion, the ability to evaluate the spatial distribution of the actual drug in real-time
could prove to be paramount in designing optimal treatment regimens, such as iodine for
synchrotron stereotactic radiotherapy or Gd in neutron capture therapy (NCT) [59]. In
NCT a stable form of Gd or boron (B) is delivered to tumors and upon irradiation with
thermal or epithermal neutrons, γ-rays and auger electrons are released by the delivered
Gd compounds [70-75]. The γ-rays and auger electrons that are emitted causing
oxidative damage to DNA that ultimately results in cell necrosis, if the Gd is extremely
close to the DNA (0-150 nm) [71]. Boron treatments are typically used due to higher
tumor uptake than Gd resulting in a more effective tumor treatment [70, 71, 73, 74]. Due
to a more complete understanding of the time dependent intranuclear localization of Gd
based compounds and the ability to see the spatial distribution using MRI, interest in Gd
based therapies has been reinvigorated[71]. Due to the increased time required for high
levels of Gd to be taken in by cells, methods for increasing the retention time of Gd in
tumors is being investigated [73]. Additionally, advances in material chemistry that improve the cellular uptake of Gd may play a significant role in NCT in the future.

While it is clear that the tissue properties play a critical role in the distribution of drug when using CED [55, 63, 65], cannula design and placement have been shown to be equally significant factors in limiting reflux and facilitating efficient distribution of drugs [54, 58, 64, 65, 76, 77]. It has been shown that cannulas with a novel step design, a regular cannula encased by a silicone outer layer, decrease reflux and have been adapted for use in non-human primates as well as for study in rats [54, 64, 77]. In both non-human primates and rats, there was a reduction in reflux and increasing Vd. This cannula design also facilitated increasing both the rate and volume of infusion [54, 64]. One interesting side-effect of increased infusion volumes is that ventricular compression begins to occur in both non-human primate and canine models. In this case, ventricular compression does not appear to result in neurological symptoms and seems to only be temporary, but are important to monitor for the development of a safe delivery methodology [78]. When investigating the effects of cannula placement for preoperative planning, a recent study by Yin et al. showed that the distance of the cannula to white matter tracts, which serve as leakage points, appeared to be the most important factor in controlling the Vd during CED [64]. The primary target for treatment of PD has been the putamen. [64] In order to get a clinically relevant response from CED based treatments, the distribution of the drug must be optimized [64]. In non-human primates, when cannulas were placed too closely to the white matter tracts of the corpus callosum, internal capsule, or the external capsule, significant leakage occurred, resulting in the loss of drug into the CSF [64, 76, 77, 79]. The resultant images obtained from the study were
analyzed to establish zones based on the distance from the white matter structures that could be used to limit leakage into the CSF and provide a 3-D coordinate system for surgical planning in human patients for treating neurodegenerative diseases such as PD as well as neuro-oncological treatments [64].

Advances in preoperative stereotactic planning [63, 64, 77], as well as the ability to visualize the Vd, may lead to more precise placement of the cannula resulting in optimized treatment of non-resectable gliomas. Despite these advances, because of the high level of expertise required for clinicians to successfully administer therapeutics via CED[80], as well as the high cost and exposure limitations inherent in MRI and CT [81], widespread use of the technique is currently limited.

<table>
<thead>
<tr>
<th>Table 2.1. Comparison of image guided delivery techniques</th>
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<tr>
<td><strong>Delivery Technique</strong></td>
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<tr>
<td>Transarterial Chemoembolization</td>
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<td>Polymer Implants</td>
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<td>Convection Enhanced Delivery</td>
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<tr>
<td>Particles</td>
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2.4. Techniques for Evaluating the Phase Inversion Process

Imaging techniques can also be used as a method for characterizing implant behavior, but only a limited number are available for characterization of ISFIs. Implant characterization provides information about implant structure and the dynamics of phase inversion. In this section, three specific modes of analysis are considered: dark ground optics, electron paramagnetic resonance (EPR), and ultrasound imaging. The fundamental principles behind each technique are discussed as well as their advantages and limitations in terms of describing the behavior of phase-inverting systems. The data extracted using these various modalities includes information used to determine water diffusion, drug release, gel formation, polymer erosion, and implant geometry. Only EPR and ultrasound can be used for continuous in vivo implant analysis, which gives insight into differences in behavior between in vivo and in vitro implants.

2.4.1. Dark Ground Optics

Dark ground optics is an imaging method developed by McHugh and colleagues which provides information regarding the mechanisms of polymer phase inversion [82-89]. This characterization technique is employed when a polymer solution is exposed to a nonsolvent bath, creating both dark ground and reflected light images [87]. The process is simple and quick, typically requiring less than three minutes of total run time and an uncomplicated data analysis [89]. From the dark ground video images, information about diffusion coefficients, morphology, liquid-liquid phase separation and gel formation can be obtained, enabling the characterization of phase inversion behavior. Using this technique Brodbeck et al. determined the protein release kinetics of a PLGA solution by
monitoring the propagation rate of the liquid demixing region [82]. Graham et al. also studied a PLGA system and used dark ground techniques to characterize the effect of formulation changes on drug release mechanisms \textit{in vitro} [85].

![Figure 2.4. Schematic of the dark ground imaging system, and a representative image showing the gel region and the diffusion fringes. “Modified from Journal of Controlled Release with permission from Elsevier, 58, Author(s), Phase inversion dynamics of PLGA solutions related to drug delivery, 233-245, Copyright (1999).”](image)

Dark ground imaging is used to track the motion of fronts during phase inversion, and can also be used to monitor changes in solvent concentration at the polymer/nonsolvent interface [90]. Figure 2.4 shows the arrangement of the imaging system. A polymer solution is situated within an optical quality quartz cell and placed in a nonsolvent bath. A thin metal foil prevents any initial interaction between the solvent and bath-side, and the phase inversion process begins following the removal of the foil when the two liquids come into contact [85]. Collimating lenses focus a filtered laser onto the quartz cell. After passing through the quartz cell, the diffraction image of the refracted laser beam is filtered onto the back focal plane of the transform lens with a circular stop. This ensures that the unrefracted light is eliminated before the image is
focused through a camera. The fringes are recorded by the camera and overlaid on the cell to visualize the relative fringe locations [87]. As water diffuses into the polymer system, interference fringes are created and dark ground video imaging records the concentration gradient that forms [91]. The interference fringes indicate the depth of water diffusion into the solution, and appear as striations in the image (Figure 1.9). Using reflected light, the region of the polymer solution which becomes phase-separated can be visualized. By monitoring reflected light and refractive index distributions, diffusion and liquid-liquid gelation fronts are identified relative to the initial thin film [87]. Both fronts move farther into the polymer region as more nonsolvent comes in contact with the solution. Figure 9 shows a dark ground image where both diffusion and gelation fronts can be seen. By tracking the position of the two fronts over time, a plot of position squared vs. time can be created for each. The diffusion fringe indicates the rate at which water enters the polymer system, and the liquid-liquid fringe reveals the rate of gelation of the system [85]. Transmitted light images can be analyzed to obtain the precipitation times [87].

Through the use of dark ground imaging, the relationship between phase inversion and implant morphology and the resultant effects on drug release have been established [85]. Graham et al. found that an increase in the gelation rate of a solution correlates to a greater initial drug burst, which suggests that drugs diffuse more quickly through a gelled system than a single-phase solution [85]. McHugh et al. also acquired data which indicates that the nonsolvent diffusion rates and gel formation kinetics ultimately affect drug diffusion through the phase-inverted polymer system [87]. This information provides a basis for the manipulation of system properties in order to control drug release.
kinetics. In addition, dark ground optical analysis gives insight into the influence of
solvent and nonsolvent compositions on the dynamics and release characteristics of
phase-inverting systems.

One limitation of the dark ground optics system is that it can only image phase-
inverting systems comprised of thin polymer films. Additionally, this technique only
applies to shorter time courses ranging from a few minutes to several hours [92]. The use
of other imaging modalities is necessary for longer-term studies such as implant
degradation analyses. *In vitro* phase inversion systems can be characterized with dark
ground optics, but no *in vivo* images can be obtained.

**2.4.2. Electron Paramagnetic Resonance Spectroscopy**

The exploration of *in vivo* behavior of phase-inverting implants is crucial to the
design of effective drug delivery systems. Common *in vivo* characterization methods
including IR spectroscopy, chromatography, and calorimetry require implant extraction
from the tissue and cannot provide continuous serial data [93]. One technique which
provides noninvasive analysis of phase inversion is Electron Paramagnetic Resonance
(EPR) spectroscopy, also called Electron Spin Resonance (ESR), and this method has
recently been employed to study ISFIs. EPR is based upon the interaction of electrons
with magnetic fields due to the magnetic moments of the electrons. These magnetic
moments cause electrons to align with the applied magnetic field within the resonator.
Electromagnetic radiation is applied to a sample, exciting the unpaired electrons at a
recognizable resonance frequency [94]. *In vivo* frequencies range from 9000 MHz to 250
MHz [95].
Continuous-wave EPR (CW-EPR) is the most effective and commonly used EPR method for in vivo characterization. This machine has a resonator chamber into which the sample is placed. The spectrometer is tuned to an optimal frequency and matched so that electromagnetic waves are not reflected back from the sample. The CW-EPR apparatus directs microwaves at the resonator cavity at the resonance frequency. The spectrometer applies a magnetic field at a value below the resonance level and slowly increases the field above the resonant frequency. This magnetic field sweep causes all unpaired electrons to switch their alignment in the field, which in turn changes the matching of the resonator cavity. Paramagnetic species are recognized because the microwaves they reflect back are detected and compiled into an absorption EPR spectrum. In order to receive a sufficiently strong signal, magnetic field modulation is used. An oscillating magnetic field with low amplitude is applied during the initial magnetic field sweep and modulates the amplitude of the signal at the resonance frequency [94]. Another frequently used EPR method is Spectral Spatial EPR imaging, which is able to make a localized assessment to determine the layer of the polymer system which corresponds to the EPR spectra [96].

The EPR spectrometer identifies paramagnetic compounds such as free radicals and is able to characterize samples based on the interaction of these species with their environment [92]. It is non-invasive, non-destructive, and continuous and therefore a time course of data can be taken from a single implant in a live animal. Discrepancies between in vitro and in vivo data can be examined to determine how drug release dynamics and implant formation are affected by the physiological conditions [97]. Mäder et al. found that for P(CPP-SA)-polymers, the drug release rate is 1.5 times faster in vitro than in vivo.
Other studies have shown that in vitro systems undergo a more complete drug release than the same systems in vivo [98]. By understanding the differences in release mechanisms inside the body, ideal drug delivery systems can be designed while taking into account variable in vivo factors such as pH, water content, and enzyme activity [94].

Many properties of polymer phase inversion systems can be studied using EPR. EPR spectra indicate several characteristics of the sample including the spatial distribution of micropolarity, microacidity, microviscosity, and nitroxide concentration [94]. These parameters provide information on the internal polymer environment from which the drug is released, and they can be tailored to modify the release behavior [97]. The drug release and polymer erosion mechanisms can be profiled by continuously measuring drug concentration and mobility through the system over time [98].

Additionally, EPR has provided a means for the in vivo measurement of polymer degradation and water penetration. Solvent/nonsolvent interaction dynamics and exchange can also be monitored due to the sensitivity of EPR to motion and polarity within an implant. Kempe et al. traced the kinetics of the exchange between the solvent NMP and water within PLGA implants both in vitro and in vivo using EPR [97]. The presence of paramagnetic species and their relative amounts can be concluded from EPR information, as well as their physical and chemical properties. EPR is effective for the characterization of multiphase systems, non-transparent samples and small-scale measurements [94]. Non-invasive and continuous in vivo monitoring of polymer systems is also possible through other means such as radioactive labeling, but EPR has the advantage of providing valuable structural information [99].
The results of EPR spectroscopy are used to determine parameters such as the hyperfine coupling parameter and rotational correlation time [92]. The hyperfine coupling parameter is calculated as the distance between the high and low field lines on EPR spectra, and is directly related to the polarity of the environment. The rotational correlation time designates the microviscosity of the environment and measures rotational movement of spin markers [97]. The presence of magnetic nuclei in a sample can cause more than one resonance and split the EPR spectrum into three lines when they interact with paramagnetic species, as seen in Figure 1.10 [94]. EPR has had many pharmaceutical applications as well, such as in vivo studies on the behavior and effects of antacids in mice [96].

Most drug delivery polymer systems are diamagnetic instead of paramagnetic and therefore are undetectable using EPR. The addition of paramagnetic compounds to the sample is often necessary for EPR detection. These species, such as free radicals, act as spin probes and are identified by the spectrometer at the resonance frequency [92]. Stable nitrooxide free radicals are common additives to polymer systems, and the concentrations of these paramagnetic materials are discovered using EPR [100]. Through varying the properties of the nitrooxide used, optimal EPR results can be obtained [93]. Copper and manganese are other examples of substances which are paramagnetic and can be added to polymer systems [94]. Paramagnetic species which are naturally occurring in living organisms include: molecular oxygen, free radical intermediates of metabolism and drugs, and metal ions in paramagnetic states. These compounds are useful for in vivo detection but are typically found in low concentrations and require a supplemental paramagnetic compound [95]. Certain nitroxides are conducive to measuring proton
activity within a polymer sample using EPR, which provides information regarding pH
distribution and microacidity [92]. In dry environments, irradiation can form free radicals
that are stable yet disappear upon contact with water. In this manner, paramagnetic
species can measure the kinetics of water penetration into an implant [94].

Figure 2.5. Schematic of the effect of polymer precipitation on the EPR signal using a tempolbenzoate
(TB) probe. “Journal of Controlled Release, 130, S. Kempe, H. Metz, K. Mäder, Do in situ forming
PLG/NMP implants behave similar in vitro and in vivo? A non-invasive and quantitative EPR
investigation on the mechanisms of the implant formation process, 220–225, Copyright (2008),

Mäder and coworkers were the first to utilize EPR for the purpose of
characterizing in vivo biodegradable implants, and this technique has proven to be
effective [99]. EPR is a desirable analytical method since low frequency spectrometers
enable monitoring of micro-viscosity which is can be related to the phase inversion
(Figure 2.5). This system can monitor implants in vivo, making EPR one of the only non-
invasive in vivo characterization techniques currently in use [91].

EPR has limitations on the type and accuracy of the data collected. EPR cannot
produce images of implant systems, and instead the data from the spectra are analyzed for
characterization. Additionally, small molecular weight (Mw) spin probes are required for EPR measurements [91].

Another complication for in vivo studies is that levels of detectable paramagnetic species in animals is often low, requiring the addition of nitroxides or other compounds. In vivo environments can absorb waves at non-resonant frequencies due to their dielectric properties, necessitating the use of low ESR frequencies to prevent this. Moreover, the EPR resonator chamber must have dimensions to accommodate the animal (usually a mouse) for in vivo studies [95]. EPR does not indicate how the in vivo environment is affected by the polymer implant, which is important to know in order to reduce negative physiological reactions in the body. Also, the size and shape of the injected polymer cannot be determined through EPR alone, so this technique is commonly paired with non-invasive MRI to give a more complete picture [97].

Since this procedure is used to track the mechanisms of polymer systems implanted into live animals, problems can arise from animal subject movement such as breathing or circulation. This additional signal noise can be as strong as the signal from paramagnetic species, and causes the spectrometer to read incorrectly. This motion-induced data can be diminished by automatic frequency control (AFC) and automatic matching control (AMC) systems. Besides changing the feedback control, an additional option to decrease physiological noise is to use longitudinally detected EPR (LODEPR). This detector does not take into account tuning or matching changes in the resonator. In LODEPR, the magnetic field is not modulated as in CW-EPR but instead the amplitude of the electromagnetic waves is altered. A solenoidal receiver coil is tuned to a frequency that is double the modulation frequency, and is used to directly detect oscillating
paramagnetic species. Since detuning and dematching of the signal are not used in LODEPR detection, physiological motion has little effect on the resultant spectrum. Despite these challenges, EPR is an effective in vivo technique [94].

2.4.3. Diagnostic Ultrasound Imaging

In addition to EPR spectroscopy, ultrasound has recently been proposed as a noninvasive in vivo characterization technique for polymer implants [91]. Ultrasonic waves have frequencies exceeding the audible range for humans (above 20 kHz) and can travel through a substance as a continuous wave or in brief pulses [101]. A transducer linearly emits short bursts of ultrasound waves into a medium and records the echoes that result from differences in elastic properties of the composite material [102]. The backscattered signal can be characterized as a function of acoustic impedance where acoustic impedance is defined as a function of density and the propagation speed of sound (eq. 1) [102] such that:

\[ Z = \rho C \]  

(1)

Z is the acoustic impedance, \( \rho \) is the density of the material, and C is the propagation speed of sound in the material [102]. Subsequently, the portion of the signal reflected back to the transducer is a function of impedance difference between two materials (eq. 2) [102]:

\[ R = \left( \frac{Z_1 - Z_2}{Z_1 + Z_2} \right)^2 \]  

(2)

where R is the intensity of the backscattered signal, \( Z_1 \) is the impedance of material 1, and \( Z_2 \) is material 2 [102]. Therefore as the polymer solution transitions from liquid to
solid, the acoustic impedance changes, resulting in the development of a backscattered signal [91].

If the material were to phase invert into a perfectly homogenous material, then one would expect to see only the edges of the implant. However, because the gelation of the polymer results in the formation of polymer lean domains composed predominantly of the nucleation of solvent which leads to pore formation intermixed with polymer rich domains, the precipitation can be imaged (Figure 2.6). Consequently, regions of high solvent concentration would lead to low signal intensity due to the homogenous nature of the polymer and solvent domains.

![Figure 2.6. Representative illustration highlighting the effect of microdomains (A) and a homogenous structure (B) on ultrasound reflection.](image)

For phase-inverting systems, the development of a signal due to changes in the acoustic resistance is caused by compositional changes of the polymer matrix. The pulse echo information is compiled into a two or three dimensional grey-scale image [102]. Within a polymer implant, the phase inverted portion reflects ultrasound waves back strongly, while the areas rich with polymer solution does not (Figure 2.7) [91]. Therefore, the ultrasound image that is generated depicts a real time picture of phase inversion and analysis of these images can be used to trace changes in the shape and composition of the implant over time.
Ultrasound is an attractive characterization technique in many respects. It is the only noninvasive imaging process that is able to collect data describing the polymer behavior \textit{in vivo} as well as \textit{in vitro}. Similar to EPR, it is noninvasive and nondestructive, allowing an entire set of \textit{in vivo} time course data to be collected using the same implant and animal and thereby reducing environmental variability and the number of animals used. Through compiling and analyzing multiple ultrasound images, data for a single implant can be extrapolated over time and quantified. While EPR requires the addition of a tracer element to collect data, ultrasound does not and it is thus a direct visualization method [91].

One shortcoming of ultrasound characterization is that it is unable to directly detect the solvent/nonsolvent exchange that occurs during phase inversion, as both dark ground optics and EPR can. Instead, properties such as polymer precipitation and swelling can be quantified to determine the inversion dynamics. Additionally, ultrasound
imaging resolution is dependent on the frequency of the transducer used, which may limit small-scale examinations of polymer implants. Combining ultrasound data with a higher resolution imaging method provides more detailed image information [91]. This technique has been utilized to relate implant formation to initial drug release kinetics, as well as to determine the effect of polymer Mw on implant behavior, as well as the effect of the payload properties on swelling and phase inversion over time [91, 103]. The following chapter will outline the development of US imaging as a technique by which effective characterization for implants formed both in vitro and in vivo (Figure 2.7). An overview of the benefits and limitations of each characterization technique can be found in Table 2.2.

<table>
<thead>
<tr>
<th>Technique</th>
<th>Benefits</th>
<th>Limitations</th>
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<tbody>
<tr>
<td>Dark Ground Optics</td>
<td>• Solvent-nonsolvent exchange can be monitored</td>
<td>• Only thin film polymer systems can be studied</td>
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<tr>
<td></td>
<td>• Simple and fast characterization</td>
<td>• Studies over short time courses only</td>
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<tr>
<td></td>
<td></td>
<td>• No in vivo imaging is possible</td>
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<tr>
<td>EPR</td>
<td>• Noninvasive, nondestructive</td>
<td>• Additional signal noise can arise from animal subject movement</td>
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<tr>
<td></td>
<td>• Can collect in vivo data</td>
<td>• No images are produced directly</td>
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<tr>
<td></td>
<td>• Provides structural information about the implant</td>
<td>• Cannot determine implant geometry</td>
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<tr>
<td></td>
<td>• Continuous data can be collected</td>
<td>• Large resonator chamber and small MW spin probes are required</td>
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<tr>
<td></td>
<td>• Solvent-nonsolvent exchange can be monitored</td>
<td>• Low EPR frequencies must be used</td>
</tr>
<tr>
<td></td>
<td>• Small scale measurements are possible</td>
<td>• No information on the in vivo environment is found</td>
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<tr>
<td></td>
<td>• Can be used to characterize multiphase systems</td>
<td>• Paramagnetic species must be added for detection</td>
</tr>
<tr>
<td>Ultrasound</td>
<td>• Noninvasive, nondestructive</td>
<td>• Cannot directly detect solvent-nonsolvent exchange</td>
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<tr>
<td></td>
<td>• Can collect in vivo data</td>
<td>• Low resolution prevents small scale imaging</td>
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<tr>
<td></td>
<td>• Does not require a probe</td>
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<td></td>
<td>• Studies can be followed over long time courses</td>
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2.5. Future Perspective

Medical imaging is rapidly transforming patient care by providing safe, fast, and reliable techniques that allow care providers to minimize the invasive nature of many therapeutic procedures (Table 2.1). One relatively novel field in which imaging is now gaining ground is drug delivery. Image guided drug delivery has great potential to revolutionize patient care by facilitating a number of related processes such as minimally invasive placement of drug eluting devices into nonresectable tumors or noninvasive monitoring of therapeutic efficacy. Constant, rapid development of improved imaging modalities such as C-arm CT imaging has made it possible to more accurately select appropriate feeding arteries for microcatheter placement in TACE to enhance treatment efficacy, while concurrently reducing the probability of toxic side effects [25, 26]. MRI advancements have not only provided a means for real-time imaging of the distribution volume of drugs and nanoparticles, but these innovations have also improved the reliability of CED as a neuro-oncological treatment option [54]. Additionally, functional DWI is proving to be a reliable way to monitor the necrosis of tumors after a variety of interventional treatments. As the field of image guided drug delivery evolves, surgical procedures will not be performed without advanced modeling and planning based on preoperative imaging. One example would be the use of MRI to determine the 3-D coordinates of the optimal placement of a cannula in order to avoid reflux into the CSF during a CED procedure. Improvements in functional imaging may also be used to develop feedback systems so that therapeutic planning can be adjusted and tailored for more personalized treatment. Not only can imaging provide clinical information about drug distribution and treatment efficacy, but the evaluation of drug eluting implant
behavior in situ can be used to elucidate the fundamental principles that control drug release behavior in vivo. This insight may then be used to improve implant design so that delivery of therapeutic agents can be more effectively delivered which could potentially lead to improved treatment efficacy and lower overall costs to both the patient and health care provider.

While image guided drug delivery in clinical settings is ever growing, the role of imaging in research is becoming equally valuable. The development of imaging techniques to provide high throughput noninvasive methods of monitoring drug delivery and treatment efficacy is a key factor in developing screening methods in industrial and laboratory settings. The use of molecular imaging techniques will provide a means of diagnosing conditions, and will also be used for targeted drug delivery and may even lead to nontoxic treatment options through the development of pro-drug based therapies. In addition to advances in targeted drug delivery, research in image guided drug delivery will play a key role in developing theranostic multifunctional agents, which will provide a means to not only personalize the treatment, but concurrently provide a noninvasive means for evaluating its efficacy.

2.6. References


Chapter 3: Noninvasive Characterization of In situ Forming Implants Using Diagnostic Ultrasound

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Luis Solorio, Brett M. Babin, Ravi B. Patel, Justyna Mach, Nami Azar, and Agata A. Exner
3.1. Introduction

The treatment of solid tumors using systemic chemotherapy alone is often unsuccessful. As discussed in the previous chapter, obstacles such as heterogeneous tumor vasculature and elevated intratumoral pressure, among many others, limit drug bioavailability at the site of action and reduce therapeutic efficacy. Approaches that increase the local drug concentration at the target site through physical or chemical targeting can potentially overcome these pitfalls. For example, surgical placement of drug eluting polymer implants directly into the tumor can minimize systemic drug exposure while simultaneously increasing the level of drug within the tumor to several times above the minimum therapeutic dose [1-5]. Phase sensitive ISFIs offer a compelling alternative to solid prefabricated implants because they can be placed through noninvasive image guided procedures [1, 5, 6].

Similar systems have been used successfully in commercial applications for the delivery of leuprolide, which is used for the treatment of prostate cancer. In the Eligard® system, the drug is suspended in the polymer solution Atrigel®, and upon injection forms a solid drug depot that can release leuprolide for up to six months [7-9]. These implants have a distinct release pattern consisting of a period of burst release followed by a period of diffusion facilitated release. As the matrix begins to degrade, the release is enhanced until the entire cache of drug has finally been depleted [10]. It has been shown that the drug release from PLGA is directly related to the rate of phase inversion, and that the burst release may be directly correlated to the rate of phase inversion and the resultant morphology of the polymer phase. Some of the variables that are expected to affect the rate of phase inversion are the molecular weight of the polymer,
polymer concentration, the hydrophilicity of the solvent, and the non-solvent composition [11, 12].

As discussed in Chapter 1, only a handful of techniques are available to evaluate the phase inversion process of \textit{in situ} forming systems. This chapter outlines the development of a novel means for evaluating the phase-inversion process of \textit{in situ} forming implants through the use of diagnostic ultrasound and subsequent quantitative image analysis.

Ultrasound refers to sound waves with a frequency above 20 kHz [13]. In diagnostic ultrasound, a piezoelectric transducer converts electrical energy into mechanical pressure waves that propagate through a material. The backscatter is a result of the impedance differences existing within a material. The resultant image is therefore an acoustic map of the mechanical interactions of the pressure waves with the object, as described by the difference in impedance with the surrounding environment [14]. Since the \textit{in situ} implants transition from liquid solution to solid implant, the change in phase alters the impedance of the implant, allowing visualization of the phase inversion process with ultrasound. The use of imaging to monitor changes within a system is well established and has several advantages over existing techniques [5, 14, 15]. Most importantly the method is nondestructive, thus the formation data, drug release data, solvent release data, and swelling data can all be obtained from the same implant. In addition, because the technique is noninvasive, the same implants can be imaged over the time scale of seconds to months, providing unprecedented longitudinal information regarding immediate formation as well as implant degradation. These processes can be measured regardless of the geometry of the implant, making it applicable to a wide
variety of systems and experiments. Finally, the evaluation can be performed in vitro as well as in vivo to provide an accurate representation of the implant properties in an undisturbed physiological environment. The goal of the current study was to demonstrate the utility of ultrasound as a noninvasive, non-destructive tool to evaluate the phase inversion processes of these implants in vitro and in vivo and to relate this information to the drug release profiles in these environments.

3.2. Materials and Methods:

3.2.1. Materials

All materials were used as received with no further purification. Poly(DL-lactide-co-glycolide) (PLGA 50:50: 2A, MW 15,000 Da, inherent viscosity 0.16dL/g; 3A, MW 29,000 Da, inherent viscosity 0.28 dL/g; 4A, MW 64,000 Da, inherent viscosity 0.46dL/g) was obtained from Lakeshore Biomaterials, Birmingham, AL. N-methyl-2-pyrrolidinone (NMP) and sodium fluorescein (MW 376.28) were obtained from Sigma Aldrich, St. Louis, MO. Agarose was obtained from Fisher Scientific, Waltham, MA. 1,1’-dioactadecyl-3,3,3’,3’-tetramethyl-indocarbocyanine perchlorate (DiI) was obtained from Invitrogen, Eugene, OR.

3.2.2. Preparation of Polymer Solutions

Solutions of 40 wt % PLGA in NMP were prepared with 2A, 3A, and 4A polymers. Polymer was added to NMP in scintillation vials and allowed to mix overnight in an incubated shaker at 37°C until the polymer had completely dissolved. Polymer solutions were stored at 4°C for up to three days before use. For release studies, sodium
fluorescein was used as a mock drug and polymer solutions were made as described previously using a 60:39:1 mass ratio of solvent:polymer:drug [16].

3.2.3. Polymer Encapsulation in Agarose

A 1% agarose solution was used to encapsulate the implant in a tissue phantom for the duration of the study. Liquid agarose was stored in a warm water bath. Warm agarose (9ml) was added to the mold and placed on a bed of ice. Before the phase transition of the agarose was complete, an additional 6ml of boiling agarose was added to the mold, and allowed to cool to below the glass transition temperature of the PLGA (43.5°C), at which time a drop of the polymer solution (25 mg) was added and the agarose was allowed to solidify (Figure 3.1).

Figure 3.1. Agarose was added to a mold and allowed to cool for 8 min (A). An additional layer of agarose was then added and allowed to cool below the glass transition temperature of the PLGA (B). Implant was formed by dropping PLGA/NMP solution into the void created by the mold (C) resulting in the final phantom (D) placed in the custom fabricated holder. Scale bar represents 1.75 cm.

The phantom was placed in a 150ml bath of 37°C diH₂O, and images were recorded after 40 min, 1 hr, 2 hrs, and then every other hour for the first 10 hrs, and once daily for the next 5 days. Four implants were examined for each molecular weight of PLGA.
3.2.4. *In Vitro Ultrasound Imaging*

The implants were imaged using a Toshiba Aplio SSA-770A diagnostic ultrasound. A 12MHz PLT-120 transducer was used with the following parameters: a dynamic range of 55, a mechanical index of 1.1, a gain of 80, and a depth of 3 cm. The transducer was fixed using a clamp, and the phantom was imaged from the bottom, and held in a constant position by a custom fabricated holder. The phantom was rotated in the holder until the center of the implant was found, and then the agarose was marked so that the same plane would be imaged throughout the study. Images were acquired after 40 min, 1 hr, 2 hrs, and then every other hour for the first 10 hrs, and once daily for the next 5 days and stored as TIFF images.

3.2.5. *In Vivo Ultrasound Imaging*

All animal experiments were carried out under general gas anesthesia. Five six week old male BDIX rats (average body weight 300g, Charles River Laboratories Inc., Wilmington, MA) were used. 1% isoflurane was used with an oxygen flow rate of 1 L/min (EZ150 Isoflurane Vaporizer, EZ Anesthesia™). Due to the consistent behavior and injectability of the 3A implants during the first 48hrs, these implants were chosen for use in the *in vivo* studies to determine if a correlation existed with the burst period of drug release and the phase inversion of the polymer implants [17]. 50ul of fluorescein loaded polymer solution was injected subcutaneously at five locations on the dorsal side of the rat using a 21-gauge hypodermic needle. The implants were imaged using a Toshiba Aplio SSA-770A diagnostic ultrasound with a 12MHz PLT-120 transducer using
the following parameters: a dynamic range of 55, a mechanical index of 1.1, a gain of 80, and a depth of 3 cm. Images were taken at 1 hr, 4 hrs, 8 hrs, 24 hrs, and 48 hrs.

3.2.6. Image Analysis

Ultrasound images are the visualization of the backscattered signal that arises due to the difference in mechanical impedance between different materials and phases. The backscattered signal is displayed as a gray-scale array with values ranging from 0-256, with 0 indicating a negligible difference in impedance from the surrounding media, in this case 1% agarose. The liquid polymer solution has low mechanical impedance and generates a negligible backscattered signal, while the phase inverted polymer causes significant reflection of the pressure waves, resulting in an image. The development of an ultrasound signal over time was interpreted as an increase in impedance due to the precipitation of the polymer.

The Gray-scale value (GV) was analyzed by measuring the mean GV of the implants over time, so that an index of the matrix impedance could be evaluated and used as a means to evaluate the change in the mechanical properties of the matrix over time. The mean GV was analyzed by first finding all pixels with values greater than zero in the implant. Then the average of the non-zero values was determined. Implants initially form a thin shell that thickens over time as the polymer phase inverts, therefore, the growth of the precipitation front can be determined by measuring the change in shell thickness over time. First, for in vitro analysis the region of interest (ROI) was isolated by using a parametric intensity based segmentation method of mixed Gaussians [18]. Gaussian intersections were used to threshold the image foreground from the background.
The image was then converted from gray scale to binary, and then the implant interior was filled to create a total area image (Figure 3.2). For *in vivo* analysis, the implants were manually segmented (five images for each implant), and a threshold value was selected using the method of mixed Gaussians after the ROI was isolated in order to remove low intensity noise. The ROI was used to create the total area image. The measurements from the five images were averaged together for each implant.

![Representative isolated gray scale image of the 3A implant (A). The implant after a threshold has been applied (B). The total area image generated from the threshold image (C).](image)

**Figure 3.2.** Representative isolated gray scale image of the 3A implant (A). The implant after a threshold has been applied (B). The total area image generated from the threshold image (C). The scale bar represents 0.25cm.

The ratio of the number of pixels that comprised the unfilled region of the threshold image to the total number of pixels in the total area image was used to determine the percent formation. In 2A implants, the internal polymer-solvent gel appeared to break through the outer polymer shell after 24 h (in a process we have called “leakage”). Thus the first 10 h of the study were used to quantitatively describe the shell growth *in vitro*. Leakage also occurred in the 3A implants, but not in the 4A implants and has been highlighted in Figure 3.3 (see arrows). For the *in vitro* images, plateau (Pr) and time delay terms (τ) of an exponential equation were fit using the sum of least squares with MATLAB, and these terms were used as a means by which the formation rate of the different polymers could be compared (Figure 3.6).

\[
F(t) = Pr\left(1-e^{-t/\tau}\right)
\]

\[
t=time
\]

\[
F(t)=\text{percent formation}
\]
Pr=Plateau value

τ=time delay

Finally, implant swelling was determined \textit{in vitro} from the total area images. Since the total area images were binary, the number of implant pixels could be determined by summation of the pixels. Then sums were normalized by dividing the number of implant pixels in the total area image at a given time point by the number of implant pixels in the total area image of the first time point. All image analysis was performed using MATLAB.

3.2.7. Image Validation

Agarose phantoms were constructed as previously described, but the polymer solution was prepared by adding a small amount (less than 0.1 mg) of a hydrophobic DiI dye during solution preparation in order to enhance contrast of the implants. The implants were then imaged with ultrasound at 2 hrs, 6 hrs, and 24 hrs. After ultrasound imaging, the implants were frozen, sliced, and photographed for comparison with the ultrasound images.

3.2.8. Drug Release

In vitro release studies were carried out by first placing agarose entrapped 3A implants (n=3) in 150 ml of 37°C diH2O, and incubating at 37°C on a rotating shaker table. At 1, 4, 8, 24, and 48 hrs, the implants were removed from the agarose phantom and degraded in 2M NaOH. The agarose was returned to the bath side and both the bath
side and implant were left overnight. The total fluorescein mass was determined by measuring the fluorescence from the bath side solution and the dissolved implant solution, then concentration was determined using a standard curve. The cumulative mass release was normalized by calculating the total mass of fluorescein in the implants.

In vivo release studies were performed by determination of residual fluorescein in a subcutaneous 3A implant at 1, 4, 8, 24, and 48 hrs (n=5). After US image acquisition at these time points, the animal was euthanized and the implants were dissected out and degraded overnight in 2M NaOH solution; fluorescein concentration was determined using a standard curve. Cumulative mass release was normalized by the theoretical mass of fluorescein in the implants. For both in vivo and in vitro studies the fluorescence was measured using a Tecan 200 series plate reader at an excitation wavelength of 485nm and an emission wavelength of 525nm.

3.2.9. Statistical Analysis

ANOVA was used to determine statistical significance (P<0.05, n=4). A Tukey multiple comparison test was used to identify significantly different groups. Error is reported as the standard error of the mean (SEM).

3.3. Results

3.3.1. Image Validation

Representative gray-scale images of PLGA implants are shown in Figure 3.3. Some implants exhibited “leakage” of the polymer into the surrounding agarose (indicated by the arrows) which was caused by an increase in hydrostatic pressure as a
result of implant swelling. The constrained nature of implants encapsulated in agarose allows a limited degree of swelling to occur before the implants reach a critical threshold that forces any remaining liquid polymer solution through the agarose along the plane with the lowest resistance. Leakage occurred within 24 hrs of the 2A implants, and could be seen as early as 72 hrs after implantation in the 3A implants. Leakage did not occur in the 4A implants (Figure 3.3).

<table>
<thead>
<tr>
<th>40 min</th>
<th>2 hrs</th>
<th>6 hrs</th>
<th>10 hrs</th>
<th>24 hrs</th>
<th>48 hrs</th>
<th>72 hrs</th>
<th>96 hrs</th>
<th>120 hrs</th>
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<td><img src="image8.png" alt="Images" /></td>
<td><img src="image9.png" alt="Images" /></td>
</tr>
</tbody>
</table>

**Figure 3.3.** Representative isolated gray scale images of the implants over time, the polymer molecular weight increases from top to bottom with 2A on the top row, 3A in the middle, and 4A on the bottom row. The scale bar represents 0.25cm. The arrow indicates where leakage has occurred.

Validation of the US images was performed by entrapping DiI loaded implants in agarose so that an US image could be obtained and compared with the actual implant (Figure 3.4). Due to the hydrophobicity of the DiI dye, the darker pink regions were assumed to be hydrophobic-rich regions that contained polymer and solvent, while the lighter regions were hydrophilic domains [17]. All US images corresponded to the sectioned implants.
3.3.2. Gray-Scale Analysis

2A polymers had a maximum mean GV of 74±3 after 10 hrs, but the mean GV decreased to 65±3 after 24 hrs in the agarose phantom. 3A polymers had a maximum GV of 82±2 after 24 hrs, but decreased to a value of 70±9 by day 3. The 2A and 3A polymers were no longer statistically different (p<0.05) after 72 hrs in the agarose phantom, with the mean GV of both polymer types decreasing. The 4A polymers attained the highest mean GV, 94±8 after 3 days, and appeared to plateau after 72 hrs in the agarose phantom, becoming statistically different from the other polymer types after 48 hrs of encapsulation in the agarose phantom (Figure 3.5A). The change in the gray-scale intensity of the implants over time can be seen in Figure 3.2.
Figure 3.5. Changes in mean GV of the three molecular weight polymers over time (A). Quantitative swelling data of the three molecular weight polymers (B). Quantitative formation data for three molecular weight polymer implants over the first 10hrs of formation (C). Formation data for the full 120 hr study (D).

3.3.3. Implant Swelling

The 2A polymer exhibited the greatest swelling within the first 24 hrs, increasing in size more than 60%. Implant cross-sectional area reached a maximum of 80% total increase after 48 hrs, and then began to shrink gradually. The 3A polymer showed an initial decrease in size, shrinking 10% after one hour in the agarose phantom, but then steadily increased in size, reaching a 20% increase within 24 hrs and an 80% increase after 96 hrs. Finally, the 4A polymer showed an initial increase in cross-sectional area to approximately 120% of the initial area within the first 5 hrs. After 5 hrs, the cross-sectional area decreased slightly and remained constant for the duration of the experiment (Figure 3.5B).
3.3.4. Implant Formation

The plateau value (Pr) from the parametric analysis was used to describe the percent of the polymer that had undergone phase inversion sufficient for creation of an echogenic signal. All three polymers had statistically different Pr values, with the 4A polymer having the highest Pr value followed by the 2A and then the 3A (Table 3.1). Both the 2A and the 4A polymers reached precipitation values above 95% 48 hrs after implantation in the agarose phantom, while the 3A polymer approached 90% precipitation after 96 hrs. A sharp increase in polymer precipitation occurred after the leakage of the polymer solution from the depot with the 2A and 3A polymers (Figure 3.5C-D).

<table>
<thead>
<tr>
<th>Polymer Type</th>
<th>Pr (%)</th>
<th>( \tau ) (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2A</td>
<td>72(^*)</td>
<td>2353±171</td>
</tr>
<tr>
<td>3A</td>
<td>64.5(^*)</td>
<td>2759±236</td>
</tr>
<tr>
<td>4A</td>
<td>85(^*)</td>
<td>1141±93(^*)</td>
</tr>
</tbody>
</table>

*plateau value indicating extent of precipitation
\(^*\) time delay
\(^*\) statistically significant difference (p<0.05) within parameter group

The time delay value (\( \tau \)) was used to aid in describing the effect of time on polymer formation. The 4A polymer had a statistically different \( \tau \) from both the 2A and 3A polymers, but the 2A and 3A polymers were not statistically different from each other (Table 3.1).

3.3.5. In Vivo Formation

3A polymer implants were evaluated in vivo for 48 hrs following injection. Precipitated polymer occupied 86±5% of the total cross-sectional area within 8 hrs and reached a maximum value of 90±6.4% after 24 hrs at which time the implant formation
had reached a plateau (Figure 3.7A). Representative gray-scale images obtained from the *in vivo* analysis are shown in Figure 6. A linear correlation was found between the *in vivo* and *in vitro* formation ($R^2 = 0.98$, Figure 3.7B).

**Figure 3.6.** A. Representative ultrasound image of the subcutaneous implant after 1 hr, the arrows indicate the location of the implant, skin, and ultrasound gel. B. isolated gray scale images of the *in vivo* subcutaneous implants over time, C. threshold image of the same implant (shown in B.). The scale bar represents 0.25cm.

**Figure 3.7.** Quantitative formation data for the 3A polymer implants *in vivo* and *in vitro* (A), linear correlation of the *in vivo* and *in vitro* formation data ($R^2 = 0.9845$, B).
3.3.6. Drug Release

The *in vitro* implants released 46±4.8% of the total drug mass within the first 8 hrs, and a total of 50±1.8% mass released after 48 hrs (Figure 3.8). In comparison, *in vivo* implants released 61±8.6% of the drug after 8 hrs and released 82±6.3% of the drug after 48 hrs (Figure 3.8B). Release from the *in vivo* and *in vitro* implants was not statistically different until 8hrs (P=0.047) at which time the release was statistically different for the duration of the study. A strong linear correlation was seen between the percent polymer precipitation and the percent mass release *in vivo* and *in vitro* ($R^2=.93$ and $R^2=.9486$, Figure 3.9).

![Figure 3.8](image1.png)

*Figure 3.8.* Cumulative release of fluorescein from 3A polymer implants encapsulated in and agarose and subcutaneously injected implants.

![Figure 3.9](image2.png)

*Figure 3.9.* Correlation of *in vivo* and *in vitro* release data with *in vivo* and *in vitro* formation (*in vitro* $R^2=0.9486$ and *in vivo* $R^2=0.93$).
3.4. Discussion

The use of diagnostic ultrasound to study the phase inversion process of in situ forming implants is a novel means to evaluate the process in a noninvasive, nondestructive manner. The primary advantage of this technique is the real time visualization of the implant formation process. Through the use of quantitative image analysis techniques, long term information regarding formation and swelling behavior for the same implant over time can be obtained. Additionally, by monitoring the phase inversion process, factors that may affect drug delivery such as polymer leakage or fibrous encapsulation may be monitored. The ability to evaluate these phenomena provides a means by which the effectiveness of the implants can be ascertained clinically.

In the current study, the utility of monitoring implant formation using ultrasound was demonstrated by analyzing the effect of polymer MW on the formation and swelling of in situ forming PLGA-NMP.

The behavior of implants embedded in agarose was monitored in a tissue phantom. Change in the implants over time can be seen in Figure 3.2. Polymer leakage can be seen after 24 hrs with the 2A polymer and after 72 hrs with the 3A polymer. While polymer leaking was not anticipated, it is suspected to have occurred due to the increase in hydrodynamic pressure that corresponded with the increase in implant swelling. Ultimately, because the implant was constrained by the agarose, residual polymer burst from the implant along the weakest plane of the agarose phantom. The driving force for this phenomenon is hypothesized to be the osmolarity of the implants. Due to the small MW, the 2A implants would have the highest number of moles at a given mass resulting in the highest osmotic force; additionally the affinity of the polymer
for the solvent would add to the osmotic affect further enhancing the osmotic drive. As a consequence, these implants would swell the fastest and be the first of the polymers to show signs of leakage. The delayed leakage seen in the 3A implants could be a result of the degradation byproducts increasing the osmolarity and subsequently increasing the water content of the implants which would result in increased phase inversion. Consequently, the sudden change in surface area may adversely affect drug delivery resulting in a large burst release of drug, which would not be detectable using any other technique to evaluate phase inversion. The leakage phenomenon could prove to be potentially problematic in environments that would enhance polymer degradation (such as the acidic environment of a tumor). The enhanced degradation may increase the implant osmolarity ultimately resulting in enhanced swelling and leakage of the implants leading to undesirable burst of drug. Therefore, in agreement with prior work it is clear that the environment in which the implant is formed plays a crucial role during implant formation and subsequent drug release process [19]. The propensity for the lower molecular weight polymers to swell highlights the importance of polymer selection when optimizing for specific delivery applications in vivo.

The average GV was intended to be used as an index to determine the degree of depot formation, with lower mean GVs corresponding to a more fluid matrix and large mean GVs correlating to fully-formed solid depots. Using this measurement, it was observed that the 4A PLGA depots attained the highest gray scale value, indicating nearly complete solvent diffusion and implant precipitation. In contrast, the mean GV of 2A implants reached a plateau after 4 hrs, suggesting a lack of further change in matrix stiffness. The 2A implants also showed a reduction in gray-scale value after 24 hrs,
which corresponded to leakage of the implant. This limited the mean GV index as a tool for determining the degree of formation. A similar decrease in mean GV was noted in the 3A depot after leakage was seen on day 3.

Implant swelling was measured as a change in cross-sectional area over time. The 2A depot increased in size drastically in 24 hrs, and a high degree of swelling was not seen with the 3A until after 72 hrs. The decrease in cross-sectional area seen in the 3A and 4A depots was most likely caused by diffusion of solvent out of the depot occurring at a faster rate than water influx and resulting in a loss in total implant volume.

Many variables can be manipulated to alter the precipitation rate of the in situ forming polymer system. Often, the desired kinetics can simply be achieved with the appropriate selection of an excipient and solvent [20]. It has been reported that implant formulations comprised of high molecular weight polymers in solvents with high water miscibility will undergo a rapid phase inversion resulting in a characteristic honeycomb like structure of diffusion pores for drug and water to travel through [12, 21]. When NMP is used as the solvent, the critical concentration of water required to induce phase inversion decreases as the molecular weight of the polymer increases, due to the change in affinity of the solvent for the polymer. Therefore, it was anticipated that the rate of polymer precipitation would occur fastest with the 4A PLGA followed by 3A, and 2A PLGA [20, 22]. A parametric analysis of the process showed that while the 4A implants did in fact form the fastest, gelation rates of the 2A and 3A implants were not statistically different. Our data indicated that the 2A polymer had undergone a greater degree of precipitation than the 3A PLGA. This disparity is attributed to the difference in initial osmotic drive. However, although the change in impedance was sufficient to induce
backscatter in the 2A implants, it is clear that this data alone cannot be used as a standalone tool to dictate when the implants have completely solidified. Instead, the combination of GV and precipitation must be used to determine the state of the implant.

*In vitro* analysis of polymer precipitation and drug release showed a high degree of correlation within the first 48 hrs. This relationship is most likely related to the release of drug during the precipitation of the polymer. Interestingly, the correlation begins to wane as the mechanism of delivery transitions from burst release to a form of diffusion mediated release. Differences were seen between the *in vitro* and *in vivo* release rates after 8 hrs, which may be attributed to the difference in surface area and more importantly convective removal of solvent and drug, which consequently enhances the polymer precipitation.

Because of the novelty of the ultrasound characterization technique, little data is currently available for direct comparison of our results. In the most applicable study, Kempe et al. noninvasively evaluated the phase inversion dynamics of an *in situ* forming implant similar to the 3A polymer using EPR [23]. While our results were similar *in vivo*, they differ with respect to the precipitation rate of the polymer *in vitro*. The deviations may be explained by the vastly different nature of the two techniques. In particular, the rate of solvent and water exchange is limited in the agarose phantom which may lead to delayed implant formation. Additionally, it is possible that the spin probe required for EPR analysis may affect the phase inversion dynamics of the implants [24]. One benefit of our technique is that phase inversion can be monitored without the need of a probe, which is required for EPR. However, EPR is able to measure the solvent exchange process which currently cannot be examined with ultrasound imaging.
3.5. Conclusion

This study has demonstrated, for the first time, that it is possible to noninvasively and nondestructively image the phase inversion process using a diagnostic ultrasound. The phase inversion data can then be directly related to the rate of drug release, providing a new means for the study of in situ forming implants in *in vivo* applications. While ultrasound has several advantages over traditional methods, it is limited by the resolution of the images. This makes ultrasound ideal for analyzing the macro-scale behavior of the implants, but traditional techniques, such as SEM analysis, can provide complimentary, higher resolution information. Additionally, while the study of implants in a constrained environment provided insight into their behavior, additional studies need to be performed in a nonconstrained system, so that the relationship between the GV and the solidification of the implant can be better understood. Overall, this novel technique is a powerful means by which *in situ* forming drug delivery depots can be monitored and studied noninvasively throughout the lifespan of the implant. Potential applications of this technique include high throughput screening for new implant development and simplified *in vitro/in vivo* correlation of implant properties. The US technique is not limited to phase sensitive *in situ* forming polymer systems and may be applied to other *in situ* forming systems, such as ones undergoing phase transition due to temperature or pH. Furthermore, the noninvasive analysis may be utilized in fields outside of drug delivery to monitor the behavior of polymer implants in tissue engineering and related applications. While the focus of this chapter was on the evaluation of phase inversion
using diagnostic ultrasound, the following chapter ultrasound imaging is used to evaluate the effect of drug properties on implant behavior.

3.6. References


Chapter 4: Effect of Cargo Properties on *In Situ* Forming Implant Behavior Determined by Noninvasive Ultrasound Imaging

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* indicates that the authors contributed equally

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4.1. Introduction

As discussed in previous chapters, the behavior of ISF implants can be altered by essentially any factor that changes the nature of the mass transfer dynamics [1-7]. For example, it has been shown that the choice of solvent and polymer can influence the rate of phase inversion and subsequent drug release. Likewise, additives such as Pluronic have also been shown to reduce the burst release of drug due to reduction of the diffusivity of the polymer-lean domains [5, 6] and properties of the drug loaded into the implant may also considerably affect the behavior of the system [7]. In this chapter, we investigated the role of a variety of drug properties on implant behavior with the help of ultrasound imaging analysis. Four drugs with different characteristics (Table 4.1) were evaluated in terms of their effect on implant phase inversion, swelling, release, and polymer degradation. To model the effect of protein loading, bovine serum albumin (BSA), a commonly released model protein, was incorporated into the polymer solution.

![Chemical structures](image)

**Figure 4.1.** The chemical structures of the drugs used in this study: (a) DiI, (b) Dox, (c) Fluorescein

The lipophilic dye 1, 1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate (DiI, Figure 4.1A) was used to evaluate the effects of loading a hydrophobic drug. Finally, to evaluate the effect of a smaller and relatively water-soluble
drug (100mg/mL), sodium fluorescein (Figure 4.1C), and the chemotherapeutic drug doxorubicin (Dox, 10mg/mL [8], Figure 4.1B) were examined. To demonstrate the utility of the ultrasound characterization technique in vivo, Dox or fluorescein loaded implants were injected subcutaneously in Sprague-Dawley rats so that the in vivo release, swelling, and phase inversion behavior could also be characterized.

**Table 4.1.** Properties of the drugs used

<table>
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<th>Fluorescein</th>
<th>Dox</th>
<th>Dil</th>
<th>BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mw (Da)</td>
<td>376</td>
<td>580</td>
<td>934</td>
<td>66,430</td>
</tr>
</tbody>
</table>

4.2. Materials and Methods

4.2.1. Materials

Poly(DL-lactic-co-glycolic acid) (PLGA) (50:50 3A, Mw 24,000 Da, inherent viscosity of 0.25 dl/g) was obtained from Lakeshore Biomaterials, Birmingham Al and used as received. N-methyl-2-pyrrolidinone (NMP), sodium fluorescein, BSA, Dox were used as received from Sigma Aldrich (St. Louis, MO). Agarose and phosphate buffered saline (PBS) were received from Fischer Scientific (Waltham, MA). DiI was obtained from Invitrogen (Eugene, OR).

4.2.2. Preparation of Polymer Formulations

All solutions were prepared using a 39:60:1 mass ratio of PLGA:NMP:drug by first suspending or dissolving the drug in NMP. Polymer was then added to the solution and allowed to incubate overnight in a 37°C shaker table at 90 RPM. Polymer solutions were stored at 4°C and used within three days.
4.2.3. *In Vitro Dissolution Studies*

Drug release profiles were evaluated by injecting 50 µl (roughly 40-50 mg) of polymer-drug solution into 10 ml of 37°C PBS (pH 7.4); implants were then placed in an incubated orbital shaker (37°C at 90 RPM). During the first 8 hours (h), solution was sampled and then replaced with fresh warm PBS at 0, 0.5, 1, 2, 4, 6, and 8 h after implant formation. After the first 24 h, the bath solution was sampled, and then completely removed and replaced by 10 mL of fresh buffer solution daily for 14 days (d). After 14 d, implants were removed from the bath solution and solubilized so that the residual drug mass could be determined. Implants loaded with fluorescein were dissolved in 2M NaOH. Fluorescein mass was determined by measuring the fluorescence in the solution samples and referenced to a standard curve of known masses on a multimode microplate reader (Tecan Ltd., Infinite 200 series) at excitation/emission wavelengths (Ex/Em) of 485/525 nm. Implants loaded with DiI and Dox were dissolved in 5 ml of NMP. DiI concentration was determined by measuring absorbance on the microplate reader at 549 nm, while Dox concentration was determined using fluorescence with an Ex/Em wavelengths of 470/585 nm. BSA release was determined using a micro-BCA total protein assay kit (Pierce Biotechnology, Rockford, IL), and residual protein mass was determined by degrading the implants in a 0.9 M NaOH solution and then neutralizing the solution with a 0.9 M HCl solution before the residual protein mass was evaluated with the micro-BCA kit. The cumulative drug release was calculated from these measurements and normalized by the actual total initial drug loading at.
4.2.4. *In Vitro Ultrasound Imaging*

Implants were imaged using ultrasound as previously described [14]. Briefly, a diagnostic ultrasound using a 12 MHz transducer (Aplio XG, Toshiba Medical Systems) was immobilized below an agarose mold so that the implant could be imaged through the Z-axis of the implant. Implants were formed by injecting 50 µL of polymer solution into 1 mL of phosphate buffered saline (PBS, pH 7.4), in an agarose phantom containing a 1 ml void. Images were taken immediately after the implant was injected into the PBS bath, as well as 0.5, 1, 2, 4, 6, and 8 h after injection during the first day. After images were taken, the implants were placed in vials containing 10 ml of warm PBS and placed on an incubated orbital shaker. Implants were then imaged once daily for a period of 10 days. Analysis of ultrasound images was performed using a custom MatLab code (MathWorks Inc., Natick, MA), first by selecting the implant shell, then thresholding the image to create a binary image, and finally summing the pixel values to determine the area of the formed polymer shell as well as the total cross-sectional area of the implant [14, 15]. The rate of phase inversion was determined by monitoring the ratio of the polymer shell to the total cross-sectional area, and the change in cross-sectional area over time was used to monitor the implant swelling [14, 15]. Finally, the first 48 h of release and phase inversion were used to evaluate the relationship between burst release and phase inversion. A mathematical fit was performed using the sum of least squares with MatLab. The equation F(x) = S₀(1 − e^{−τx}) + mx was used, where X represents the percent of drug release, F(x) = percent formation; S₀ = initial polymer precipitation, (%); τ = time...
delay, \( \left( \frac{1}{\% \text{release}} \right) \); and \( m \) is the proportionality constant relating the change in drug concentration to the phase inversion, \( \left( \frac{\%}{\% \text{release}} \right) \).

4.2.5. Erosion and Degradation Study

Changes in implant mass with respect to time were monitored by first recording the initial mass of implants formed by injecting 50 \( \mu l \) of polymer solution into 10 ml of warm PBS (pH 7.4), which were then kept in an incubated shaker (37\( ^\circ \)C at 90 RPM). In order to maintain sink conditions, 1 ml of bath solution was removed daily and replaced with warm, fresh PBS. Implants were removed from the bath solution at 1, 4, 7, 10, 14, 17, and 21 d. Implants were first frozen and then lyophilized for 4 days, and the final implant mass was then recorded.

Changes in the polymer Mw were then evaluated by dissolving the lyophilized implants in tetrahydrofuran (THF), filtered using a 0.45 \( \mu m \) syringe filter, and analyzed using gel permeation chromatography (GPC) (Agilent Technologies 1200 Series). The detectors used were refractive index and variable wavelength (Both from Agilent Technologies 1200 Series) and the columns used were 2 of AM GPC Gel linear/10u and an AM GPC Gel Guard column/10u in series (American Polymer Standards Corp). The flow rate of 1ml/min was used and the results were compared to a polystyrene standard.

4.2.6. Scanning Electron Microscope Imaging and Analysis

Implant microstructure was evaluated by first preparing implants by injecting 50 \( \mu l \) of polymer solution into 10 ml of warm PBS. Initially, 5 ml of bath solution was
removed and replaced with 5 ml of warm PBS at the conclusion of the 8 h in order to maintain sink conditions. After the first 24 h, 1 ml of bath solution was removed daily and replaced with 1 ml of warm, fresh PBS to keep sink conditions constant. At predetermined time points (3d, 7d, 14d after implantation), the implants were removed from the bath solution, imaged using ultrasound, and then freeze fractured over dry ice. The fractured samples were then lyophilized for 96 h, and then mounted to an aluminum stub and sputter coated with 5 nm of Pd. The coated samples were then imaged using a Quanta 200 3D ESEM with an acceleration voltage of 3.5 kV and a hole size of 10.

4.2.7. In Vivo Analysis

Animal studies were performed following previously described techniques in accordance with the standards established by the Case Western Reserve University Institutional Animal Care and Use Committee [14, 15]. Briefly, ten 10 week old male Sprague-Dawley rats (two rats per time point, with five implants per rat) which had an average body weight of 295±15 g (Charles River Laboratories Inc., Wilmington, Ma) were anesthetized using 1% isoflurane at a flowing oxygen rate of 1L/min (EZ150 Isoflurane Vaporizer, EZ Anesthesias™). Implants were formed subcutaneously by injecting 50-70 µl of polymer solution under the dorsal skin flap with an 18-gauge hypodermic needle. Subcutaneous injection was chosen because the current clinical application of a similar formulation (Eligard) is administered in the same manner. The implants were then imaged using the same ultrasound scanner and transducer at 1, 4, 8, 24, and 48 h after implantation. Animals were euthanized at each time point, and the implant was dissected out, followed by dissolution of the implants in order to evaluate the
residual drug remaining. Fluorescein and Dox fluorescence was measured using a Tecan 200 series plate reader with Ex/Em wavelengths of 485/525 nm for fluorescein and 470/585 nm for Dox and then referenced to a standard curve.

4.2.8. Statistical Analysis

Statistical significance was determined using a one-way analysis of variance (ANOVA, p<0.05). A Tukey multi-comparison test was used to evaluate differences between groups, with all analysis performed using Minitab (Minitab inc., State College, PA). Unless otherwise noted, all data were reported as mean ± standard deviation.

4.3. Results

4.3.1. In Vitro Drug Release

ISFIs have characteristic release kinetics consisting of three distinct phases of release: burst (0-24 h), diffusion (between 24h and 216 h), and degradation (216-336 h). During the initial period of release, implants loaded with Dox had the highest overall dissolution of drug followed by fluorescein and BSA, with 27.2±1.8%, 23.2±3.2%, and 21.4±1.4% respectively (Figure 4.2). DiI had a significantly lower burst, releasing only 0.2±.03% during that 24 h period (Figure 4.2). The mean mass of drug released per day during the diffusion phase, was statistically greater for Dox and fluorescein (3.2±1.1%)

![Figure 4.2. Cumulative mass release of drug from the ISFI into PBS (pH 7.4).](image-url)
and 2.7±1.2% respectively), than both BSA and DiI (1.0±0.3% and 0.03±0.02%). During the degradation phase, drug dissolution increased relative to the diffusion phase for both fluorescein and DiI (6.5±0.8% and 0.2±0.1%), while decreasing for Dox and BSA (1.5±0.3% and 0.2±0.1% respectively). Release percentages are summarized in Table 4.2.

Table 4.2. Mean release per phase of release

<table>
<thead>
<tr>
<th>Release</th>
<th>Fluorescein</th>
<th>Dox</th>
<th>DiI</th>
<th>BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burst (%)</td>
<td>23.2±3.3</td>
<td>27.2±1.8</td>
<td>0.2±0.04</td>
<td>21.4±1.5</td>
</tr>
<tr>
<td>Diffusion (%)</td>
<td>2.7±1.2</td>
<td>3.2±1.1</td>
<td>0.03±0.02</td>
<td>1.0±0.3</td>
</tr>
<tr>
<td>Degradation (%)</td>
<td>6.5±0.8</td>
<td>1.5±0.3</td>
<td>0.2±0.1</td>
<td>0.2±0.1</td>
</tr>
</tbody>
</table>

4.3.2. Ultrasound Characterization and Image Validation

Figure 4.3. Representative gray-scale ultrasound images of implants acquired over a period of 240 hours; each row is representative of implants loaded with different drugs: (a) Fluorescein, (b) DiI, (c) BSA, and (d) Dox. The scale bar represents 2.5 mm. Pore formation is indicated with arrows.

Representative gray-scale images of the implants are shown in Figure 4.3. Initially, the implants formed a thin shell, and as phase inversion progressed, gray-scale intensity increased as a result of acoustic impedance differences occurring as the polymer precipitated. Implants loaded with fluorescein and DiI formed pores near the center of the implants which increased in diameter over time (Figure 4.3). For implants loaded
with BSA and Dox, these pores were present initially, but did not expand over time (Figure 4.3). Image validation was performed by comparing ultrasound images to pictures of the cryosectioned implants and the SEM micrograms. These same implants were compared with 7 day images at a higher magnification. Hyperechoic regions were generated as a result of the heterogeneous microstructures formed during phase inversion, with isoechoic regions corresponding to polymer lean domains within the implant microstructure (Figure 4.4).

**Phase Inversion**

Initially, polymer precipitation occurred rapidly with only negligible changes in echogenicity occurring after 4 h, with DiI loaded implants showing the greatest rate of precipitation, transitioning from an initial phase inverted area occupying 29.3±6.0% to a plateau value of 78.2±6.1% (Figure 4.5A, 4.5D). Dox and BSA loaded implants both had an increased initial echogenic signal that occupied a larger portion of the cross-sectional area (50.9±7.1% and 58.8±6.0%) and reached a statistically equivalent plateau 4 h after implantation to the DiI loaded implants (Figure 4.5A, 4.5D). Conversely, fluorescein loaded implants had an initial precipitation area of 22.0±11.0% initially, and reached a plateau of 58.7±13.2%, showing a similar rate of change to both BSA and Dox loaded implants (Figure 4.5A, 4.5D).
Figure 4.5. (a) Quantitative formation data acquired in the same implants over the course of 12 d, (b) corresponding quantitative swelling data, (c) correlation of phase inversion and drug release, (d) phase inversion over the course of the first 6 h.

After 24 h the implants continued to precipitate, but more gradually, with DiI loaded implants reaching a maximum precipitated area 48 h after implantation, fluorescein loaded depots 96 h after implantation, Dox and BSA loaded implants reaching maximums after 144 h (84.0±5.6%, 88.8±5.8%, 92.7±2.0%, and 95.1±4.1%). The polymer area remained constant for 3 days after phase inversion was complete. For implants loaded with DiI, fluorescein, and Dox phase inversion was followed by a decrease in precipitated area during the duration of the study, and to a much smaller extent with BSA loaded implants (Figure 4.5A).

**Implant Swelling**

Implants loaded with DiI initially shrank, losing 15.0±7.4% of the original cross-sectional area, with no change observed until after 8 h in solution (Figure 4.5B). After 8 h, the implants began to swell, and approached the original cross-sectional area after 24 h. Swelling continued until a maximum of 31.2±9.8% greater than the original cross-
sectional area was reached after 144 h in buffer. An initial decrease in implant cross-
sectional area was also observed with both Dox and fluorescein loaded implants 
(14.6±2.5% and 4.8±5.3% respectively). Depots loaded with Dox began to approach the 
original size after 4 h in buffer, while the fluorescein loaded implants only took 1 h. Both 
implants loaded with Dox and those loaded with fluorescein continued to swell, with Dox 
loaded implants increasing an additional 70.7±15.8% relative to the original cross-
sectional area after 120 h and fluorescein loaded implants increasing 75.6±23.5% after 
168 h (Figure 4.5B). BSA loaded implants did not shrink initially, but after an initial 
period of expansion increasing in size by 11.85±9.3% after 1 h and only negligible 
changes were observed over the course of next 24 h. After 24 h in solution the implants 
began to swell, with area increasing an additional 66.7±10% relative to the original 
cross-sectional area after 120 h in buffer (Figure 4.5B).

**Correlation of Phase Inversion to Drug Release**

A nonlinear relationship was developed correlating the phase inversion to the 
burst phase of drug release (Figure 4.5C). The value $S_0$ was used to evaluate the initial 
echogenicity of the implants. Fluorescein had the lowest $S_0$ value, followed by Dox, then 
DiI, and finally BSA (Table 4.3). The $\tau$ value was added to account for the transition to 
linearity, with DiI and Dox each having $\tau$ values greater than 1, indicating a rapid 
transition into the linear region (Table 4.3). Finally, the slope was used to evaluate the 
sensitivity in the linear region.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Fluorescein</th>
<th>Dox</th>
<th>DiI</th>
<th>BSA</th>
<th>Dox In Vivo</th>
<th>Fluorescein In Vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td>$S_0$</td>
<td>28.1</td>
<td>51.3</td>
<td>72.0</td>
<td>83.4</td>
<td>48.4</td>
<td>46.2</td>
</tr>
<tr>
<td>$T$</td>
<td>0.9</td>
<td>14.9</td>
<td>51.6</td>
<td>0.6</td>
<td>1.0</td>
<td>3.4</td>
</tr>
<tr>
<td>$M$</td>
<td>1.5</td>
<td>1.1</td>
<td>25.1</td>
<td>-0.2</td>
<td>0.1</td>
<td>-0.1</td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.97</td>
<td>0.98</td>
<td>0.56</td>
<td>0.99</td>
<td>0.97</td>
<td>0.98</td>
</tr>
</tbody>
</table>
4.3.3. Erosion and Degradation

Initially as a result of NMP loss, mass loss was above 40% for all implant formulations, with no statistical difference between the implants for the first 10 d. After 10 d, implants loaded with DiI and those loaded with fluorescein began to show increased mass loss relative to those implants loaded with Dox or BSA (28.4±2.7%, 29.9±3.3%, 35.3±1.5%, 34.7±1.4% respectively) (Figure 4.6A). Changes in the weight average Mw showed that implants loaded with fluorescein had an initially elevated level of polymer degradation, with the mean Mw reduced by 39.7±12.2% after only one day (Figure 4.6B). Implants loaded with either Dox or BSA showed reduced degradation relative to the implants loaded with either DiI or fluorescein after 1 week in solution, but after 14 days all implant formulations had undergone a similar level of hydrolysis (Figure 4.6B).

![Figure 4.6.](image)

**Figure 4.6.** (a) Cumulative erosion data, measured as percent mass loss over time from ISFI formed in PBS (pH 7.4). (b) Degradation analysis measured as change in the weight average Mw over time, with data normalized by the Mw of the polymer before exposure to PBS.

4.3.4. Implant Microstructure Analysis

Comparison of the microstructure that forms as a result of the phase inversion dynamics show that implants loaded with Dox, fluorescein, and BSA all have a similar cell like microstructure with highly interconnected porous interior (Figure 4.7A, C, and...
D). Conversely, while macroporous structures were formed in the DiI loaded implants, they appear to be less interconnected with large regions of polymer separating the polymer-lean domains (Figure 4.7B). After 7 d in buffer, porosity appears to increase for the fluorescein loaded implants (Figure 4.7E). Porosity appeared to have increased for both the BSA loaded implants and the Dox loaded implants, but the increase in pore size was not as substantial (Figure 4.7G and H). Implants loaded with DiI showed a less defined structure after 7 d in buffer (Figure 4.7F).

4.3.5. In Vivo Characterization

Polymer precipitation rapidly reached a plateau within the first hour of implantation, and no significant changes were observed over the course of the next 48 h.
(Figure 4.8). No significant difference in phase inversion was observed between implants loaded with Dox or those loaded with fluorescein. Dox dissolution was significantly higher *in vivo* than *in vitro*. *In vivo*, 42.8% of the drug was lost 1 h after implantation, but release slowed after 1 h, with 58.5% of drug released after 24 h. After 24 h, there was a second burst of release, with 73.4% of the drug lost after 48 h. Release of fluorescein was similar to that of Dox, but fluorescein had a slightly lower initial burst of drug, losing only 28.7% 1 h after implantation, but increased to 47.5% after 8 h. A second burst was observed with 68.3% of fluorescein lost after 24 h and 73.5% after 48 h. Implants loaded with Dox decreased in cross-sectional area for the first 24 h, but showed a modest increase after 48 h. Implants loaded with fluorescein showed a decrease in size over the first 8 h, but swelling was observed after 24 h (Figure 4.9).

**Figure 4.9.** (a) Cumulative mass release of drug from implants injected subcutaneously under the dorsal skin flap of Sprague-Dawley rats over the course of 48 h, (b) quantitative swelling data for the same implants, (c) quantitative formation data for implants formed subcutaneously, (d) changes in mean gray-scale data over time.
4.4. Discussion

Ultrasound characterization is a noninvasive means by which implants can be evaluated nondestructively in a number of different environments, providing real-time images of polymer precipitation. This nondestructive characterization technique can not only be used to evaluate the effect of injection site on implant behavior, but also how the host responds to the implant [14, 15]. Previous studies have focused on the effect of environment on phase inversion and swelling using a single mock drug, fluorescein [14, 15]. The focus of this study was to evaluate how various drug properties alter the phase inversion dynamics, swelling, and release properties of the implants. Evaluation of the implant phase inversion showed that polymer precipitation occurred in two steps; first the formation of the polymer shell, which occurred within the first four hours. Then a slower transition which occurred over the time scale of days, as the implant interior slowly transitioned into a stable matrix. The outer shells of the implants were highly echogenic, with a distinctly different microstructure from the interior domain (Figure 4.4). In the case of implants loaded with DiI or fluorescein, a pore formed in the implant center after the implants phase inverted, which gradually increased in size over time. Pore formation also occurred with BSA and Dox loaded implants, but the pore did not increase in diameter over time. We hypothesize that the pore is initially a result of entropically driven phase separation, and increases in size due to the polymer degradation, which was slower for both BSA and Dox loaded implants. Additionally, fluorescein, BSA and Dox resulted in a similar microstructure due to their comparable rates of phase inversion. DiI showed a significantly different microstructure, with large macrovoids due to the drug’s insolubility in water consequently affecting the implant’s phase inversion dynamics.
Drug hydrophobicity was a significant factor altering the implant swelling. The implants loaded with the hydrophobic dye DiI showed an initial reduction in cross-sectional area. This indicates that the influx of water into the implant was lower than the efflux of solvent from the implant.

When comparing the burst release to phase inversion, a mathematical relationship was developed to account for the initial echogenicity of the precipitating polymer, providing a means by which the drug release could be approximated noninvasively. While implants loaded with Dox had a higher initial precipitation, the phase inversion and drug release occurred at near equivalent rates. Interestingly the relationship between phase inversion and drug release for implants loaded with BSA was negative, which we hypothesize to be a result of the crystallized BSA dissolving as water diffused into the matrix during the phase inversion process. While the correlation coefficient was above 0.97 for implants loaded with Dox, fluorescein, or BSA, this relationship did not hold for DiI loaded implants. The poor correlation is suspected to be a result of the low mass of drug that is released by these implants. While a relationship exists between polymer precipitation and burst release, because of the rapidly reached plateau in phase inversion and elevated levels of drug release, the sensitivity was lower than what was observed in vitro.

When the erosion and degradation behavior of the implants was evaluated, rapid mass loss was observed for both DiI and fluorescein loaded implants, but the rate of erosion was slower for the implants loaded with Dox and BSA. While this result was not anticipated, it has been observed that the internal environment of PLGA microparticles is acidic with a pH ranging from 2-5 [16]. It has also been reported that BSA has a
maximum buffering capacity of pH 3.5 [17], indicating that BSA may be acting as a buffer and delaying polymer degradation. In the case of Dox loaded implants, we speculate that as a consequence of the low pH, Dox becomes positively charged. Over time as buffer diffuses into the implants and the acidic by-products diffuse out, internal pH increases closer to 5 [16], (above the pKa of the polymer [18]) resulting in a net negative charge state within the matrix, leading to the formation of an ionic complex that retards both the mass loss of drug and polymer degradation. The potential for ionic complexation is not limited to Dox. In addition to the buffering capacity of BSA at low pH, the isoelectric point is between pH 4.7-4.9 [13], which indicates that the protein will be in a cationic state when loaded in the polymer matrix. The resultant electrostatic interactions may be an additional contributing factor altering the release and degradation profile of BSA loaded implants. As neither DiI nor fluorescein would interact with the matrix, release is elevated as the polymer degrades.

Release in vivo has been shown to be elevated relative to the release observed in vitro [14, 15, 19]. Interestingly, beyond the high initial burst, secondary burst releases occurred in accordance with observed increases in polymer cross-sectional area (Figure 4.9). The resultant changes in implant geometry may enhance drug release in two ways, by first increasing the surface area for drug diffusion and secondly by introducing convective forces within the implant elevating drug release.

The noninvasive technique utilized in this study was able to successfully quantify the relative longitudinal implant behavior in vitro and in vivo and offer some insights into the complex relationship between drug properties and their effects on implant formation and erosion. Nonetheless, some limitations remain. One point of concern is the effect of
drug itself on the ultrasound signal. In the majority of cases, since ultrasound characterizes phase inversion through monitoring changes in polymer matrix echogenicity, the addition of drug should not affect the characterization process. Indeed in the case of drugs that readily dissolve in the polymer solution, no interference was observed. However, in the case of drugs with low solubility in the polymer solution, there was an elevated initial echogenic signal. We hypothesize that this elevated signal is a result of the non-dissolved agent causing an acoustic impedance mismatch between the liquid polymer and crystallized drug, leading to hyperechoic regions within the liquid interior of the implant. Since BSA is not soluble in NMP, the resultant polymer suspension led to the hyperechoic domains. While Dox is soluble in NMP, elevated concentrations of the drug have been show to result in the formation of aggregates [10-12], which would then act as a scattering agent within the liquid interior of the implant. Despite the initial elevated background signal, in all cases relative polymer precipitation could still be observed and successfully quantified.

4.5. Conclusion

Advances in medical imaging have led to the development of tools for accurate placement of devices in the body as well as methods for monitoring treatment efficacy. In the field of drug delivery, these tools can also be used in order to better understand how a delivery system is behaving in the body, so that deviations in device behavior can be better understood. This study demonstrated that ultrasound imaging could be used to monitor implant behavior regardless of the drug loading both in vitro and in vivo. The ability to noninvasively monitor the behavior of implants in their unperturbed
environment, whether *in vitro* or *in vivo* provides a means by which the interactions between the drug, the matrix, and the injection site can be more fully understood. In this study a relationship was established between phase inversion and drug release which provides a means by which burst release of implants can be approximated. Additionally, alternative imaging techniques, such as MRI, could prove useful in further investigation of implant behavior, providing insight into other characteristics such as solvent exchange. As the image-guided drug delivery field evolves, these techniques could in the near future be utilized in the clinic to better understand and subsequently improve *in situ* forming implant performance and hopefully broaden their application scope.

4.6. References

Chapter 5: Noninvasive Characterization of the Effect of Varying PLGA Molecular Weight Blends on In Situ Forming Implant Behavior Using Ultrasound Imaging

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* Authors contributed equally to this work
5.1. Introduction

Parameters that affect the drug release profile of injectable implants have been discussed in previous chapters, and include: solvent polarity, polymer concentration, and polymer type [1-20]. Through the use of poorly miscible solvents which lead to a slowly formed, dense polymer matrix, implants with a near zero order release have been developed [8, 10, 21, 22]. Despite the optimal release profile, their use has been somewhat limited clinically due to their high viscosity which limits the injectability of the polymer solution [9, 19, 22]. Conversely, fast phase inverting systems have optimal viscosity, but have been shown to release a large burst of drug due to formation of a heterogeneous polymer matrix with a highly interconnected porous network [10, 17, 23-26]. In order to limit the burst release, excipients have been used [4, 9, 25]. These additives reduce burst by decreasing the diffusivity coefficient of drug through the porous network within the depot [9, 10, 25]. While they have shown to be effective in vitro, there efficacy is somewhat limited in vivo [26]. In this chapter we discuss how changes in the polymer matrix can be used to alter implant behavior.

By varying the polymer molecular weight (Mw) drug release and phase inversion can be modified for implants formed both in vitro and in vivo [10, 18, 25-27]. Previous research has demonstrated that the average Mw of PLGA influences both the release profile and implant morphology [3, 15, 16, 25, 26, 28], with lower Mw implants having greater overall release and phase inversion than the higher Mw counterparts [3, 15, 16, 28, 29]. These differences have been attributed to the higher hydrophilicity and degradation rate of the lower Mw polymers [3, 10, 15-17, 28]. For many therapeutic applications, the ideal release profile for such an agent would have an intermediate release than what is obtained from the pure polymer types. The aim of this study was to
demonstrate that using PLGA Mw blends could be a simple way to optimize the release of a small-molecule, hydrophilic drug without the use of additional excipients. Blends of different Mw PLGA polymers were investigated both in vivo and in vitro. Effect of the blends on the rate of implant formation, evolution of implant morphology, polymer degradation and erosion were investigated using a noninvasive imaging technique developed previously by our group [26, 30]. Briefly, diagnostic ultrasound was used to visualize and quantify phase inversion in real-time. As the liquid implant formulation begins to solidify upon contact with water, the acoustic impedance of the polymer solution changes, and a higher proportion of incoming ultrasound waves are backscattered. These changes in acoustic impedance can be used to visualize the rate of phase inversion, providing insight into changes in implant morphology both in vitro and in vivo. This is of interest since phase inversion of ISFIs has been thought to govern much of the early drug release properties [10, 17, 23, 31], yet the undisrupted behavior of ISFI has been difficult to quantify. In this study we show a direct relationship between specific morphological parameters and alterations in degradation, erosion and subsequent drug release profiles resulting from PLGA Mw variations.

5.2. Materials and Methods

5.2.1. Materials

All materials were used in the state obtained from the respective companies. Poly (DL-lactide-co-glycolide) (PLGA 50:50 2A, MW 15kDa, inherent viscosity 0.16dl/g; 3A, MW 29kDa, inherent viscosity 0.28 dl/g; 4A, MW 53kDa, inherent viscosity 0.46 dl/g) was obtained from Lakeshore Biomaterials, Birmingham, AL. N-methyl pyrrolidinone
(NMP) and sodium fluorescein (MW 376.28) were obtained from Sigma Aldrich, St. Louis, MO. Agarose was acquired from Fischer Scientific, Waltham, MA.

5.2.2. Preparation of Polymer Solutions

Fluorescein was used as a mock low Mw mock hydrophilic drug. Polymer solution was prepared using a mass ratio of 39:60:1 PLGA to NMP to fluorescein. First, fluorescein was dissolved in NMP for 1 hr on an incubated orbital shaker (37°C, 90 RPM). Next, PLGA was added to the NMP:fluorescein solution and allowed to dissolve overnight in the orbital shaker protected from light. Blend formulations of 1:1 molar ratios of the three different Mw polymers were produced for investigation (15kDa:29kDa, 15kDa:53kDa, 29kDa:53kDa). Additionally, formulations consisting of pure non-blended polymer were produced (15kDa, 29kDa, and 53kDa).

5.2.3. Ultrasound Characterization

Implants were imaged using ultrasound as previously described [30, 32]. Briefly, an agarose phantom containing a 1 ml void, was filled with phosphate buffered saline (PBS, pH 7.4). Then polymer solution (44.3±3.6 mg) was injected into the PBS. Images were taken immediately after injection into PBS, and then after 0.5, 1, 2, 4, 6, and 8 h during the first day (d), then daily for 10 d. Images were obtained using a 12 MHz transducer (Aplio XG, Toshiba Medical Systems). Implants were kept at 37°C on an incubated orbital shaker in 10 ml of warm PBS for the duration of the study. Image analysis was performed using a custom MatLab code (MathWorks Inc., Natick, MA) which selected the implant shell, applied a threshold to the entire image to create a binary
image, and summed the pixel values to determine the area of the formed polymer shell and the total cross-sectional area of the implant [26, 30, 32]. The rate of phase inversion was quantified using the ratio of the polymer shell area relative to the total cross-sectional area, and the change in cross-sectional area over time was used to monitor implant swelling [26, 30, 32]. Linear regions in the swelling profile were determined by evaluating derivatives of the swelling plots. A 3-D ultrasound tomography analysis was performed by embedding implants in a 1% agarose phantom. The phantom was then placed on a MSL series linear stage (Newmark systems, MSL-25-11), with the US transducer fixed above the actuated stage. Images were taken every 200 µm through the volume of the implant. The 2-D ultrasound data was then reconstructed into a 3-D volume using AMIRA (Visage Imaging GmbH) (Movie 5.1).

5.2.4. Drug Release

50 µl of implant solution (44.3±3.6 mg) was injected into a 10 ml 37°C PBS bath (pH 7.4). Implant mass was recorded immediately, and then 1 ml of the sample solution was removed and then replaced by 1 ml of warm fresh PBS in order to maintain sink conditions. Samples were taken on the first day at 0, 0.5, 1, 2, 4, 6, and 8 h, then once daily for 14 d, and on days 16, 18, and 21. After 21 d, the implants were removed and added to 5ml of 2M NaOH overnight in order to determine the mass of any residual drug. Fluorescence was measured using a Tecan Ltd, Infinite 200 Series plate reader, with excitation wavelength 485nm and emission wavelength 525nm. The
concentration of fluorescein was measured by comparison to known established standard concentrations, and the cumulative drug release was then calculated from the obtained fluorescence data. The diffusion and degradation phases of release were determined by evaluating derivatives of the release plots. As previously described, drug release and phase inversion were correlated using a non-linear mathematical fit to $F(x)=S_0(1-e^{-\tau x})+mx$ [32]. $F(x)$ is the percent phase inversion, $x$ represents the percent drug release ($\%$), $S_0$ represents the initial polymer precipitation ($\%$), $\tau$ is the time delay $\left(\frac{1}{\% \text{ release}}\right)$, and $m$ is the proportionality constant that relates phase inversion to drug release $\left(\frac{\%}{\% \text{ release}}\right)$.

5.2.5. Erosion and Degradation

Implant erosion was determined by monitoring the change of implant mass with respect to time. After implant formation, the initial mass was recorded. 1 ml of bath-solution was discarded daily and replaced with 1 ml of fresh PBS solution to maintain sink conditions. At each respective time point (every other day for 21 d) the implant was removed from solution, frozen, then lyophilized for 5 d, and the final implant mass was recorded. The degradation was monitored by evaluating changes in the weight average $M_w$ over time as previously described [32]. The implants were prepared for GPC analysis by first dissolving the lyophilized implants in tetrahydrofuran (THF) and then filtered using a 0.45µm syringe filter. $M_w$ was determined relative to narrow polystyrene standards using an Agilent 1200 series liquid chromatography system, a refractive index and variable wavelength detector, and two American Polymer Standards linear bed GPC columns (Mentor, OH) at a flow rate of 1 ml/min.
5.2.6. In Vivo Analysis

All animal studies were performed as previously described following protocols approved by the Case Western Reserve University Institutional Animal Care and Use Committee [26, 30]. Briefly, five 24-week old male Sprague-Dawley rats with an average body weight of 565±58 g (Charles River Laboratories Inc., Wilmington, MA) were anesthetized using 1% isoflurane with an oxygen flow rate of 1 l/min (EZ150 Isoflurane Vaporizer, EZ Anesthesias\textsuperscript{TM}). Implant solution (50 to 70 µl) was injected under the dorsal skinflap in five locations using an 18-gauge hypodermic needle. The depots were then imaged using ultrasound with a 12 MHz transducer 1 h, 4 h, 8 h, 24 h, and 48 h after implantation. Drug release was determined by dissecting out the implants after euthanasia, then degrading the implants in 5 ml of 2 M NaOH overnight. The mass of fluorescein was determined by comparison to a standard curve using a Tecan 200 series plate reader at an excitation wavelength of 485 nm and an emission wavelength of 525 nm [26, 30].

5.2.7. Statistical Analysis

Statistical significance was determined using ANOVA (p<0.05, N=4 for in vitro studies and N=5 for in vivo studies). Significant difference among groups was established using a Tukey multiple comparison test, with analysis performed using Minitab. Unless otherwise noted, data is reported as mean ± standard deviation.
5.3. Results

5.3.1. Ultrasound Imaging

Immediately after injection of the implant solution into PBS, a thin polymer shell could be visualized in all implants as a result of mass transfer events increasing the acoustic impedance mismatch of the polymer and the surrounding PBS (Figure 5.1). After the initial shell formation, the implants continued to transition into solid depots, which resulted in an increase in ultrasound backscatter within the interior of the implants (Figure 5.1). After phase inversion had completed, a pore formed in the center of the implants that continued to expand over the duration of the experiment. The time required for a pore to develop was inversely related to the polymer Mw (Figure 5.1).

5.3.2. Phase Inversion

In all cases, the implants rapidly precipitated reaching a period of limited change within the first 8 h of exposure to the bath solution. Formulations using either 15 kDa or 29 kDa PLGA achieved a pseudo-steady state after 4 h in the bath-side solution (precipitating at 77.8±1.9% and 56.0±1.5% of the total cross-sectional area respectively), with implants formulated using 15 kDa PLGA precipitating significantly more than all
other formulations. Implants using the 53 kDa PLGA, 15 kDa:29 kDa, or 15 kDa:53 kDa polymers all reached a plateau after 6 h in PBS with the precipitated polymer occupying 53.7±3.3%, 50.2±3.4%, and 62.2±3.1% of the cross-sectional area respectively. Finally, depots consisting of 29 kDa:53 kDa PLGA reached a plateau of 63.7±5.3% after 8 h in the bath-side solution (Figure 5.2).

After the initial rapid period of phase inversion, final polymer precipitation varied with the polymer used, with maximum polymer precipitation taking as little as 3 d or as long as 9 d. Implants containing 15 kDa PLGA underwent faster phase inversion than implants without the low Mw polymer. Implants formulated using only 15 kDa PLGA completed phase inversion the fastest, reaching a maximum precipitated area of 84.5±4.0% within 3 days. The mixture of 15 kDa PLGA with 29 kDa PLGA increased the amount of time for phase inversion, reaching a maximum of 89.3±1.7% after 6 d. Both implants formulated using the 15 kDa:53 kDa blend and with 29 kDa PLGA alone completed phase inversion after 7 days, reaching a maximum of 83.4±9.5% and 90.1±2.0% respectively (Figure 5.2). The blend of 29kDa:53kDa PLGA required 8 d to completely phase invert, with the
precipitated polymer occupying 92.2±0.6% of the cross-sectional. Finally, the implants formulated using only 53 kDa PLGA completely phase inverted after 9 d in PBS, reaching the maximum precipitation (89.3±6.3%) (Figure 5.2).

5.3.3. Swelling

The 15 kDa implants initially increased in size, expanding to 1.26±0.16 times the original cross-sectional area within 30 m in solution, with negligible changes occurring within the first 24 h after the initial expansion. Between 24 and 48 h in buffer, implants significantly increased in size, increasing to 1.74±0.15 times the initial cross-sectional area, expanding by 47.3% within that 24 h period. Between 48 and 120 h, implants continued to swell an average rate of 18.6% per day. After 120 h in buffer, the implants showed no significant changes in cross-sectional area, remaining at 2.36±0.04 times the initial area, for the duration of the study (Figure 5.3). Implants formulated using the 29 kDa PLGA achieved a pseudo-steady state 2 h after injection into the bath-side solution, increasing 1.19±0.01 times larger than the original cross-sectional area. After 24 h in solution, the implants increased an average of 10.2% per day (from 1.20 to 1.81 times the original area). After 192 h the implants showed a rapid decrease in cross-sectional area, shrinking 58.7% over the final 48 h of

Figure 5.3. Change in implant cross-sectional area over time, for the 15 kDa, 29 kDa, and 53 kDa implants (A); and 15kDa:29kDa blend, 15kDa:53kDa blend, and 29kDa:53kDa blend (B)
the study (Figure 5.3). A 3-D reconstruction of an implant formulated using the 29 kDa polymer demonstrates the expansion and collapse of the drug eluting depot over the course of 12 d (Figure 5.4). Implants formulated using the 15kDa:29kDa blend achieved an initial plateau after 4 h in buffer. After 24 h, the cross-sectional area of depots formulated using the 15kDa:29kDa polymer blend increased at a rate of 14.8% per day, but began to decrease in size after 192 h in the bath-side solution (Figure 5.3).

![Figure 5.4. 3-D reconstruction of a 29 kDa implant over the course of 12 days, the interior green region is the hypoechoic domain.](image)

<table>
<thead>
<tr>
<th>Table 5.1. Parameters characterized using ultrasound imaging</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ultrasound Characterization</strong></td>
</tr>
<tr>
<td>Maximum Phase Inversion (d)</td>
</tr>
<tr>
<td>Maximum Phase Inversion (%)</td>
</tr>
<tr>
<td>Rate of Swelling (%)</td>
</tr>
</tbody>
</table>

All implant formulations using the 53 kDa polymer, initially decreased in size. Depots formulated using only the 53 kDa PLGA began to approach the original area after 24 h in solution (0.98±0.04 times the initial area), while the 15 kDa:53 kDa implants required 48 h and the 29 kDa:53 kDa PLGA depots approached the original cross-sectional area after 2 h in solution. Implants formulated using the 53 kDa polymer expanded an average of 9.4% per day after 24 h in solution, and began to decrease in size after 192 h (Figure 5.3). Between 48 h and 168 h, depots formulated using the blend of 15 kDa:29 kDa PLGA began to increase at a rate of 5.5% per day. While the implants
formulated using the 29 kDa:53 kDa PLGA blend increased at a rate of 7.4% per day after 8 h in solution for the duration of the study. Results are summarized in Table 5.1.

5.3.4. Drug Release

Phase sensitive ISFIs have release profiles that typically consist of three phases: burst, diffusion, and degradation. During the initial burst period of release, which occurs over the course of the first 24 h, implants containing the 53 kDa polymer had the statistically highest release of mock drug (Figure 5.5). The 29kDa:53kDa blend had the highest overall burst, followed by implants with only the 53 kDa PLGA, and then the 15kDa:53kDa (burst release of 31.0±3.0%, 30.6±2.3%, and 29.6±2.1% respectively). Implants formulated with using only the 29 kDa polymer averaged a lower burst release than the other implant formulations, followed by the 15 kDa and the 15kDa:29kDa formulations (22.7±2.9%, 24.8±1.7%, and 27.1±3.2% respectively).

![Cumulative in vitro release of fluorescein over time](image)
Table 2: Average release of fluorescein at each release phase

<table>
<thead>
<tr>
<th>Release</th>
<th>15 kDa</th>
<th>29 kDa</th>
<th>53 kDa</th>
<th>15kDa:29kDa</th>
<th>15kDa:53kDa</th>
<th>29kDa:53kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burst (%)</td>
<td>24.8±1.7</td>
<td>22.7±2.9</td>
<td>30.6±2.3</td>
<td>27.1±3.2</td>
<td>29.6±2.1</td>
<td>31.0±3.0</td>
</tr>
<tr>
<td>Diffusion (μg)</td>
<td>5.2±0.4**</td>
<td>2.6±0.7**</td>
<td>1.2±0.6**</td>
<td>2.2±0.6</td>
<td>1.8±0.4</td>
<td>2.0±0.3</td>
</tr>
<tr>
<td>Degradation (%)</td>
<td>-</td>
<td>4.1±0.4</td>
<td>3.2±0.6</td>
<td>4.1±0.3</td>
<td>3.5±1.0</td>
<td>4.4±0.7</td>
</tr>
<tr>
<td>Lock Down (%)</td>
<td>83.6±3.1*</td>
<td>76.9±6.4</td>
<td>70.5±5.6**</td>
<td>79.9±6.2</td>
<td>76.9±6.7</td>
<td>81.4±5.0+</td>
</tr>
</tbody>
</table>

* indicates that the 15 kDa polymer had a statistically different burst than the 29kDa:53kDa PLGA blend
** indicates that the pure polymer types had statistically different diffusion release from each other
£ indicates statistically different diffusion release from the blends
+ indicates a statistical difference between 15 kDa and 53 kDa implants
± indicates the statistical difference between the 53 kDa and 29kDa:53kDa blend

The duration of diffusion and degradation phases was dependent on the Mw of the polymer. The transition between phases was determined by evaluating the derivative of release. The numerical derivative doubled for all formulations except for implants formulated using only the 15 kDa PLGA. Implants fabricated using the 29 kDa polymer alone, began to show elevated release after 7 d in the bath-side solution; this transition occurred after 9 d for implants formulated using the 53 kDa polymer. Blend formulations that contained the 15 kDa polymer transitioned into the degradation phase after 6 d in PBS, but the 29kDa:53kDa blend took 8 d (Figure 5.5). While implants formulated using the 15 kDa polymer did not have distinct degradation and diffusion phases, the rate of drug dissolution per day was statistically greater for the 15 kDa polymer than any of the other implant formulations over the entire time course of the study (5.2±0.4 %/d).

Among the implant formulations with distinguishable phases of release, those formulated with the 29 kDa polymer, the 15kDa:29kDa blend, and the 29kDa:53kDa blend had a statistically greater rate of dissolution than implants using the 53 kDa polymer and the 15kDa:53kDa blend (Table 5.2). During degradation facilitated release, all implant formulations with distinguishable release phases had statistically equivalent release kinetics (Table 5.2). The release characteristics are summarized in Table 5.2.
5.3.5. Correlation of Release with Phase Inversion

The percent of drug released was correlated with phase inversion during the burst phase of release (Figure 5.6). The $S_0$ parameter was used to describe the initial echogenicity of the implants, with the 15 kDa polymer implants having the highest value, followed in order of decreasing echogenicity by the 29 kDa polymer, the 29kDa:53kDa polymer, the 15kDa:53kDa polymer, the 53kDa polymer, and the 15kDa:29kDa blend (Table 5.3). The transition to the linear region, $\tau$, was fastest with implants formulated using the 53 kDa polymer and the associated blends (Table 5.3). The 15kDa:29kDa implants transitioned more rapidly than implants formulated using either the pure 15 kDa polymer or 29 kDa PLGA (Table 5.3). All implant formulations except for those formulated using only the 15 kDa polymer had a sensitivity greater than 1 within the linear range.

Table 5.3. Parameters evaluated for mathematical fit of phase inversion and drug release

<table>
<thead>
<tr>
<th>Variables</th>
<th>15kDa</th>
<th>29kDa</th>
<th>53kDa</th>
<th>15kDa:29kDa</th>
<th>15kDa:53kDa</th>
<th>29kDa:53kDa</th>
<th>In Vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td>$S_0$</td>
<td>79.4</td>
<td>31.9</td>
<td>25.3</td>
<td>24.1</td>
<td>27.6</td>
<td>29</td>
<td>1.0</td>
</tr>
<tr>
<td>$\tau$</td>
<td>0.2</td>
<td>0.6</td>
<td>0.8</td>
<td>0.7</td>
<td>1.0</td>
<td>1.2</td>
<td>-0.1</td>
</tr>
<tr>
<td>$m$</td>
<td>0.1</td>
<td>1.3</td>
<td>1.1</td>
<td>1.1</td>
<td>1.4</td>
<td>1.4</td>
<td>1.6</td>
</tr>
<tr>
<td>R²</td>
<td>0.97</td>
<td>.95</td>
<td>.99</td>
<td>.96</td>
<td>.99</td>
<td>.98</td>
<td>.99</td>
</tr>
</tbody>
</table>

5.3.6. Erosion and Degradation

A rapid initial decrease in mass occurred for all implant formulations, with an average mass loss of $53\pm1.3\%$ within 24 h after injection into the bath-side solution. Implants formulated with 15 kDa PLGA had the highest rate of erosion, reaching a residual mass
of 38.0±2.1% within 5 d in buffer (Figure 5.7).

Erosion was a slower process for all other polymer formulations, requiring 7 d to achieve a residual mass lower than 40% for the 15kDa:29kDa polymer implants (35.3±1.0%), 8 d for the 29 kDa implants (37.6±2.8%), 10 d for both the 15kDa:53kDa blend and the 29kDa:53kDa blend (37.7±2.2% and 37.2±1.2%), and 12 d for the 53 kDa implants (35.0±0.5%). Polymer degradation was faster in larger Mw polymers and decreased with the initial Mw (Figure 5.8).

5.3.7. In Vivo Formation and Drug Release

Polymer precipitation occurred rapidly, reaching a plateau within the first 4 h of implantation, with negligible changes occurring for the remainder of the study (Figure 5.9). The average gray-scale value was significantly lower in vitro than in vivo over all
time points, and followed a similar trend as
phase inversion (Figure 5.10). The release of
fluorescein was significantly higher in vivo
than in vitro releasing 25.3±2.6% of the
mock drug released within the first hour after
implantation, increasing to 42.0±4.5% after 4
h, with significantly less release occurring
during the following 4 h. A secondary burst
of drug was released between 8 h and 24 h
after implantation, with negligible changes
occurring for the duration of the study.

Correlation of the burst release and phase inversion resulted in an initially linear
relationship, with a sensitivity greater than 1 (Table 5.3).
5.4. Discussion

ISFI systems provide a minimally invasive platform for the sustained local delivery of drugs, while concurrently improving patient compliance and minimizing fabrication costs. Due to the inherent flexibility of this implant system, any number of therapeutic agents can be administered and loading can easily be customized. In order to better tailor the implant system for the desired application, it is important to understand the role of implant composition, particularly the Mw of the polymer used in the ISFI. The purpose of this study was to evaluate the effect of blending various Mw polymers to develop implants with customizable intermediate properties.

Since ISFIs can be characterized noninvasively with ultrasound, the transition of ISFIs from a liquid solution to a solid drug eluting depot could be observed longitudinally in a single implant. All implants initially formed a polymer shell immediately after contact with an aqueous environment, followed by a more gradual transition within the interior of the depot. In all cases, the implants formed a pore within the center of the depot that increased in diameter over time (Figure 5.1). The formation of macrovoids has been reported in asymmetric membranes manufactured using phase inversion with solvents that are highly miscible with the nonsolvent [33-37]. In the case of asymmetric membrane formation, the phase inversion occurs in two steps [36]. First, a dense polymer shell forms that creates a barrier for diffusion, then the rate of phase invasion slows down leading to the development of counter diffusion gradients between the solvent and nonsolvent [36]. This process leads to the development of macropores within these membranes as the less stable solvent-rich interior separates into polymer lean domains and more stable polymer rich regions [36]. In the case of ISFIs using NMP (a
highly water miscible solvent), we can see the rapid formation of the shell, followed by
the slower development of echogenic signal within the interior of the implant. Therefore,
the central pore observed may be the formation of a macrovoid within the center of the
implant at the location where the solvent concentration is the highest.

A consequence of the polymer shell and the implant size is the potential for
development of a pH gradient in addition to the solvent gradient. It has been reported
that when implants are large and fabricated using bulk eroding polymers, the interior of
the implants degrades more rapidly than the portion exposed to the bath-side solution
[38-40]. Since the polymer shell is in constant contact with the bath-side, the newly
formed acidic oligomers from the degrading polymer shell are rapidly lost to the
surrounding solution [38, 40]. Conversely, the acidic products formed within the interior,
are not readily released to the exterior solution due to the low diffusivity of the polymer
shell. This difference in oligomer retention results in the development of a pH gradient
that leads to the autocatalysis of the polymer within the implant interior and an increase
in implant osmolarity [38-41]. The presence of these degradation products would
ultimately lead to the complete solidification of the phase inverting implant by increasing
the diffusion of water into the interior, which would stabilize the inner most domains.
While the initial pore formation may be a result of a solvent gradient, the dynamic nature
of the central pore is most likely a function of the polymer autocatalysis.

As with the formation of the central pore, phase inversion was also a function of
the polymer Mw. The effect of polymer Mw on phase inversion has been hypothesized
to be a result of implant osmolarity, polymer hydrophobicity, affinity for solvent, and a
decreased diffusivity of the outer shell [10, 30, 42]. Therefore, implants formulated using
lower Mw PLGA phase invert more rapidly than implants formulated with higher Mw polymers. As a consequence of the relationship of Mw to phase inversion, implants formulated using blends had phase inversion profiles that were intermediate to the pure polymer types (Figure 5.2). Additionally, polymer swelling was inversely related to the polymer Mw, with the blends having intermediate swelling profiles relative to the pure polymer types. The only exception being the 15kDa:53kDa blend, which had a swelling profile comparable to the 53 kDa PLGA implants (Figure 5.3). This difference in swelling behavior could be a result of residual solvent concentration within the polymer changing the initial implant osmolarity, a result of shell diffusivity, or a consequence of polymer hydrophobicity.

When the implant erosion was evaluated, there was an initial rapid release of solvent leading to an elevated mass loss during the first 24h for all polymer formulations. The erosion profile is in stark contrast to what is observed from preformed polymer implants where only negligible changes in the implant mass occur initially followed by a period of rapid erosion. After initial solvent loss, a similar process is observed with ISFIs, in that the onset of erosion (residual mass less than 39% of the initial mass) is delayed. The lag time for erosion increased with Mw, with the blends having intermediate erosion profiles relative to implants formulated using the pure polymer (Figure 5.7).

With preformed systems fabricated using bulk eroding polymers, the onset of erosion has been explained as an effect of the percolation threshold. In percolation theory, the polymer is treated as a lattice of interconnected points [38, 43]. Over time, as the oligomers are lost to the bath-side solution, the interconnectivity of the lattice is lost.
This results in a critically low number of nodes within the lattice, and elevated pore interconnectivity [38, 43]. Erosion begins when the interconnected network of pores leads to the surface of the implant, which results in the rapid release of the internal degradation products to the solution. We hypothesize that this process is also occurring with the ISFIs [38]. Interestingly, the onset of erosion corresponds to both the final solidification of the depots, and the sharp increases in the rate of drug release, which we speculate to be a result of the percolation phenomenon. Polymer degradation occurred more rapidly than anticipated, which we speculate may be a result of the implant size, an effect of the residual solvent, or even the elevated surface area due to the highly interconnected porous interior. The rate kinetics increased with Mw, which is in agreement with the findings of Wu et al., who hypothesized that the elevated degradation kinetics were a result of an increased number of reaction sites on the larger polymers [44].

The effects of polymer Mw on release were more complex than anticipated. Burst release from implants appeared to be controlled by the polymer with the higher burst. While, diffusion release was anticipated to be an average of the polymers used, the release profile instead appeared to closely follow the behavior of the polymer with the higher Mw. Finally degradation facilitated release appeared to be controlled by the lower Mw polymer. It was also observed that as the polymer ratio approached 10:1, intermediate behavior was lost (data not shown). After the degradation phase of release, a lockdown occurred in all implants, which we hypothesize may be a result of latent crystallization of the implants as a result of polymer degradation, resulting in a decrease in diffusivity and an increase in affinity for the drug and polymer [45].
Due to the high initial rate of phase inversion relative to drug release, a nonlinear relationship was developed to correlate drug release and phase inversion [32]. A rapid transition to linear behavior occurred for all polymer formulations except for implants containing the 15 kDa PLGA (Figure 5.10). When implants were formed in vivo, the nonlinearity was lost, and both phase inversion and release occurred rapidly. The elevated rate of release has previously been observed, but the use of the 15kDa:53kDa polymer blend resulted in a statistically lower overall release when compared with previous studies using 29kDa PLGA implants. These findings may be a result of lower initial swelling of the polymer blend.

5.5. Conclusion

ISFIs provide a unique platform which is simple to manufacture, has a diverse range of potential applications, and can be administered through a minimally invasive injection and monitored using medical imaging. In this study, molar ratios of the polymer used in the implant formulation were altered so that the effects of matrix composition on implant behavior could provide an alternative means by which the release from these depots can be tailored to fit a desired application. Results confirmed that intermediate implant behavior could be achieved by altering the molar ratio of the polymers used in the depots. Evaluation of polymer erosion data along with concurrent visualization of the implants provided insight into the longitudinal effect of autocatalysis and changes in the outer shell diffusivity on drug release, swelling, and phase inversion. Noninvasive characterization of implants using ultrasound imaging was able to provide unique insight into the behavior of these systems in situ, which may not only be used to
evaluate ISFI systems, but may be extended to preformed polymer systems as well. Advances in these techniques such as the ability to monitor the implants in 4 dimensions (X,Y,Z, and time), may substantially improve characterization. Currently, the parameters that alter the release kinetics in vivo are poorly understood, but through the use of medical imaging, the multivariable system may be simplified. Evaluation of solvent exchange using MRI, or SPECT/PET analysis to evaluate macrophage activity may lead to improved understanding as to what effect the host has on implant behavior in situ.

5.6. References


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Chapter 6: Effect of Injection Site on In Situ Formation and Drug Release In Vivo

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Luis Solorio*, Ravi B. Patel*, Hanping Wu, Tianyi Krupka, and Agata A. Exner
*Authors contributed equally to this work
6.1. Introduction

The ISFI systems have several advantages compared to traditional pre-formed implant systems. As discussed in previous chapters, because of their injectable nature, implant placement is less invasive and painful for the patient thereby improving comfort and compliance. Additionally the manufacturing process required for fabrication is relatively mild and straightforward making ISFI systems particularly suitable for delivery of fragile protein and peptide therapies. However, due to the complexities of the phase inversion process, poor in vitro – in vivo correlations, potential solvent toxicity [1], and difficulty in controlling burst drug release [2], relatively few products using this delivery system have made it to market.

In the present study, release of a model drug agent, fluorescein, from varying Mw PLGA formulations was examined in both in vitro and in vivo environments. Fluorescein has a similar low Mw (376.28 Da) and hydrophilicity to other active chemotherapeutic agents such as carboplatin (371.25 Da). Additionally, using a nontoxic model drug agent allowed us to test multiple implants within a single animal without incurring toxicity, thereby reducing the total number of animals needed for in vivo experimentation. Since the composition of the external environment, in this case the tissue surrounding the ISFI, can greatly affect phase inversion and drug release, implant behavior was examined in a variety of in vivo environments including in subcutaneous (SC), non-necrotic tumor, and necrotic tumor tissue. In addition, drug release from ISFIs implanted into tumors treated with radiofrequency (RF) ablation therapy was examined. Application of chemotherapeutic drug delivery implants as adjuvant chemotherapy post ablative treatment has been an area of significant research interest in recent years, and the
potential for ISFIs to be used for this application was explored [3-5]. To correlate the dependence of drug release with the phase inversion process in an unperturbed \textit{in vivo} environment, a novel ultrasound implant imaging method recently described by Solorio et al. was utilized [6]. Finally scanning electron microscopy was used to examine the structure and morphology of phase inverted implants in differing \textit{in vitro} and \textit{in vivo} environments. Results from this study should lead to an improved understanding of the behavior of ISFI systems in a variety of environments and provide additional insight into the mechanisms leading to the discrepancy between \textit{in vitro} and \textit{in vivo} implant formation and drug release.

6.2. Methods

6.2.1. Materials and polymer solution preparation

ISFI polymer solutions were comprised of poly(D,L-lactide-co-glycolide) (PLGA) with a 50:50 lactide/glycolide (L/G) ratio from Lakeshore Biomaterials (Birmingham, AL), 1-methyl-2-pyrrolidone (NMP) from Sigma (St. Louis, MO), Pluronic P85 (P85, Mw: 4600 Da), a tri-block co-polymer of PEO-PPO-PEO donated by BASF Corp. (Shreveport, LA), and sodium fluorescein (Sigma), which was used as a mock low Mw drug molecule (Mw 376.28). All solid components of the mixture were dissolved in the NMP solvent overnight at 37°C in an orbital incubator-shaker (New Brunswick Scientific, C24) at 80 rpm and stored at 4°C after a homogenous solution was obtained. The relative mass percentage of each component in polymer solution was 60/36.5/2.5/1 for NMP, PLGA, P85, and fluorescein respectively. To examine the effect
of PLGA Mw on drug release, three different formulations comprised of PLGA were prepared with the PLGA polymer having a mean Mw of 18, 33, and 50 kDa.

6.2.2 In vitro drug release

In vitro drug release from varying Mw ISFI polymer formulations was examined using two different methods. The first method utilized a typical continuous, cumulative drug dissolution study which has been previously described, and shall be further referred to as the continuous time point (CTP) release method [7]. Briefly, 50 μl of polymer solution for each formulation (n=4) was injected into a bath solution of 12 ml of PBS (pH 7.4) and placed in an orbital incubator-shaker set at 37°C and 80 rpm. The bath solution was then sampled (100 μl) at several experimental time points (ETPs) (1 h, and 1, 2, 4, and 7 days) post injection to determine drug release profiles. The concentration of drug released into the bath solution samples was quantified using a fluorescence plate reader (Tecan Ltd., Infinite 200 series) with an excitation/emission (Ex/Em) wavelength of 485/535 nm and referenced to a standard curve. The sensitivity, accuracy, and linearity of fluorescence quantification were 20 pM fluorescein, 98%, and 99%, respectively. Cumulative drug release was calculated by normalization to the total injected drug mass determined by post experimental degradation of the implant. For this method, cumulative drug release was monitored for all time points for each trial with the same implant.

The second method was similar to in vivo drug release trials and will be referred to as the discrete time point (DTP) drug release method. 50 μl of polymer solution for each formulation was injected into a 12 ml bath solution of PBS (pH 7.4) and placed in an orbital incubator-shaker set at 37°C and 80 rpm. However instead of sampling the
bath solution at each ETP the implant (n = 4 per time point) was removed from the bath solution and degraded in 2 ml of 2 M NaOH for 24 h. Degraded implant solutions were then neutralized and diluted with PBS (pH 7.4), and drug concentration was calculated using a fluorescence plate reader and standard curve for reference. Cumulative drug release was calculated by normalizing the mass of drug remaining in each implant to the initial drug loading of the implant.

6.2.3. In vivo subcutaneous drug release

Drug release from ISFIs in a SC environment was examined in fifteen 11 week old male BD-IX rats (average body weight 320 g, Charles River Laboratories Inc., Wilmington, MA). All experimental protocols were approved by the Case Western Reserve University Institutional Animal Care and Use Committee (IACUC). Rats were anesthetized with 2% isoflurane and an O2 flow rate of 1 L/min (EZ150 Isoflurane Vaporizer; EZ Anesthesia, Palmer, Pa). Five 50 μl implants loaded with 1% fluorescein were injected and distributed evenly under the dorsal SC skin-fold of each animal. For each sampling time point, implants comprised of each varying Mw PLGA polymer formulation (n=5), were distributed across 3 animals. At each ETP post implant injection, animals were euthanized, and the implants were removed and degraded in 2 M NaOH solution. The degraded implant was neutralized, diluted in PBS at pH 7.4, and the fluorescence of drug remaining in the implant was quantified using a fluorescence plate reader as described in the previous section. The cumulative drug released from the implant was calculated by using the known implant drug loading mass and normalized for any small variations in injection volume as determined by experimental measurement.
6.2.4. Tumor inoculation and intratumoral drug release

Drug release from ISFIs in tumors was examined in ten 11 week old male BD-IX rats (average body weight 330 g, Charles River Laboratories Inc., Wilmington, MA) with SC rat colorectal carcinoma cell line (DHD/K12/TRb) tumors. Cells for tumor inoculation were prepared using a previously described protocol and were inoculated into animals 6 weeks prior to implant treatment [3]. Two tumors were inoculated bilaterally, approximately 1 cm inferior to the scapula and 1 cm lateral to the midline, while the other two tumors were bilaterally inoculated approximately 1 cm superior to the iliac crest and 1 cm lateral to the midline. Once tumors had fully matured, rats were anesthetized, and the tumor site was prepared by shaving and cleansing with alcohol and iodine. Tumor size was measured by calipers (R.B.P.), and the average tumor diameter was found to be 1.16 ± 0.16 cm for all groups. Any tumors that were not developed by this time or remained too small to be injected with implant were excluded from the study. Remaining animals received an injection of 50 μl of 33 kDa PLGA ISFI solution loaded with 1% fluorescein. To determine drug release from ISFIs in tumor tissue, implants were removed at 1 h (n=6), 1 d (n=6), 2 d (n=6), 4 d (n=4), and 7 d (n=6) post injection. Drug release was quantified as described in Section 6.2.3. Post mortem location of the implant was qualitatively examined to determine whether it was located in a necrotic cavity or within a non-necrotic tissue space and compared with earlier notations.

6.2.5. Tumor ablation and drug release in ablated tumors

In addition to measuring drug release from ISFIs in the SC and intratumoral environments, release into ablated tumor tissue that has undergone coagulative necrosis
was examined [8]. DHD/K12/TRb rat colorectal carcinoma tumors were inoculated and grown to a mean diameter of $1.20 \pm 0.42$ cm in ten 11 week old BD-IX rats using the protocol described in the previous section. Animals were then once again anesthetized, and the tumor area was shaved and sterilized for RF ablation treatment. A modified 1 cm exposed tip 20-gauge non-internally cooled electrode was inserted into the tumor mass and a monopolar RF current was applied to the tumor using a 480-kHz RF generator (3E; Radionics, Burlington, Mass) and an abdominal grounding pad. Tumors were ablated with a power of 2-4 watts with a constant electrode temperature of $80^\circ$C for 3 minutes. After ablation, the electrode was removed, and 50 μl of 33 kDa PLGA ISFI solution was immediately injected into the ablated tumor volume through the ablation track. Implants were removed at each ETP post treatment and analyzed using the method described in Section 6.2.3.

6.2.6 In vivo ultrasound implant formation imaging

Implant formation was examined using a previously established ultrasound technique [6]. Briefly, animals were anesthetized, and each implant was imaged using a 12 MHz PLT-120 transducer on a Toshiba Aplio SSA-770A diagnostic ultrasound machine with the following parameters: dynamic range of 55, mechanical index of 1.1, gain of 80, and 3 cm depth (Figure 6.1A). At least five ultrasound still images of each implant in their respective tissue environment were taken at each ETP.
6.2.7 Image analysis

Ultrasound image analysis of *in vivo* implant formation was conducted using a methodology recently developed by Solorio et al [6]. Briefly for each condition, ROIs from five implant images (Figure 6.1B) were manually segmented, and a parametric segmentation technique utilizing the method of mixed Gaussians was used to find a threshold value to remove low intensity noise from each implant image (Figure 6.1C). The summation of the number of pixels in the selected ROI was used to calculate the total implant area (Figure 6.1D). Next the implant area that had solidified into solid matrix, pixels above the set threshold was found (Figure 6.1E). The percent precipitation of the implant was calculated by finding the ratio of the number of pixels above the threshold, to the total number of pixels in the implant area. The percent of implant precipitation was found for all ETPs.

6.2.8 Scanning electron microscopy

The structural morphology of each implant formulation *in vivo* and *in vitro* was examined using scanning electron microscopy (SEM). *In vitro* and *in vivo* implant samples were prepared by injecting 50 μL of each polymer formulation into either a PBS bath or SC tissue respectively. Implant samples were then removed from their bath.
solution at one (n=3) and seven (n=3) days post injection, freeze fractured in liquid nitrogen, and subsequently lyophilized for 24 h. All prepared samples were then bonded to an aluminum stub and sputter coated with 5 nm of Pd. SEM micrographs were then obtained using a Quanta 200 3D ESEM with an acceleration voltage of 2 kV and a hole size of 3.5.

6.2.9. Statistical Analysis

A one-way ANOVA test was used to determine statistically significant (p<0.05) differences between experimental groups. Specific differences between groups were identified using a Tukey multi comparison test. All statistical tests were carried out with MatLab using the statistics tool box. Errors were reported as +/- standard error of the mean (S.E.M.).

6.3. Results

6.3.1. In vitro drug release

The accuracy of obtaining a drug release profile by degrading several different implants at various time points after injection into a bath solution versus sampling the bath solution from a single implant dissolution study was examined in vitro. The results from both methods show that low Mw PLGA implants released drug slower than high Mw PLGA implants during the burst

Figure 6.2. The cumulative percent of the loading fluorescein mass released from varying Mw PLGA ISFIs in vitro is shown using the CTP (A) and DTP (B) release study methods.
release phase (0-24 h) (Figure 6.2, Table 6.1). Statistically significant differences seen between implant groups are similar for both drug release methods with low Mw 18 kDa PLGA implants having a significantly lower percent of drug release at the 1 h (p<0.001) and 24 h (p<0.01) compared to higher Mw 50 kDa PLGA implants. However after 24 h, the drug release from 18 kDa PLGA ISFs was no longer significantly different than the other implant formulations, with no further significant differences occurring for the duration of the study. The 33 kDa PLGA formulation exhibited a significantly lower (p<0.01) release at 1 h compared to the high Mw 50 kDa PLGA samples for both release methods. No other significant differences were noted for the 33 kDa PLGA formulation.

To examine the similarity of drug release profiles obtained from each method, a correlation coefficient value correlating the drug release profile obtained from the CTP and DTP methods, R², was found to be greater than 0.9 for all groups.

6.3.2. In vivo drug release and implant formation

Drug release from varying Mw PLGA implants was examined in vivo in a SC environment. Here, drug release displayed a trend opposite of that observed in vitro. Namely, low Mw PLGA implants released drug at a faster rate than high Mw PLGA implants in SC tissue.
ISFIs (Figure 6.3A). Low Mw 18 kDa PLGA implants released a significantly (p<0.05) greater percent of their initial loading dose than higher Mw 33 and 50 kDa PLGA implants at 1 h, 1, 2, and 4 d. Additionally, the burst release at 24 h from in vivo implants was significantly greater than that of implants in vitro for 18 and 33 kDa PLGA polymers.

To examine the effect of implant formation on drug release, ultrasound was used to quantify implant phase change. These formation studies showed that the larger Mw 33 and 50 kDa PLGA implants have a lower rate of matrix precipitation than the lower Mw 18 kDa PLGA implants (Figure 6.3B). The 33 and 50 kDa PLGA implants have a significantly lower (p<0.05) percentage of solidified matrix during the burst drug release time points (1 h, 1 d). In addition when implant precipitation was correlated to drug release for all implant formulations, the correlation coefficient was found to be greater than 0.92 for all cases (Figure 6.3C). Finally the total implant area was examined for each PLGA formulation, and results show that implant cross-sectional area decreased over time for all formulations (Figure 6.3D).

6.3.3. Intratumoral drug release and implant formation

The mean drug release from implants injected into solid tumors was slightly faster than

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**Figure 6.4.** The cumulative percent of the loading fluorescein mass released from 33 kDa PLGA ISFIs in varying in vitro and in vivo environments (A) is shown. Differences in drug release from implants in necrotic and non-necrotic tumors is also displayed (B).
release observed in a SC environment (Figure 6.4A). However, due to the large variability in tumor tissue density with several implants having been injected into a necrotic tumor cavity while others were injected into very dense tumor tissue, the release profile in tumors was quite variable. When the drug release profile was further refined into necrotic tumor and non-necrotic tumor groups, it was seen that drug release in necrotic tumor was slower than in non-necrotic tumor (Figure 6.4B). Significant differences (p<0.05) between non-necrotic and necrotic tumor implant groups were observed at 2 and 4 days post injection.

The rate of formation in tumors was also found to be slightly faster than in an \textit{in vivo} SC environment (Figure 6.5). However, tissue scabbing of some tumors resulted in low image quality, resulting in a reduced number of trials and an inability to separate the data sets into necrotic and non-necrotic subgroups. Nonetheless, when the tumor formation curve was correlated to intratumoral drug release a correlation coefficient of 0.95 was found.

6.3.4 Ablated tumor drug release and implant formation

Drug release from implants injected into tumors treated with RF ablation was investigated as well. No significant differences were noted between drug release from ISFIs in ablated tumors in comparison to unablated tumors at time points greater than 1 h (Figure 6.4A). However, the 1 h burst release in
ablated tumors was significantly greater than that seen in all other *in vivo* environments. Yet within 24 h, all other trials except for necrotic tumor had reached a similar level of release as ablated tumor, and no significant differences were found between groups at time points greater than 1 h. Additionally, ISFIs implanted in ablated tumor had a higher percent of formation at the 1 h time point in comparison to unablated tumors (Figure 6.5). The correlation, $R^2$, between drug release and implant formation in tumors was found to be .93.

### 6.3.5. Scanning electron micrographs of *in vivo* and *in vitro* implants

*In vitro* and *in vivo* implant samples were prepared for SEM using implant samples injected into their respected environments and retrieved 1 day and 7 days post injection. Due to incomplete implant precipitation and likely a large residual solvent concentration after one day post injection, *in vitro* lower Mw 18 and 33
kDa PLGA implants were destroyed during the freeze-drying process of SEM sample preparation. However, \textit{in vitro} higher Mw 50 kDa PLGA implants were sufficiently precipitated at this same time period and their structure could be compared \textit{in vivo} implant samples at day 1. Qualitative observation of these implants shows that matrix morphology of \textit{in vitro} implants is more homogenous than what is observed \textit{in vivo} (Figure 6.6). In addition the overall porosity of \textit{in vitro} implants is greater than \textit{in vivo} 50 kDa PLGA implants at one day. By day 7, all \textit{in vitro} and \textit{in vivo} implant samples had sufficient time to precipitate enough to survive sample preparation. In contrast to the 1 day 50 kDa PLGA samples, \textit{in vitro} implants had a comparable porosity to \textit{in vivo} implants. Moreover the \textit{in vitro} lower Mw 18 kDa PLGA implants had a greater porosity than the higher Mw 50 kDa PLGA implants, however the \textit{in vivo} implants of both these formulations had a similar level of porosity (Figure 6.7).

6.3.6. Implant shape in varying \textit{in vivo} environments

\textbf{Figure 6.8}. Photographs (A-E) of 33 kDa PLGA ISFIs at day 7 post injection \textit{in vitro} (A, F) and \textit{in vivo} SC (B, G), necrotic tumor (C, H), non-necrotic tumor (D, I), and ablated tumor (E, J) environments and their corresponding cross-sectional area using US imaging (F-J)

In addition to the bath side components such as dissolved proteins, salts, and acids present in the external tissue surroundings, another factor that likely affects implant
formation and drug release is the final shape of the injected implant in different environments. For example, in an in vitro environment our implants conformed to a uniform spherical shape (Figure 6.8A), while in an in vivo SC environment the injected implant had a flat disc like shape upon removal (Figure 6.8B). Intratumoral implants had a variable shape that was globular (Figure 6.8C + 6.8D) and sometimes consisted of multiple lobes. Additionally the cross sectional area of implants in a necrotic cavity (Figure 6.8C) was greater than that of implants in a non-necrotic tumor location (Figure 6.8D). Finally, since implants in ablated tumors were injected directly into the ablation track formed during RF treatment, these implants conformed to a rod-like shape that filled the ablation track cavity (Figure 6.8E). Ultrasound images of the cross-sectional area of implants in each environment also show how different environmental effects have on implant shape (Figure 6.8F-J).

6.4. Discussion

In vitro drug dissolution studies have shown that release from ISFIs is dependent on a variety of factors such as solvent type [9], polymer Mw [7], excipient factors [2], and external bath side effects [10]. However the effect these differing variables have on the rate of implant formation may hold the key to understanding the variability of drug release from ISFIs. Implant formation or precipitation occurs by a process of solvent exchange whereby as water influx into the implant occurs and water miscible solvent leaves the system, the hydrophobic polymer will precipitate out of solution. Previous studies by Graham et al, have shown that in vitro implant formation examined using dark ground imaging (DGI) correlated to observed drug release from differing ISFI
formulations with slower forming implants releasing drug slower than faster forming implants [10]. The proposed reason for this effect as hypothesized by McHugh et al., is that the mobility of the drug agent is reduced in the more viscous interior non-phase inverted core than in the porous water rich precipitated matrix shell [11]. Therefore the solvent efflux increases and implant precipitation increases, drug dissolution and release would thereby be increased. In our current study, we found the rate of implant precipitation to be strongly correlated, (R^2>0.90) for all cases, to the rate of drug release. Because of this relationship, ultrasound imaging of the implant formation process can be a useful technique to noninvasively monitor and predict drug release in a variety of in vivo environments [6]. This technique may be particularly beneficial for monitoring ISFIs injected into cancerous lesions due to the high degree of inter-patient tumor variability and differences in tissue density and architecture. In the current study, the external environmental effects on ISFIs in a variety of in vitro and in vivo surroundings were examined.

First, drug release from ISFIs in varying in vivo environments was compared to their release in vitro. For all in vivo tissue environments, drug release from ISFIs was found to be significantly greater than their corresponding in vitro release. In addition, the rate of implant formation was found to correlate with the rate of implant drug release with in vivo ISFIs solidifying much quicker than what has previously been shown in vitro [6]. While this finding is consistent with previous studies using ultrasound to examine implant formation [6, 12], other studies using electron paramagnetic resonance (EPR) to determine the rate of implant formation have found little difference between in vivo and in vitro implant formation [12]. One reason for the difference seen between our method
and the one used for EPR may be due to the use of a spin-probe in EPR technique that may affect the phase inversion dynamics of the ISFI polymer solution [12]. In vitro implant formation studies using dark ground imaging (DGI) have also shown similar results to ours with slower forming implant formulations releasing their drug slower than faster forming ISFIs [9]. However while implant formation has been correlated in vitro and in vivo using EPR and ultrasound, studies directly correlating drug release from these ISFIs in a similar manner have not been conducted to our knowledge. Previous examination of similar implants types in vivo have only measured serum plasma drug levels with several studies concluding that in vivo release was in good agreement with release profile seen in vitro [13, 14]. Since serum plasma drug concentrations are dependent on several physiological factors such as drug adsorption, metabolism, and clearance that occur after the drug is released, a direct comparison between in vitro and in vivo environments is difficult. Therefore this may account for any discrepancies seen between our results and previous findings in the literature [12].

Concurrently, the effect of polymer chain Mw on drug release was investigated in both an in vitro and a SC in vivo environment. However since there are significant difficulties in performing a continuous release study in vivo, first the accuracy of an adapted method (discrete time point method, DTP) was tested in comparison to a traditional continuous cumulative release study. For the DTP method, the release profile was obtained by determining the percent of drug remaining in implants removed from bath solution and degraded at each time point. The in vitro results described in Section 6.3.1 show that the DTP method has a release profile comparable to that obtained using a more traditional continuous release method. The results from these experiments also
corroborate previously established findings whereby lower Mw PLGA ISFI formulations release drug slower than higher Mw ones [15]. Interestingly, the opposite trend was seen when these same formulations were injected into an in vivo SC environment with higher Mw PLGA formulations releasing drug slower than lower Mw ones. While data in the literature on the effect of polymer Mw on in vivo drug release is limited, a study by Ravivarapu et al. has found a similar trend whereby lower Mw PLGA ISFIs injected into the SC skin fold of canines released drug faster than higher Mw implants as measured by plasma drug concentration [14].

The contradictory effect of polymer Mw on drug release in vivo and in vitro is a perplexing finding; however examination of implant formation in these two environments may provide some answers. In vivo (Section 6.3.2) the higher Mw 33 and 50 kDa PLGA formulations precipitate significantly slower than the lower Mw 18 kDa PLGA formulation. However, a study by Solorio et al. using similar US formation methods found that in vitro, 18 kDa PLGA implants formed slower than 50 kDa PLGA implants [20]. Moreover the authors found that 18 kDa PLGA implants underwent greater swelling resulting in a significantly greater volume expansion over time compared to higher Mw 50 kDa PLGA implants in vitro, and in some cases would even burst through agarose tissue phantoms when embedded inside them [6]. This in vitro swelling effect suggests that there is greater water influx than solvent efflux, and lower Mw 18 kDa PLGA implants may retain a greater percent of their solvent and drug than higher Mw 50 kDa implants which have a faster formation and release rate. Conversely, this expansion effect was not seen in vivo with all implants having a declining total cross-sectional implant area over time as determined by US imaging. These findings suggest that there is
a decoupling between the rate of water influx and solvent efflux from the implants, with solvent ejection occurring faster than water movement into the implants resulting in the decrease in total implant cross-sectional area. Implants that retain solvent will likely precipitate slower and thereby have slower drug release than implants which form faster as described above. Lending credence to this hypothesis is the fact that in vivo implants survived SEM preparation, while 18 and 33 kDa in vitro implants dissolved in the residual solvent once the water had been removed from the implant following lyophilization. Meanwhile, the in vitro PLGA 4A sample and all of the 1 day in vivo samples were able to withstand processing for SEM suggesting that these implants had a low concentration of residual solvent at this time point and were more fully precipitated. The greater porosity of in vitro implants in comparison to their corresponding in vivo counterparts shown by SEM in Section 6.3.5 also suggests that in vitro implants expand and swell more than in vivo implants. Moreover, previous studies examining phase separation of varying Mw PLGA polymeric implant solutions have shown that lower Mw PLGA polymers undergo a two-part phase inversion process whereby these polymers first form into a soft hydrogel and later solidify into a stiffer polymer matrix [10]. Studies by Patel et al. have also shown that in vitro higher Mw PLGA polymers have a lower solubility than lower Mw PLGA polymers, and a lower critical water concentration threshold is required to induce their precipitation out of solution [7]. The ability of low Mw PLGA implants to retain their solvent, solubilized polymer, and drug in vitro may lead to a persistent osmotic effect that results in implant swelling.
Figure 6.9. Deviation of in vivo–in vitro drug release from varying Mw ISFIs over time.

\[
\text{Dev} = \frac{\% \text{ in vivo release} \ (t) - \% \text{ in vitro release} \ (t)}{\% \text{ in vitro release} \ (t)}
\]

In vivo, the ability of the implant to expand is likely limited due to interstitial pressure or compressive forces exerted on the implant by the surrounding tissue. Therefore due to a mechanically induced convective force, caused by the in vivo environment, solvent and drug efflux are prone to be increased, and the rate of implant formation and drug release are greater than the in vitro counterparts. This hypothesis is further supported by comparing the burst release of the in vitro and in vivo for the high Mw implants. During the first hour post injection the in vivo high Mw PLGA implants show only a modest increase in the amount of drug released relative to the in vitro implants (Figure 6.9). This effect is presumably due to limited swelling tendency of the higher Mw implants during this time [4]. In contrast, lower Mw PLGA implants have been shown to swell and expand much more significantly than high Mw PLGA implants post injection into an aqueous phase [4]. Consequently, the convective force created by the mechanical pressure of the dorsal skin fold pushing on the swelling implant causes a larger mass of drug to be released immediately after injection in vivo. This trend is lost over time as the implants continue to precipitate and the osmotic forces inducing implant expansion are reduced. The tendency of varying Mw PLGA implants to expand or compress in in vitro and in vivo environments is summarized in Figure 6.10A.
Finally drug release and implant precipitation of ISFIs in different in vivo tumor environments was examined. Due to differences in the external tissue environment of necrotic, non-necrotic, and ablated tumors a different drug release profile was seen for each. The drug release from necrotic tumors was very similar to drug release in subcutaneously placed implants. Although the implant shape and likely composition of the external interstitial fluid are different for both environments, both have a limited tissue density allowing the implant to expand. The mechanical pressure exerted by the dorsal skin fold on the implants resulted in their compression and flattening. Similarly, necrotic tumors have been shown to have high interstitial pressure [16], which can constrain the implant swelling or expansion similar to the force generated by the dorsal skin fold. In contrast, implants placed in non-necrotic and ablated tumors, which have a greatly increased tissue density and have been found to be much stiffer than normal tissue [17-19], exhibited a faster drug release (Figure 10B). The space available for these implants to swell and expand was even more restricted and resulted in smaller implant sizes. Surprisingly, implants placed in ablated tumors had a greater 1 h burst release than in non-necrotic tumor. However this may be explained by increased tissue stiffness, even in comparison to non-necrotic tumor tissue, and edema that has been shown to occur post ablative treatment [17]. In addition, since

Figure 6.10. The tendency of varying Mw PLGA implants to expand or compress in vitro and in vivo (A) and the effect of in vivo tissue compliance on drug release from ISFIs (B) is summarized.
implants were injected into ablated tumors only 1 min post ablation, residual heat at the injection site may play a role in implant formation and drug release behavior. By the 24 h time point, the drug release observed in non-necrotic tumors was similar to ablated tumor implants. Examination of implant precipitation for each \textit{in vivo} environment demonstrated that faster precipitating implants released drug faster than slower forming counterparts.

While the role of tissue mechanical forces remains our primary hypothesis to explain differences between in vitro and in vivo drug release from ISFIs, various other factors may play a role including the water content of the surrounding medium, implant shape and surface area, and tissue perfusion. However if the water content of the surrounding medium was playing a major role in the observed drug release profiles, it would be expected that implants placed in more water rich environments would precipitate and release drug faster. Therefore, in vitro ISFIs would be expected to precipitate the quickest and have the fastest drug release since this environment has the greatest water content. Similarly, if differences in tissue perfusion were the primary factor affecting drug release, release in the relatively avascular necrotic tumor cavity would be expected to be slower than in the more vascular subcutaneous space due to increased drug elimination and a larger gradient driving force for drug release. However in our studies, we found in vitro implant precipitation and drug release to be the slowest. In addition, no differences in behavior were found between implants placed either subcutaneously or in a necrotic tumor cavity.

As for implant surface area, qualitative observation of implant shape (Figure 6.8) did confirm that implants in SC tissue and ablated tumor tissue which were shaped as a
thin flat disk and long thin rod had a greater surface area to volume ratio in accordance with their faster drug release in comparison to spherically shaped in vitro implants. However implants in SC tissue (thin flat disc shaped) which exhibited similar behavior to intratumoral ISFIs (spherical or globular shaped), would be expected to have a greater surface area to volume ratio. Therefore while implant shape and surface area may contribute to differences in drug release from ISFIs placed in different tissue environments, our findings suggest that implant shape is constrained by the available space at the injection site. While the data presented in these studies has only hinted at this phenomenon, future work comparing release from ISFIs of similar shape in various constrained environments should be conducted to validate this hypothesis. Another limitation of this study is that release of only one agent, fluorescein was examined, and release behavior of other agents with differing solubility, Mw, or charge may be quite different. Since the focus of these studies was on environmental effects on drug release, only release of one agent fluorescein was examined, however release of other agents should be considered in future studies.

6.5. Conclusion

While modulation of drug release from ISFIs has incited significant research interest over the past decade, most in vitro drug release experiments have focused on the effects of drug type, polymer solution formulation components, and external bath side effects. Our findings suggest that in an in vivo environment, local compressive mechanical forces and interstitial fluid pressures may play an even greater role in determining drug release from ISFIs. This is especially true in the case of low Mw
polymer implants that have a greater tendency to swell and are inclined to precipitate slower \textit{in vitro}. Higher Mw polymer implants which have been shown to precipitate faster with a stiffer matrix and tend to swell significantly less than smaller Mw PLGA polymers can be used to mitigate the local environmental mechanical effects. Another interesting byproduct of ISFI behavior \textit{in vivo} that has not yet been explored but may be of significant interest for future studies is the potential use of these implants for convective drug transport. The tissue mechanical forces and interstitial fluid pressure acting on the implant increase solvent and drug efflux as well as implant precipitation and may also increase local tissue drug penetration. This process in a tumor volume could be of significant interest for local chemotherapeutic drug delivery.

6.6. References


Chapter 7: Conclusions and Future Directions

7.1. Conclusions and Implications in Image Guided Drug Delivery

Advancements in medical imaging technology have resulted in a growing number of new applications, including image guided drug delivery. This minimally invasive approach provides a comprehensive answer to many challenges with local drug delivery. Future evolution of imaging devices, image-acquisition techniques and multifunctional delivery agents will lead to a paradigm shift in patient care. These therapies are particularly useful to the field of interventional oncology, where elevation of tumor drug levels, reduction of systemic side effects and post-therapy assessment are essential. By using medical imaging techniques for drug delivery applications, a variety of diseases can be treated with minimal systemic involvement while concurrently monitoring treatment efficacy and the behavior of the delivery vehicle.

In Chapter 3, the development of a noninvasive technique for evaluating the \textit{in situ} behavior of phase inverting polymer systems using diagnostic ultrasound was described. Phase inverting polymer systems have historically been used for industrial applications, as asymmetric membranes for microfiltration of bacteria and reverse osmosis, but their use has rapidly expanded in other areas. In medicine, they have been used as matrixes for drug delivery and tissue engineering scaffolds. The use of phase inverting systems for the controlled release of therapeutic agents is of interest due to the injectable nature of the implants, which provides a less invasive means of physically placing the implant at or near the site of action. The release rate of drugs from these systems is often related to the rate of implant formation. Until the completion of this work, only a limited number of techniques were available that could monitor phase
inversion, and none of these methods could be used to visualize the process directly and noninvasively.

In this thesis, diagnostic ultrasound was used to visualize and quantify the process of implant formation in a phase inversion based system both in vitro and in vivo. Concurrently, sodium fluorescein was used as a mock drug to evaluate the drug release profiles and correlate drug release and polymer precipitation. Implants comprised of three different molecular weight poly(lactic-co-glycolic acid) (PLGA) polymers dissolved in 1-methyl-2-pyrrolidinone (NMP) were studied in vitro and a 29 kDa PLGA solution was evaluated in vivo. The implants were encapsulated in a 1% agarose tissue phantom for five days, or injected into a rat subcutaneously and evaluated for 48 hrs. Quantitative measurements of the gray-scale value (corresponding to the rate of implant formation), swelling, and precipitation were evaluated using image analysis techniques, showing that polymer molecular weight has a considerable effect on the swelling and formation of the in situ drug delivery depots. A linear correlation was also seen between the in vivo release and depot formation (R²=0.93). Chapter 3 demonstrated, for the first time, that ultrasound can be used to noninvasively and non-destructively monitor and evaluate the phase inversion process of in situ forming drug delivery implants, and that the formation process can be directly related to the initial phase of drug release dependent on this formation.

It has been reported that the kinetics of this phase transition have a direct effect on drug release, and can be altered through factors that change the mass transfer events of the solvent and aqueous environment, including properties of the entrapped active agent. In Chapter 4, the effect of payload properties on implant phase inversion, swelling, drug
release, and polymer degradation were evaluated. Poly(DL-lactide-co-glycolide) implants were loaded with either: sodium fluorescein, bovine serum albumin (BSA), doxorubicin (Dox), or 1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate (DiI).

Fluorescein and Dox were released at near equivalent rates throughout the diffusion phase of release but due to differing drug-matrix interactions, Dox loaded implants released a lower mass of drug during the degradation phase of release. DiI was not readily released, and due to increased depot hydrophobicity, resulted in significantly lower swelling than the other formulations. The initial echogenicity was higher in Dox loaded implants than those loaded with fluorescein, but after the initial precipitation, phase inversion and drug release occurred at near equivalent rates for both Dox and fluorescein loaded implants. Nonlinear mathematical fitting was also used to correlate drug release and phase inversion, providing a noninvasive method for evaluating implant release ($R^2 > 0.97$ for Dox, BSA, and fluorescein; DiI had a correlation coefficient of 0.56), which provides a noninvasive means for approximating the release of drug during phase inversion from these implants.

Since precise, reproducible control of drug release from ISFIs is essential to their successful clinical application. Chapter 5 investigated the effect of varying the molar ratio of different molecular weight (Mw) poly(D,L-lactic-co-glycolic acid) (PLGA) polymers within a single implant on the release of a small Mw mock drug (sodium fluorescein) both in vitro and in vivo. Implants were formulated by dissolving three different PLGA Mw (15, 29, and 53kDa), as well as three 1:1 molar ratio combinations of each PLGA Mw in 1-methyl-2-pyrrolidinone (NMP) with the mock drug fluorescein.
Since implant morphology and microstructure during ISFI formation and degradation is a crucial determinant of implant performance, and the rate of phase inversion has been shown to have an effect on the implant microstructure, diagnostic ultrasound was used to noninvasively quantify the extent of phase inversion and swelling behavior in both environments. Implant erosion, degradation, as well as the \textit{in vitro} and \textit{in vivo} release profiles were also measured using standard techniques. A non-linear mathematical model was used to correlate the drug release behavior with polymer phase inversion, with all formulations yielding an $R^2$ value greater than 0.95. Ultrasound was also used to create a 3D image reconstruction of an implant over a 12 day span. In this study, swelling and phase inversion were shown to be inversely related to the polymer Mw with 53kDa polymer implants increasing at an average rate of 9.4%/day compared with 18.6%/day in the case of the 15 kDa PLGA. Additionally the onset of erosion, complete phase inversion, and degradation facilitated release required 9 d for 53 kDa implants, while these same processes began 3 d after injection into PBS with the 15 kDa implants. It was also observed that PLGA blends generally had intermediate properties when compared to pure polymer formulations. However, release profiles from the blend formulations were governed by a more complex set of processes and were not simply averages of release profiles from the pure polymers preparations. This study demonstrated that implant properties such as phase inversion, swelling and drug release could be tailored to by altering the molar ratio of the polymers used in the depot formulation.

While \textit{in situ} forming drug delivery implants offer an attractive alternative to pre-formed implant devices for local drug delivery due to their ability to deliver fragile drugs,
simple manufacturing process, and less invasive placement. The clinical translation of these systems has been hampered, in part, by poor correlation between \textit{in vitro} and \textit{in vivo} drug release profiles. In Chapter 6, the behavior of poly(D,l-lactide-co-glycolide) (PLGA) ISFIs were examined \textit{in vitro} and \textit{in vivo} after subcutaneous injection as well as injection into necrotic, non-necrotic, and ablated tumor to better understand the effect of environment on implant behavior. Implant formation was quantified noninvasively using an ultrasound imaging technique. Drug release of a model drug agent, fluorescein, was correlated with phase inversion in different environments.

Results demonstrated that burst release \textit{in vivo} was greater than \textit{in vitro} for all implant formulations. Drug release from implants in varying \textit{in vivo} environments was fastest in ablated tumor followed by implants in non-necrotic tumor, in subcutaneous tissue, and finally in necrotic tumor tissue with 50\% of the loading drug mass released in 0.7, 0.9, 9.7, and 12.7h respectively. Implants in stiffer ablated and non-necrotic tumor tissue showed much faster drug release than implants in more compliant subcutaneous and necrotic tumor environments. Finally, implant formation examined using ultrasound confirmed that \textit{in vivo} the process of phase inversion was directly proportional to drug release. These findings suggest that not only is drug release dependent on implant formation but that external environmental effects, such as tissue mechanical properties, may explain the differences seen between \textit{in vivo} and \textit{in vitro} drug release from \textit{in situ} forming implants.

\textit{In situ} forming implant systems are an exciting field of study, and have been successfully used to treat diseases that range in severity from prostate cancer to periodontitis. These systems provide a compelling alternative to preformed polymer
implants, and may prove to be paramount in overcoming the intrinsic obstacles of the physical targeting of polymer implants for the local delivery of therapeutic agents. The combination of medical imaging and drug delivery provides a unique path by which longitudinal evaluations of the delivery system, the patient, and treatment efficacy can all be monitored noninvasively. This approach provides key insight into the effect that the host has on the delivery vehicle, providing a method by which complex systems can be systematically evaluated. Through a more systematic approach in implant design, optimal treatment regimens which improve patient comfort, and reduce side effects, can be realistically achieved.

7.2 Future Directions

The use of diagnostic ultrasound to characterize the behavior of implants \textit{in situ} has provided valuable insight into the parameters that effect drug release. While the correlation of phase inversion with drug release can be used to approximate burst release after implantation, noninvasive evaluation of long term release is currently limited. The use of functional imaging techniques such as ultrasound elastography can be used to

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure7_1.png}
\caption{Strain data from 15 kDa PLGA ISFIs obtained from elastography imaging (A) compared with modulus information obtained from nonconfined compression testing (B).}
\end{figure}
Ultrasound elastography is a promising technique for noninvasive evaluation of mechanical properties because it is a method by which strain distribution can be determined through the cross-correlation of A-mode scans (which is a measurement of echo amplitude with respect to time) pre and post compression of a material [1]. The strain is determined by first estimating the time shift between A-line pairs before and after the material is compressed [1]. The time shift can be identified as the maximum peak in the cross-correlation plot of the A-line pairs, which can then be used to calculate the strain using the following relationship [1]:

\[ \Delta S_i = \frac{(t_{i+1} - t_i)}{2 \left( \frac{\Delta Z}{c} \right)} \]

In this equation \( S_i \) is the strain, \( t \) is the time (and the difference is calculated using the maximum of the cross-correlation plot), \( \Delta Z \) is the displacement distance of the compression, and \( C \) is the speed of sound through the material [1]. The output of the functional imaging is a strain map that can be used to solve for the modulus of the material. We hypothesize that a decrease in the polymer elastic modulus will occur as a result of degradation (Figure 7.1) and that this change can then be used to evaluate longitudinal release. Additionally, due to the effect of the injection site stiffness on drug release, ultrasound elastography can be used to evaluate the mechanical properties of the target location to insure that the appropriate polymer formulation is used for the specific application.

The utilization of additional imaging techniques that can evaluate solvent exchange as well as morphological changes may provide key insight into additional factors that can alter the release profile \textit{in situ}. One especially promising technique is the
use of diffusion weighted magnetic resonance imaging (MRI) to evaluate the change in
diffusivity of the implants over time (Figure 7.2A). Diffusion weighted MRI imaging the
diffusivity of water within a medium. In tissues or a polymer matrix the free diffusion of
water is limited by the surround environment, and this analysis obtain the apparent
diffusion coefficient of water within the region [2]. When a bi-polar gradient (or a
diffusion gradient) is applied to moving water molecules signal is lost due to incomplete
rephrasing by the second gradient [2]. The signal loss can be used to calculate and
apparent diffusion coefficient (ADC) which is a measure of the water diffusivity [2].
Another technique that may be useful for developing a system for predicting in vivo
behavior includes fluorescence molecular tomography (FMT) (Figure 7.2B). FMT
imaging evaluates the volumetric distribution of fluorophores within a tissue, by applying
excitation light from multiple locations. Charge-coupled devices (CCDs) are used to
detect the emitted light from the source. The projections obtained from the CCDs are
then used to develop a 3-D reconstruction of the fluorophore distribution volume [3].

### Figure 7.2.
(A) Proton density (PD) and calculation of apparent diffusivity coefficients (ADC) from
diffusion weighted MR study. Implants were imaged at 2 and 24 hrs, day 3, 5 & 7. (B) FMT image of an
implant loaded with PC-4, embedded in an agarose phantom.

One unique feature of ISFIs is that their size (which enhances the autocatalytic
degradation leading to increased implant osmolarity) in addition to the presence of
residual solvent leads to implant swelling. When the expansion of these implants is
constrained, the resultant swelling can lead to leakage of the residual solvent solution into the surrounding environment (Figure 3.2). The presence of these additional forces can also be used to improve the spatial distribution of drug due to the development of mechanically induced convective forces which can be used to elevate the distribution volume and elevate the rate of phase inversion (Figure 7.3). Development of a system which takes advantage of these properties may result in an implant system that overcomes the biggest hurdle for local delivery, which is the poor penetration distance of the drug from the implant.

Both DiI and BSA were shown to be effective in altering the behavior of implants. The hydrophobicity of DiI reduced the porosity of the implants, and BSA was shown to reduce the first order degradation rate constant by 16%. Future studies can focus on the use of these molecules as additives in order to further tune the release profile of implants by decreasing the diffusivity with the addition of DiI and altering the degradation profile with the addition of BSA(Figure 7.4). These additives may provide a method for achieving a more injectable zero order release system (relative to SPI systems), or play a role in the development of a dual release system. Additionally, the
use of steroids such as dexamethasone, may provide a means by which the inflammatory response may be reduced, which may reduce the in vivo secondary burst release.

Finally, future studies evaluating treatment efficacy, as well as safety are important steps in translating this system from bench to bedside. Despite the safety record of the Gliadel® wafer, any implantable device has the potential cause the formation of embolisms (due to the contact of blood with the polymer which would initiate the clotting cascade), the lungs should be evaluated for the presence of blood clots. Studies should also be conducted so that a comparison of the blood plasma levels for the released drug with those of drug within the lesion after implantation to insure that there is minimal systemic involvement. Through careful evaluation of the multitude of parameters involved in this system, optimal implant formulations can be developed for a near limitless number of applications.

7.3 References

Bibliography


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Appendix

A.1 Agarose Phantom Release Study

Material:

1% Agarose solution 55°C
0.5g of Agarose
50ml diH₂O
60/40 PLGA 2A, 3A, and 4A
Agarose Mold

Method:

1. Place the mold on a bed of ice. Pour 9ml of 55°C agarose into the mold and let it sit a total of 8 minutes in the mold.

2. After 3 minutes begin heating 50ml of a 1% agarose solution (will boil after 5 minutes).

3. After the 8 minutes is up, remove the lid, put the mold on the stand, and add 6ml of boiling 1% agarose solution to the molded first layer and let cool for 1.5 minutes. At 9:30 the agarose solution should be below 50°C, and polymer can be added.

4. Load a syringe with polymer and record the mass.

5. Add a drop of polymer (~50-100ul) to the cooling liquid layer and allow for the drop to fall into the pit created by the mold. After the polymer has been added quickly cover the syringe tip to avoid adding additional polymer drops. Then move the phantoms back onto the ice.

6. Record the mass of the syringe, after the injection.

7. Allow the agarose phantom to set.

8. Remove the phantom and place into the warm release solution (37°C, 150ml of diH₂O, gentle shaking) for 10 minutes, and image the phantom after the 10 minutes.

9. Begin release study, image the agarose phantom everytime a sample is taken.
A.2 Implant Phase Inversion Protocol

Protocol for **US Imaging:**

Materials:

- PLGA
- NMP (N-methyl pyrrilidone)
- 1% Agarose
- Fluorescein
- Phosphate Buffered Saline
- 2M NaOH

Steps for making Sheets

1. Prepare PLGA solution in NMP (60/39/1, NMP, PLGA, Fluorescien)
2. Make 1 L of 1% agarose solution
3. Prepare 30 Agarose phantoms (15 ml in molds allowing to cool)
4. Store phantoms in container to eliminate evaporation of water
5. Prepare pipette tips by removing 1.5 cm from end using a razor
6. Fill 20 ml scintillation tubes with 10 ml of PBS and put in incubator
7. Incubate polymer and PBS over night at 37°C in the incubated shaker
8. Tare the scale with the PBS filled scintillation tube
9. Place agarose phantom on US (12 Mhz transducer, Luis Polymer preset)
10. Add 50ul of of polymer to the agarose phantom and store 5 images
11. Transfer the implant to the scintillation tube
12. Mass the implant
13. Remove 1 ml of buffer solution and replace with 1 ml of fresh buffer
14. Image at 30 min, 1 h, 2 h, 4 h, 6 h, and 8 h by repeating step 9 and transferring implant to the agarose phantom and storing 5 images
15. After each time point image is taken repeat step 11 and 13.

16. Repeat step 9, then transfer implant to phantom for daily image.

17. After image has been taken repeat steps 11 and 13.

18. Process images using matlab for gray-scale value changes, changes in cross-sectional area, and phase inversion.
A.3 Implant Release Study Protocol

Protocol for Release:

Materials:

PLGA
NMP (N-methyl pyrrolidone)
1% Agarose
Fluorescein
Phosphate Buffered Saline
2M NaOH

Steps for making Sheets

1. Prepare PLGA solution in NMP (60/39/1, NMP, PLGA, Fluorescien)
2. Label 1.5ml centrifuge tubes for time points through the first 6 h of release
3. Prepare pipette tips by removing 1.5 cm from end using a razor
4. Fill 20 ml scintillation tubes with 10 ml of PBS and put in incubator
5. Incubate polymer and PBS over night at 37\(^\circ\)C in the incubated shaker
6. Tare the scale with the PBS filled scintillation tube
7. Add 50ul of polymer to the tared scintillation tube and record time of injection
8. Mass the implant
9. Remove 1 ml of buffer solution and replace with 1 ml of fresh buffer
10. Repeat step 9 through first 8 h after injection into PBS
11. After 24 h, completely remove the 10 ml of buffer, keeping 1 ml for analysis, and replace with 10 ml of fresh warm buffer.
12. Repeat step 11 for duration of experiment.
13. At the final time point, add implant to plastic container and add 5ml of 2M NaOH to degrade.
14. Measure the fluorescein concentration of the solution against the standard curve
   (Standard curve in PBS for time points, and standard curve in 2M NaOH for degraded sample).

15. Plot vs time.
A.4 Implant Erosion Study Protocol

Protocol for Erosion:

Materials:

- PLGA
- NMP (N-methyl pyrrilidone)
- 1% Agarose
- Fluorescein
- Phosphate Buffered Saline
- 2M NaOH

Steps for making Sheets

1. Prepare PLGA solution in NMP (60/39/1, NMP, PLGA, Fluorescien)
2. Label 1.5ml centrifuge tubes for time points cutting off lid locks and poking a hole into the lid.
3. Mass and record each of the centrifuge tubes.
4. Prepare pipette tips by removing 1.5 cm from end using a razor
5. Fill 20 ml scintillation tubes with 10 ml of PBS and put in incubator
6. Incubate polymer and PBS over night at 37°C in the incubated shaker
7. Tare the scale with the PBS filled scintillation tube
8. Add 50ul of of polymer to the tared scintillation tube and record time of injection
9. Mass the implant
10. Repeat steps 6-8 for each time point and replicate in the study.
11. After 5 h, remove the 5 ml of buffer, and replace with 5 ml of fresh warm buffer.
12. At each time point completely remove the buffer and transfer the implants into the premassed centrifuge tubes and record wet mass.
13. Freeze implants overnight and lyophilize for 4 d.
14. After lyophilization, remass tubes with dried mass of implant

15. Record the loss of mass over time and normalize by the initial implant mass
A.5 Implant Degradation Study Protocol

Protocol for **Degradation:**

Materials:

- PLGA
- NMP (N-methyl pyrrilidone)
- 1% Agarose
- Fluorescein
- Phosphate Buffered Saline
- 2M NaOH

Steps for making Sheets

1. Take implants used for erosion study after the dry mass has been recorded.
2. Dissolve in THF for at least 2 h into a 10 mg/ml concentration.
3. After the implants have dissolved, syringe filter at least 1 ml into GPC vials using a 0.45 µm filter.
4. Label vials and run on GPC at 25°C for 40 minutes and compare output spectra peaks with standard curve of known Mw.
5. Plot log(Mw) over time, and fit to find the 1st order degradation kinetics.
A.6 Implant 3D Imaging Study

Protocol for 3-D Imaging:

Materials:

PLGA
NMP (N-methyl pyrrilidone)
1% Agarose
Fluorescein
Phosphate Buffered Saline
2M NaOH

Steps for making Sheets

1. Prepare PLGA solution in NMP (60/39/1, NMP, PLGA, Fluorescien)
2. Make 1 L of 1% agarose solution
3. Prepare Agarose phantom (has long well with 5 divets for implant placement)
4. Store phantom in container to eliminate evaporation of water
5. Prepare pipette tips by removing 1.5 cm from end using a razor
6. Fill 20 ml scintillation tubes with 10 ml of PBS and put in incubator
7. Incubate polymer and PBS over night at 37°C in the incubated shaker
8. Tare the scale with the PBS filled scintillation tube
9. Add 50ul of of polymer to the scintillation tube
10. Mass the implant
11. Add implants to agarose phantom, and add 50 ml of 40°C agarose covering the implants
    and creating a smooth flat surface allowing agarose solidification for 20 min.
12. Set-up ultrasound by fixing the transducer on the agarose phantom, and using the Luis
    PLGA preset
13. Find the implant center
14. Open the linear stage software, and move the stage until the implants are out of view

15. Move stage 200 μm steps and record image at each step until out of view of the implants

16. Remove phantoms from the implants and return to scintillation tube

17. Repeat steps 11-16 for each time point

18. Process images using matlab for gray-scale value changes, changes in cross-sectional area, and phase inversion.

19. Process images using Amira 5.1 to get 3D information and generate 3D figures.
A.7 Subcutaneous release studies

Materials:

- 20 syringes with 70-80 µl of polymer solution
- Anesthesia Equipment with extra O₂ tanks and isoflurane
- Ultrasound Machine, transducer, and gel pad for tumor imaging.
- Shaving clippers and vacuum cleaner

Animal Groups:

**Total of 5 animals with 4 tumors each**

Drug release and *in vivo* formation time points taken (1h, 4h, 8h, 1d, 2d)

5 implants per animal

Animal Methods:

1. Syringes will be weighed and numbered
2. Animals will be weighed on scale
3. Animals will be induced with 3% isoflurane with a Oxygen flow rate 1 L/min.
   ○ After induction, animals will be fitted with a mask with 1.5% with Oxygen flow rate of 1 L/min
4. The injection site will be shaved for imaging
5. Clip ear of rat and record rat information in lab notebook
6. Injection sites will be cleaned with iodine
7. Implants will be injected into the tumor by pinching skin, and inserting the needle into the subcutaneous space, and solution injected
8. Keep syringe in subcutaneous space for 30 s before removing
9. Record weight of used syringe and needle and subtract from original weight to get injection mass.
10. Ultrasound implant at 1 h, 4 h, 8 h, 1 d, and 2 d after implant injection.
11. Record in lab notebook the location of injection.
12. Make sure animals are doing ok once they wake up from anesthesia.
13. Animal will be sacrificed after last imaging time point and implant will be dissected out.
14. Resected implants and drug loaded polymer solution will be placed in 2M NaOH overnight, and measured against a standard curve to determine the release based on the maximum drug loading.