Multisystem Drug Delivery System:
Micelles Encapsulated in Hydrogels

BY
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THESIS
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>API</td>
<td>Active Pharmaceutical Ingredient</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium Persulfate</td>
</tr>
<tr>
<td>CMC</td>
<td>Critical Micelle Concentration</td>
</tr>
<tr>
<td>Cur</td>
<td>Curcumin</td>
</tr>
<tr>
<td>DDH$_2$O</td>
<td>Deionized Distilled Water</td>
</tr>
<tr>
<td>Dil</td>
<td>1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate</td>
</tr>
<tr>
<td>DiO</td>
<td>3,3'-Dihexadecyloxacarbocyanine Perchlorate</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic Light Scattering</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
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<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
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<tr>
<td>DOX</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s Phosphate-Buffered Saline</td>
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<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
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<tr>
<td>Em</td>
<td>Emission</td>
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<tr>
<td>EO</td>
<td>Poly(ethylene oxide)</td>
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<tr>
<td>EPR</td>
<td>Enhanced Permeation and Retention</td>
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<tr>
<td>Ex</td>
<td>Excitation</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<td>FRET</td>
<td>Fluorescence Relative Energy Transfer</td>
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<tr>
<td>GI</td>
<td>Gastrointestinal</td>
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<tr>
<td>HLB</td>
<td>Hydrophilic-Lipophilic Balance</td>
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<tr>
<td>IC50</td>
<td>Median Inhibition Concentration</td>
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<td>Abbreviation</td>
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<tr>
<td>Indo</td>
<td>Indomethacin</td>
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<td>IV</td>
<td>Intravenously</td>
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<td>kDa</td>
<td>Kilodaltons</td>
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<td>Log P</td>
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<td>Multidrug Resistant Proteins</td>
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<td>NSAID</td>
<td>Non-Steroidal Anti-Inflammatory Drug</td>
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<td>PBC</td>
<td>Pluronic® block copolymers</td>
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<td>PEG</td>
<td>polyethyleneglycol</td>
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<td>PEGDA</td>
<td>Poly(ethylene glycol) Diacrylate</td>
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<td>Poly(propylene oxide)</td>
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<td>Polyvinylidene Fluoride</td>
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SUMMARY

Micelles, a nanoparticle system composed of self-assembled amphiphilic polymer chains, have been used in the drug delivery field for the encapsulation of active pharmaceutical ingredients (API). The aim of encapsulation is to extend overall drug circulation time in the body. Micelles also greatly aid in the solubilization of hydrophobic drugs. However, there are severe drawbacks to using micelles alone for drug delivery including rapid disassembly when diluted below a critical micelle concentration (CMC) in the bloodstream and concentration and elimination in the kidneys and liver if injected intravenously. Hydrogels are hydrophilic crosslinked networks of polymer chains which act as an implantable depot by which small particles such as micelles may be implanted and released over time. By encapsulating micelles in the hydrogel, we can control and extend drug release over time as well as concentrate micelles at the treatment site. This gives cells time to more easily access and take up these drug loaded micelles. Our work pertains to the development and characterization of multiscale drug delivery system in which drug loaded Pluronic® micelles were encapsulated within a polyethylene glycol diacrylate (PEGDA) hydrogel. APIs of different hydrophobicities are loaded into Pluronic® micelles which in turn are loaded into and released over time by diffusion from PEGDA hydrogels.

Micelles made up of different Pluronics® and encapsulating APIs of differing hydrophobicities were characterized before use in this drug delivery system. Particle sizing using dynamic light scattering (DLS) showed as the hydrophobicity of the drug increases, micelle size decreases. Similarly, as the hydrophobicity of the drug increases, drug loading efficiency of the micelle also increases. Drug release experiments using drug-loaded micelle
demonstrated that the more hydrophobic the drug is, the slower the rate of release from the hydrogel/micelle system. We hypothesize it is because a more hydrophobic drug is held more tightly to the core of the micelle and therefore takes more time to travel out of the interiorly bound micelle out of the hydrogel.

Hydrogels made up of 3.4 kDa, 10k Da, and 20 kDa molecular weight were used for the encapsulation and release of drug loaded micelles. We found that higher molecular weight PEGDA created hydrogels of larger mesh size using an experiment based off of Archimede’s Principle and the Flory-Rehner Swelling Equations. Hydrogels of higher molecular weight (and therefore mesh size) released drug-loaded micelles at a faster rate than hydrogels of lower molecular weight. This data suggests that a larger polymer mesh size allows for faster micelle diffusion out of the hydrogel.

Pluronic® F-127/L101 mixed micelles were shown to be significantly larger compared to Pluronic® F-127 micelles (about 5x fold). Addition of larger micelles or increasing the loaded amount of micelles within hydrogels significantly increased hydrogel mesh size. Increased hydrogel mesh size led to increased rate of micelle release from the hydrogel. Pyrene loaded micelle release were used to model whole intact drug-loaded micelle release as well as the ability of micelle to reassemble outside of the hydrogel. Intact micelle from hydrogel release was further confirmed by releasing FRET loaded micelles from hydrogels and measuring buffer fluorescence at 484 nm excitation and 565/501 nm emission. Unlike pyrene loaded micelles, FRET loaded micelles cannot assemble and then reassemble outside of the hydrogel without losing their intrinsic fluorescent properties. Franz cell experiments demonstrated the drug’s ability to leave the micelle and pass through the hydrogel if the entire intact micelle is too large to pass through the mesh intact. DOX loaded micelle release data showed this system follows
Higuchi’s Model or diffusion based drug release. U87-MG human glioblastoma cells were treated with the releasate of 3.4 kDa, 10 kDa, or 20 kDa molecular weight hydrogels containing no micelles, empty Pluronic® micelles, DOX HCl, or DOX-loaded Pluronic® F-127 micelles as an to test for overall system effectiveness in killing tumor cells. Using an MTS assay, significant cellular cytotoxicity was observed in groups treated with DOX micelle/DOX HCl loaded hydrogels and increased with higher molecular weight PEGDA.

To summarize, platform composed of drug loaded Pluronic® micelles encapsulated in a PEGDA hydrogel was developed and characterized for localized, extended drug delivery. This is the first time that these two polymer materials (both Pluronics® and PEGDA) were combined to create a drug delivery device. More importantly, this is the first time the mechanism of drug-loaded micelle release from hydrogel have been studied and more specifically, that intact micelles can be released intact after encapsulation in a porous cross-linked polymer system. These released drug-loaded micelles in turn have been shown to have similar cytotoxicity to tumor cells compared to the hydrophilic form of the drug itself. These results show a promising future not only for this system itself but for all devices capable of releasing micelles.
CHAPTER 1
INTRODUCTION

1.1 Recent Advances in Drug Delivery

Currently, it has been estimated that about 40% of approved drugs in the market and about 90% still in development are poorly water soluble [1]. Because the human body is made up of 65% water, this often leads to poor biodistribution and rapid elimination by the GI system [2, 3]. Many drugs when directly ingested or injected intravenously are degraded by the body (either through the harsh pH of the stomach or elimination by the endocrine system) before reaching the site of action [3]. These drugs may then accumulate in certain organs and cause damage to normal tissue thereby leading to serious adverse side effects in patients [3]. This is the main motivation for developing novel drug delivery systems. What is considered an effective drug delivery system is one which will deploy medication to certain affected areas of the body, will enhance the efficiency of the API, and its mechanism of delivery can be easily controlled by a chemical or physiological trigger [4]. The Food and Drug Administration (FDA) in recent years have aided in the exploration of a number of different ways to aid in drug delivery including use of tablets, capsules, films, and emulsions to name just a few examples. These drug delivery methods can be administered to the patient in a variety of ways: orally, by inhalation, by IV injection, and topically through the epidermis and each method has its own advantages as well as disadvantages [5].

It has been estimated that it takes well over a decade and approximately $120 million to bring a new API to the market. As a result since the 1950s, rising costs have seen a reduction in FDA approval of new chemical compounds. In fact, approximately 40% of all US marketed drugs in year 2000 was due to novel drug delivery systems [6]. Ever since the discovery of lipid
vesicles known as liposomes to encapsulate and solubilize hydrophobic drugs in the 1960s, nanotechnology has completely altered the landscape of the pharmaceutical industry [7]. There are numerous definitions for what nanotechnology actually comprises but the most accepted is the branch of science which investigates and manipulates materials of below 100 nm in size in at least one dimension [8]. Two key advantages characterize all of nanotechnology: a high surface to volume ratio which is advantageous when attempting to modify surface properties for drug delivery and a very discrete electronic structure compared to bulk materials which makes nanoparticles useful for imaging purposes [8]. Today, a variety of nanoparticles from nanorods to metal-based nanoparticles made up of inorganic and organic materials with or without targeting moieties has been under investigation and has shown promising results in a variety of scientific areas [9].

The three model drugs utilized in this study differ vastly in their solubility as can be guessed by their chemical structures (Figure 1.1). In order to denote solubility of the compound in water, logP or the partition coefficient is utilized. LogP is defined as the ratio of concentrations of a unionized compound that can be dissolved in octanol over what can be dissolved in water. Hence, a higher logP value means the compound is more lipophilic and therefore less water soluble [10, 11]. Doxorubicin (DOX) with a logP value of 0.92 [12] is the most water soluble drug out of the three due to having the most alcohol groups in its molecular structure (Figure 1.1A) and is a chemotherapeutic for the treatment of lymphomas, Hodgkin’s disease, and several types of solid tumors [12]. DOX functions as an antineoplastic and is most commonly delivered intravenously (IV) as a hydrochloride salt. Side effects however from IV injection is very severe with the worst being life-threatening heart damage [13]. In order to concentrate the drug at the treatment site and therefore reduce side effects, the FDA approved
Doxil, a pegylated liposome form of DOX and Myocet, a nonpegylated liposome form [14]. These two forms were able to effectively reduce cardiotoxicity. However, there is a current shortage of Doxil starting in 2011 due to pharmaceutical quality control issues and thus other attempts to create a viable doxorubicin delivery system is currently under study [15].

Indomethacin (indo) is a non-steroidal anti-inflammatory drug (NSAID) for the general treatment of fever, stiffness, and swelling. Having a logP value of 4.27, indo is the most hydrophobic drug compared to DOX and curcumin which contains several benzene rings but only one alcohol group (Figure 1.1B). Indo is usually packaged as a tablet along with several emulsifiers and taken orally [16, 17]. Recent studies have shown that co-delivery of indo with chemotherapeutics increases tumor cell response to treatment [18, 19]. Finally, curcumin with a logP value of 2.92 [20] is considered sparingly soluble in water (Figure 1.1C) and like Indo has been shown to reduce inflammation and act as a chemopreventative. Interestingly, curcumin is derived from turmeric and is commonly used as a flavoring and coloring agent in a variety of foods. Thus, not only is it already commonly eaten but used in alternative medicine in Southeast Asia and India for the treatment of inflammation, skin wounds, cough, and hepatic/biliary disorders. In vitro, it has been shown to prevent cell proliferation in colon and breast cancer cells at high concentrations [21, 22]. Curcumin’s chemopreventative properties stems from its ability to inhibit free radical generation, inhibit cytochrome P450, and induce glutathione S transferase [22].
1.2 Micelles

Since their seminal introduction as drug delivery carriers in the early 1980s, micelles have received a great deal of attention. Micelles are nanoparticle systems composed of amphiphilic lipids or polymers that self-assemble [23, 24]. There are both attractive and repulsive forces that occur between the amphipathic polymers and lipids that control the properties of micelles in solutions [25, 26]. At a critical micelle concentration (CMC), formation of micelles occurs as a result of attractive forces that promote association of parts of the free lipids or polymers [27]. These forces result in formation of nanocarriers with a shielded inner core [28]. These small hydrophobic cores have allowed for micelles to effectively solubilize and shield poorly water-soluble drugs [29, 30]. Repulsive forces occurring as a result of steric
interactions between the polar end groups limit the size of micelle growth and allow micelles to preferentially associate in the spherical core-shell structures [31]. Hydrophobic interactions of the nonpolar chains in the core allow for repulsion and exclusion of solvent volume, which further reduces the size of micelles [31]. These interactions allow for nano-sized carriers (<100 nm), which are very desirable for parenteral and intravenous administration [23, 32, 33]. Below is a summary of micellar drugs approved by the FDA and currently available in the market (Table 1.1). Of particular note is SP1049C, DOX encapsulated in a Pluronic® F-127/L-61 micelle, which was granted orphan status in 2015 for the treatment of advanced prostate cancer. Alexander Kabanov and his team, who invented SP1049C and who pioneered the development of Pluronic® micelles for the treatment of cancer, discovered that some hydrophobic Pluronics® have the ability to block overexpressed drug efflux transporters on tumor cells and therefore prevent drug resistance. By encapsulating a chemotherapeutic agent inside Pluronic® micelles, Kabanov observed rapid depletion of cancer stem cells as well as overall sensitization to the drug again over time [34] [35]. Similarly, he and his team developed the method of mixing different Pluronics® to form micelles of varying sizes [36]. This technology forms the basis for our own micelle system as will be later described.

**Table 1.1. Micelle Encapsulated Drugs approved by the FDA.** Table adapted from [37].

<table>
<thead>
<tr>
<th>Name</th>
<th>Polymer Type</th>
<th>Treatment for</th>
<th>Approved</th>
<th>Marketed by</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adagen</td>
<td>PEGylation</td>
<td>Severe combined immunodeficiency disease</td>
<td>1990</td>
<td>Sigma-Tau Pharmaceuticals</td>
</tr>
<tr>
<td>Cimzia</td>
<td>PEGylation</td>
<td>Crohn's Disease</td>
<td>2008</td>
<td>UCB</td>
</tr>
<tr>
<td>Copaxone</td>
<td>Polymer composed of amino acids</td>
<td>Multiple Sclerosis</td>
<td>1996</td>
<td>Teva Pharmaceuticals</td>
</tr>
<tr>
<td>Eligard</td>
<td>PLGH</td>
<td>Prostate Cancer</td>
<td>2002</td>
<td>Tolmar Pharmaceuticals</td>
</tr>
<tr>
<td>Drug</td>
<td>PEGylation Type</td>
<td>Condition</td>
<td>Year</td>
<td>Company</td>
</tr>
<tr>
<td>------------</td>
<td>----------------</td>
<td>------------------------------------</td>
<td>------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>Macugen</td>
<td>PEGylation</td>
<td>Neovascular age-related muscular degeneration</td>
<td>2004</td>
<td>Bausch and Lomb</td>
</tr>
<tr>
<td>Mircera</td>
<td>methoxy PEG</td>
<td>Symptomatic anemia from chronic kidney disease</td>
<td>2007</td>
<td>La Roche Inc</td>
</tr>
<tr>
<td>Neulasta</td>
<td>PEG conjugation</td>
<td>Chemotherapy induced neutropenia</td>
<td>2002</td>
<td>Amgen</td>
</tr>
<tr>
<td>Oncaspar</td>
<td>PEGylation</td>
<td>Acute Lymphoblastic Leukemia</td>
<td>1994</td>
<td>Sigma-Tau Pharmaceuticals</td>
</tr>
<tr>
<td>Pegasys</td>
<td>PEGylation</td>
<td>Hepatitis C</td>
<td>2002</td>
<td>Genentech</td>
</tr>
<tr>
<td>PegIntron</td>
<td>PEGylation</td>
<td>Hepatitis C</td>
<td>2001</td>
<td>Merck</td>
</tr>
<tr>
<td>Renagel</td>
<td>Polyamine</td>
<td>Chronic Kidney Disease</td>
<td>2000</td>
<td>Sanofi</td>
</tr>
<tr>
<td>Somavert</td>
<td>PEGylation</td>
<td>Acromegaly</td>
<td>2003</td>
<td>Pfizer</td>
</tr>
<tr>
<td>SP1049C*</td>
<td>Pluronic® F-127/L-61</td>
<td>Prostate Cancer</td>
<td>2015</td>
<td>Supratek Pharma</td>
</tr>
</tbody>
</table>

*-Granted orphan status in 2015 (Orphan status refers to a disease that effects less than 200,000 individuals in the United States each year).

**Figure 1.2. Molecular structure of Pluronic® (or poloxamers).** Pluronics® are a block copolymer consisting of a central hydrophobic chain of polyoxypropylene (PPO) flanked by two hydrophilic chains of polyoxyethylene (PEO). Letters a and b represent number of repeating units of PPO and PEO for that specific Pluronic® and will decide the water solubility (the more subunits of PEO it has the more hydrophilic it will be). For example, a hydrophilic Pluronic® F-127 has 100 subunits of PEO and 65 subunits of PPO, Pluronic® P-105 which is not as...
soluble has 74 subunits of PEO and 56 subunits of PPO, and hydrophobic Pluronic® L-61 has only 3 subunits of PEO and 30 subunits of PPO.

Certain properties of micelles have made them an especially desirable delivery carrier in the context of cancer [32, 38]. A micelle’s inner hydrophobic core allows for the encapsulation and solubilization of poorly water-soluble chemotherapeutics, such as doxorubicin [32, 39, 40]. This allows for delivery of such molecules in a shielded micelle. The size of micelles is advantageous for cancer therapy because it allows them to be given by intravenous injection and theoretically endows them with an inherently longer circulation time [23, 24].

More specifically, block copolymers (such as the Pluronics® used in this project) composed of alternating hydrophobic and hydrophilic segments have the advantages of having a fairly small size distribution and high drug loading capacity (Figure 1.2). Size and surface properties of these micelles can be easily modified and determine the distribution of particles in the body. Compared to surfactant micelles, polymeric micelles have lower CMC [41], are more stable in solution [41], and a lower rate of disassociation in the body allowing for better drug encapsulation/retention [42]. Most block copolymer micelles are in the tens of nanometer size range even with loaded drug which allows for easy cellular uptake and better retention at the tumor site through the enhanced permeation retention (EPR) effect [40].

Unfortunately, a micelle’s Achilles heels often lie in its rapid dissolution in biologic solutions and nonspecific uptake by the reticuloendothelial systems (RES) [23-26]. Physical interactions allow micelles to assemble at the critical micelle concentration, but when intravenously injected and introduced into a much larger volume, this thermodynamically driven stability is lost and micelles disassemble and release their cargo [43, 44]. This leads to fast
elimination of components and sometimes non-specific dose dumping, which reduces micelle and drug efficacy and results in adverse drug events [23-25, 43].

1.3 Hydrogels

Methods for localized and controlled release of micelles would be advantageous. Hydrogels have been widely investigated and utilized for controlled drug delivery applications [45]. Hydrogels have meshlike networks, modulatable by several polymer specific parameters, such as molecular weight, amount of crosslinking, charge, and polarity [46-48]. Hydrogels have been used as devices to deliver a variety of therapeutics including: small molecules, proteins, and viruses, [49-52] and have also been shown to hold great promise as matrices for localized and sustained delivery of nanoparticles [53, 54]. The hydrogel’s network, the mesh size of which can be easily adjusted, allows for diffusion-based delivery [45, 46]. Additionally, hydrogels mimic extracellular matrix and have modulatable shape and physical characteristics, which makes them advantageous for localized delivery [45, 46]. Hydrogels are able absorb huge amounts of water at a fast rate and exist as either a dehydrated or a swollen state (Figure 1.3). When the hydrogel is in a dehydrated state, its polymer chains are in close proximity and there is little room for the diffusion of molecules. As the hydrogel swells, the space in between polymer chains increase, the mesh size increases as a result, and more solute molecules are able to diffuse in and out of the hydrogel system [55]. Equilibrium swelling is finally reached when the elastic restorative forces of the hydrogel network (mainly the crosslinking points) equals all osmotic forces [48, 56]. It is at this state where the mesh size is at a maximum that loaded drugs or in this case drug-loaded micelles can most freely diffuse of the hydrogel.
Figure 1.3. Hydrogels relaxed state (top row) or lyophilized (bottom row).

Orange colored hydrogels contain DOX-loaded Pluronic® F-127 micelles (first column) while clear hydrogels contain either no micelles (middle column) or empty Pluronic® F-127 micelles (third column).

The timeline as presented below (Figure 1.4) describes the history of hydrogels and clearly shows that although the term itself has been around for over a hundred years, the use and development of novel hydrogel systems using polymers only started in the 1960s. Wichterle and Lim was first to develop hydrogels as we know them today—a crosslinked polymer network composed of poly(2-hydroxyethyl methacrylate) (pHEMA) for use in soft contact lenses [57]. The next 40 years saw a new era of developing hydrogels out of different materials from synthetic (pHEMA, PEG and PVA) to natural (cellulose, hyaluronic acid, chitosan) for a variety of different uses including cell scaffolds for tissue engineering, contact lens material, various biomedical devices, and even in more mundane everyday objects such as toys or plant packing material [1]. First generation hydrogels were crosslinked using chain-addition reactions by vinyl
monomers and were made up of simple polymers such as poly (acrylamide) (PAM) [58], pHEMA [59], poly(vinyl alcohol) (PVA) [60] or poly(ethylene glycol) (PEG) [61]. pHEMA hydrogels have been used mainly in the making of soft contact lenses since its discovery 50 years ago [1, 59]. Recent advances in increasing mechanical stability as well as oxygen transport has led to the expansion of uses for pHEMA hydrogels for controlled drug delivery of contraceptives, vasoconstrictors, anti-inflammatories, cytostatics, and antibiotics [62].

Hydrophilic materials such as PVA or PEG are typically chemically crosslinked and have been shown to be to be useful as swelling-controlled systems for drug delivery. These two materials originally gained attention because of their perceived biocompatibility and resistance to protein adsorption [63, 64]. Second generation hydrogels were developed in the 1970s and were characterized by the ability to respond to certain environmental stimuli such as temperature or pH changes [65]. Of particular interest in this category are in-situ forming hydrogels that are able to gel at body temperature. This allows implantation of the hydrogel to be a minimally invasive procedure [66]. Pluronic® F-127 hydrogels are considered thermoreversible, nontoxic, and have been used for the delivery of anesthetics, antimicrobials, hormones, and chemotherapeutics [67, 68]. By definition, thermoreversible means that Pluronic® F-127 first exists as a micellar solution at concentrations above 15-20% w/v and at 4°C. Upon heating to 20°C, this solution undergoes a sol-gel transition as the micelles arrange themselves into a more crystalline structure [69]. Further cooling causes a reverse change where the gel breaks down again into micelles and then unimers [70]. Numerous studies have shown Pluronic® F-127 hydrogels can dissociate into drug-loaded micelles over time in the body [67, 70-72]. This adds another controllable factor for use in extended drug delivery. However, several drawbacks of Pluronic® F-127 hydrogels such as not being biodegradable, weak mechanical properties and
structural instability due to the hydrophobic PPO block have led to the development of PEG and PLA in current thermoreversible hydrogels [73, 74]. Third generation hydrogels were developed in the mid-1990s and were unique in that scientists employed stereocomplexation, metal-ligand coordination, and peptide interactions as crosslinking methods [1]. This improved the mechanical strength of hydrogels as well as allowed for better control of release kinetics in-situ [1, 75, 76]. Most recently, the term “smart hydrogel” was coined to describe hydrogels that can be crosslinked at physiological conditions with limited toxicity and which are mechanically strong (such as PEGDA used in this system for example) [75]. “Smart hydrogels” are also used to describe hydrogels as part of a multicomponent drug delivery system (i.e. a micelle in a hydrogel device as described here). [1] Incorporating a nanoparticle such as a micelle into a hydrogel can allow better control of drug release kinetics. For instance, larger nanoparticles encapsulated small molecule drugs will be retained longer in the hydrogel system, eliminate burst release, and extend release over time. This would increase drug bioavailability in the body and allow cells enough time to take up the drug. Other advantages to a nanoparticle/hydrogel composite system include increasing solubility of a hydrophobic drug and using the nanoparticle to shield and protect the drug during hydrogel formation [1] [77]. As already described in the sections above, the three drugs indo, DOX, and curcumin used in this delivery system are hydrophobic. Solubilization by encapsulating the drug in a nanoparticle before release from a hydrogel would enhance drug stability in the bloodstream.
More specifically, hydrogels in our drug delivery system will be made up of poly(ethylene glycol) diacrylate or PEGDA. The structure of PEGDA is presented (Figure 1.5). PEG is a hydrophilic, biocompatible material that has already been FDA approved to be used in a variety of medical devices namely liquid bandages and sutures [78, 79]. This polymer is considered non-immunogenic, non-toxic at molecular weights above 400, very slow to degrade, and is inherently resistant to protein adsorption making it a very good blank slate for drug delivery [63, 64]. By substituting terminal hydroxyl groups with acrylates, PEGDA can be easily crosslinked using initiators to form hydrogels. Decreasing the PEG chain length in between crosslinking sites has already been shown to increase the mechanical modulus as well as decrease mass transport. [80]
Figure 1.5. Molecular structure of PEGDA. PEGDA is available in many different molecular weights depending on the number of PEG subunits and each chain is flanked by two acrylate groups which can be crosslinked.

1.4 Nanoparticle in Hydrogel Systems

In recent years, hybrid drug delivery systems have been gaining in popularity. Specifically, reservoir based systems where a drug-loaded nanoparticle is encapsulated in a hydrogel and implanted near or at the target site allows for the dual advantages of using both a micelle and a hydrogel. Such a device will allow for a localized concentration of drugs, reducing the side effects associated with whole body treatment, and control both the dose and timing for treatment [54]. In designing a nanoparticle/hydrogel drug delivery device, one needs to consider multiple factors but ultimately the characteristic most critical is how to control rate of drug release. There are currently two options: active or passive targeting. In active targeting, an external stimuli such as light, temperature, or pH change is applied to either the hydrogel or nanoparticle or both which allows for a structural change resulting in drug release. Shah et al in a recent study published in 2014 bound a model drug (camptothecin) to a phototriggerable compound which in turn is bound to silica nanoparticles. A PEG based hydrogel matrix serves to concentrate and hold the nanoparticles at the target site (in which case is a malignant brain tumor line). The authors showed that only upon exposure to UV light is the camptothecin
released and that drug release kinetics can be easily controlled. [54] Similarly, another recent study also published in 2014 by Gao et al encapsulated cationic liposomes bound with gold nanoparticles in an acrylamide gel. When the gel is applied topically, the cationic liposomes will only fuse with bacteria at acidic pH making it capable of killing pathogens that thrive in such environments [81, 82]. While the liposomes act as the actively targeting component, the authors found through modeling that the particles are released from the hydrogel in a purely diffusion based way (fits Higuchi’s Model best).

The majority of nanoparticle/hydrogel systems (including ours) are still based on passive targeting. Nanoparticles are first incorporated into the hydrogel using 5 main methods: hydrogel polymerization in a nanoparticle suspension [83, 84], physical incorporation of the nanoparticle after gelation [85, 86], loading the hydrogel with reactive nanoparticle precursors which can assemble into nanoparticles in the matrix [87, 88], and crosslinking reactive groups on the nanoparticles to form hydrogels [89, 90]. While the majority of these studies have focused on metallic based nanoparticles (for their durability during the encapsulation process), other polymeric nanoparticle/hydrogel composites have been developed including those with carbon nanotubes, melanin, micelles, liposomes, and nanogels. Heo et al in 2014 delivered gold nanoparticles encapsulated in a photo-curable gelatin hydrogel to mesenchymal stem cells in order to encourage osteogenic differentiation. These nanoparticles had no targeting moieties and slowly diffused out of the hydrogel (which was slowly degraded by collagenase) to the site of action. [91] There have been several published papers using metal-based nanoparticles embedded in a thin hydrogel film as antimicrobial coatings [53, 92, 93]. Metals were known to be extremely toxic to yeast and most bacteria at low concentrations for a long time in history. Ancient vessels made out of copper or silver were used for food preservation and water
disinfection have been found dating to the time of Persian kings [94, 95]. Metal-based nanoparticles possess natural antifouling, anti-inflammatory [96], and anti-angiogenic properties [97] against bacteria that may develop resistance over bactericidal agents over time [53]. As such, both gold and silver nanoparticles have been incorporated as part of nanoparticle/hydrogel hybrid systems for purposes ranging from wound and burn dressings [98] and dental fillings to coatings for cooking utensils [99]. In using metal nanoparticle/hydrogel systems, many factors such as mechanical toughness, biocompatibility, swelling ratio, and stimuli responsiveness must be carefully considered [82]. Because metals are not biodegradable, efforts have shifted in recent years to making the nanoparticles or hydrogels out of natural materials such as gum acacia, dextran, gelatin, and chitosan for use in implantable dressings [87, 100].

A great advantage to incorporating metal-based nanoparticles into hydrogel systems is the overall stability of the nanoparticles and ease in obtaining uniform distribution within the hydrogel network [82]. A recent review described three structural designs that exist for all nanoparticle/hydrogel systems: nano or micro-sized hydrogels encapsulating nanoparticles, nanoparticles noncovalently immobilized in the hydrogel network, or nanoparticles covalently immobilized in the hydrogel network. Five main methods for overall nanoparticle encapsulation within the hydrogel were described: forming the hydrogel around a nanoparticle suspension, embedding the nanoparticles in the hydrogel matrix post-gelation, crosslinking the nanoparticles to form the hydrogel, incorporating reactive nanoparticle precursors inside of a hydrogel, and finally using unique gelator molecules in the hydrogel [82, 101]. The incorporation of “softer” particles such as micelles or liposomes inside of hydrogels is still a very nascent field and possible nanoparticle damage during the hydrogel polymerization process remains problematic. While the release of solid metal nanoparticles from the hydrogel can be easily tracked through
microscopy, it is not so simple to observe the release and ultimate destination of micelles or liposomes from the hydrogel in situ. However, micelle/hydrogel systems offer many advantages which each component of the system cannot provide alone namely better control of drug release kinetics in drug delivery. For instance, Zhang et al was able to increase drug loading, extend bioavailability, and incorporate hydrophobic drugs inside a hydrogel by using a dendritic nanoparticle/hydrogel hybrid system [102, 103]. Wu et al loaded Dexamethasone polymeric micelles into PEG-PCL-PEG thermosensitive hydrogels for use as post-surgical adhesive bandages. Their studies showed the ability of the hydrogels to adhere to damaged tissue and increase retention time of the anti-inflammatory drug by localizing the release of drug-loaded micelles [104]. Miyazawa used drug-loaded micelles encapsulated in hydrogels to differentiate dental pulp stem cells into odontoblasts. His simvastatin-loaded micelles of approximately 500 μm diameter in size were released from gelatin hydrogels after hydrogel collagenase degradation and showed better activity than simvastatin alone [105]. A closer example to our own glioblastoma disease model was demonstrated by Fourniols et al in a recent journal article published in 2015. The authors loaded temozolomide into PEG-PCL micelles which were then encapsulated in a photo-polymerizable polyethylene glycol dimethacrylate (PEG-DMA) injectable hydrogel. Sustained release of temozolomide from the hydrogel/micelle system was measured for up to a week and showed activity in-vivo towards the brain tumor. Furthermore, the authors showed the hydrogel could be polymerized very easily in situ and that the mechanical properties of the gel were biocompatible with brain tissue [106]. More examples of the need for nanoparticle/hydrogel hybrid systems will be further discussed in chapter 4. Nevertheless in all of these studies, the authors have focused on drug release rather than drug-loaded micelle release as well as the therapeutic aspect of the system. This ignores the many added advantages of the
drug being released in a micellar form versus alone (as will be discussed in chapter 4). We have also yet to understand the mechanisms for drug or drug-loaded micelle release which would help us adjust certain drug, micelle, and hydrogel properties in order to optimize drug release.

1.5 Structure of the Thesis

Herein, we examine the stability of micelles released from hydrogels composed of polyethylene glycol diacrylate (PEGDA). We hypothesize that a multi-scale drug delivery system, which combines the advantages of two forms of passive targeting, that of the micelle and the hydrogel, will allow for the solubility of a hydrophobic chemotherapeutic as well as provide extended release of intact drug loaded micelles by the hydrogel. Likewise, adjusting hydrogel as well as drug or micelle properties such as particle size and mesh size will alter micelle from hydrogel release. We will prove that intact drug-loaded micelles are released from the hydrogel instead of just the drug itself. Lastly, released drug-loaded micelles will be tested against a glioblastoma multiforme cell line U87-MG to show therapeutic effect. Over all, this project will be divided into 3 main aims (Figure 1.6):
Figure 1.6. Overview of the multiscale drug delivery system consisting of drug-loaded micelles encapsulated within a hydrogel for passive targeting.

Aim 1: Hydrogel Formation and Characterization

1.1 Determine the effects of two different initiators in hydrogel mesh size, drug release, and toxicity

1.2 Determine whether PEGDA molecular weight effects hydrogel mesh size and therefore drug release

Aim 2: Micelle Formation and Characterization

2.1 Comparing different Pluronic® micelles in terms of stability, CMC, size, DOX and Indo encapsulation efficiency, release from hydrogel, and cytotoxicity.

2.2 Characterization of drug-loaded Pluronic® F-127 micelles in terms of size, drug loading efficiency, and cytotoxicity
2.3 Understanding whether micelle drug type or presence of micelles in the hydrogel effects certain hydrogel properties such as mesh size and rate of drug release

2.4 Assessing cytotoxicity of DOX-loaded micelles released from PEGDA hydrogels

Aim 3: Proving Intact Micelles Are Being Released from the Hydrogel

3.1 Modeling of drug-loaded release from hydrogel

3.2 Determine whether micelles are intact while encapsulated inside the hydrogel

3.3 Determine whether intact micelles are being released from hydrogels using pyrene

3.4 Determine whether intact micelles are being released from hydrogels using FRET and understanding the effect of polymer concentration on micelle release

3.5 Determine the rate of unencapsulated drug-loaded micelles passing through a hydrogel using Franz Cell

3.6 Determine whether intact micelles that have not been encapsulated are able to pass through a hydrogel using Franz Cell
1.5 References


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CHAPTER 2
UNDERSTANDING HYDROGEL PROPERTIES AND THEIR EFFECTS ON
DRUG/MICELLE RELEASE

2.1 Introduction

Hydrogels have been widely investigated for use as drug delivery matrices due to certain unique physical properties, such as their permeable nature, high water content, and shape [1-3]. Their modulatable nature makes them very advantageous systems for many applications [1, 2]. Polymer choice, molecular weight, crosslinking method, and shape can affect the way the hydrogel functions [4]. Poly(ethylene glycol) diacrylate (PEGDA) is our polymer of choice because it is considered a biocompatible polymer and a number of FDA approved biomedical devices on the marketplace use PEGDA [5, 6]. Additionally, PEGDA can be easily cross-linked to form hydrogels, which uniquely mimic the extracellular matrix environment, [7, 8] making it suitable for most biologic applications.

Because micelles encapsulated in PEGDA hydrogels are likely released through a diffusion-based method, [9-11] we sought to understand the effect of PEGDA molecular weight and hydrogel mesh size, and their effects on micelle release. Three molecular weights of PEGDA have been analyzed. We hypothesized that higher molecular weight PEGDA would produce a hydrogel with larger mesh size as the PEG chain between cross-linking points increases. Likewise, the addition of micelles to the hydrogel is expected to interfere with potential cross-linking points further increasing hydrogel mesh size. A larger hydrogel mesh size would in turn enable faster release of encapsulated micelles and could dramatically impact the kinetics profile of this system [12-15]. Based on literature, some initiators such as TEMED/APS have been reported to be more cytotoxic than Irgacure 2959 and yet work much more quickly in
hydrogel polymerization without the need for UV light [16, 17]. However, the effects of each of these initiators if any on mesh size and subsequently micelle/drug release have yet to be elucidated. In this chapter, we consider the effect of use of different initiators in hydrogel mesh size, subsequent drug-loaded micelle release, and cytotoxicity. Finally, because higher molecular weight PEGDA is expected to release a greater amount of drug-loaded micelles at a similar time, it is expected that the releasate from 20k PEGDA hydrogels will be more cytotoxic to tumor cells short-term than the releasate from 3.4k or 10k PEGDA hydrogels. We tested this hypothesis in this chapter by conducting an MTT assay using U87-MG glioblastoma cells treated with the releasate from different molecular weight hydrogels encapsulating Dox-loaded Pluronic® F-127 micelles.
2.2 Methods

2.2.1 Materials

Doxorubicin hydrochloride was purchased from AK Scientific (Union City, CA, USA). PEGDA molecular weight 3.4, 10, and 20 kDa were purchased from Laysan Bio Inc (Arab, AL, USA). Pluronic® F-127 and Pluronic® L-101 were generous donations from BASF (Chicago, IL). Irgacure 2959 was purchased from Ciba Specialty Chemicals Corp (Tarrytown New York), N-N-N’-N’-tetramethylenediamine was from Sigma (St. Louis, Montana), and ammonium persulfate was from Acros Organics (New Jersey, USA). Methylthiazol Tetrazolium (MTT) assay kit was purchased from Sigma (St. Louis, Montana).

2.2.2 Hydrogel Formation

In order to encapsulate drug loaded micelles in hydrogels, 25 mg of lyophilized, purified micelles was combined with 0.15 g of PEGDA in 920 µL deionized water. Hydrogels were polymerized by adding 35 µL of 20% w/v stock solution of ammonium persulfate and 45 µL of 20% w/v stock solution of N-N-N’-N’-tetramethylenediamine or 0.5% w/v Irgacure 2959 (dissolved in a small amount of ethanol) to the mixture. The entire solution was then poured into a rubber mold and allowed to polymerize for 30 min at 37°C. For Irgacure 2959, hydrogels were left under UV light for a minimum of 8 hours for complete polymerization [18, 19]. Hydrogels were then removed from the mold and cut into 8 mm diameter, 2.5 mm thickness cylinders using biopsy punches for subsequent experiments (Figure 2.1).
Figure 2.1. Diagram of hydrogel formation. Pre-polymerized PEGDA mixture was poured between 2 glass slides with a rubber spacer of 2.5 mm thickness. In order to shape the hydrogels into cylinders, 8 mm diameter biopsy punches were used.

2.2.3 Hydrogel Mesh Size Determination

A hydrogel’s mesh size governs rate of diffusion, what size solutes may pass in and out of the gel, and the mechanical strength of the hydrogel [12, 20]. It is defined as the space bordered by polymer chains as well as crosslinking sites at a given point in time [20]. A hydrogel of a larger mesh size can allow solutes to easily diffuse through (Figure 2.2). Hydrogels of a smaller mesh may entrap and hold large solute molecules within the hydrogel core and therefore drastically slow rate of diffusion.
Figure 2.2. Schematic overview of hydrogels with two different mesh sizes. 
Polymer crosslinking points are also labeled.

As a hydrogel swells and the solvent penetrates the space in between polymer chains, its physical characteristics change from a glassy state (stiff and brittle) to a rubbery state. An increase in radius of gyration as well as the length of polymer between crosslinking points increases during swelling. Equilibrium swelling occurs when the stress of swelling equals the retractive force of elasticity at these crosslinking sites [21]. Volumetric swelling ratio (Q) can be used in the Flory-Rehner Theory which assumes that polymer chain lengths follow a Gaussian distribution, all crosslinks are tetrafunctional, and that the hydrogel structure is neutrally changed. This theory applies to a hydrogel submerged in a nonsolvent. In order to calculate mesh size, we must first calculate the average molecular weight between crosslinks ($M_c$) as derived in Equation 2.1 [1, 22].

$$\frac{1}{M_c} = \frac{2}{M_n} \left( \frac{\nu V_1}{\ln(1-\nu_{2,s}) + \nu_{2,s} + X_1\nu_{2,s}^2} \right) \left[ (\nu_{2,s})^{1/3} - \nu_{2,s}/2 \right]$$

Equation 2.1
Mc is the number average molecular weight between crosslinks, Mn is the number average molecular weight of polymer before crosslinking, v is specific volume of the polymer, V1 is the molar volume of solvent, v2,s is polymer volume fraction in the swollen state, and X1 is the Flory solvent interaction parameter. We can calculate V2,s and V2,r using Equation 2.2 and Equation 2.3.

\[
V_{2,s} = \frac{\frac{m_{s,b}}{P_b}}{\frac{m_{d,a}}{P_p}} \quad \quad V_{2,r} = \frac{\frac{m_{t,b}}{P_b}}{\frac{m_{d,a}}{P_p}}
\]

Equation 2.2 and 2.3

Where m_{s,b} is the mass of the fully swollen hydrogel in a nonsolvent, P_b is the density of the solvent, m_{d,a} is the mass of the xerogel in air, P_p is the density of the polymer, and m_{s,b} is the mass of the hydrogel in a relaxed state in a nonsolvent. Once Mc is found, we can relate that variable to mesh size (\xi) using Equation 2.4 as derived by Peppas and Merrill [11]:

\[
\xi = v_{2,s}^{-1/3}[12M_c/M_l]^{1/2}
\]

Equation 2.4

Where M_r is the molecular weight of each repeating polymer unit and l is the bond length between 2 carbons.

Hydrogel mesh sizes were determined using 3,400, 10,000, or 20,000 Da molecular weight PEGDA made into hydrogels and containing drug-loaded micelles. Hydrogels were weighed while suspended in butanol (a nonsolvent), allowed to swell in fresh phosphate buffered saline until equilibrium was reached (defined as less than 5% change in weight from the previous day when weighed in air), and then weighed in butanol again. Using Archimedes’ principle, hydrogel volume was calculated by dividing weight in butanol over the density of butanol [23].
Hydrogels were freeze-dried using a Labonco lyophilizer for 24 hours and then weighed in air. Hydrogel weight values obtained were used to calculate the mesh size of hydrogels containing micelles using the Flory-Rehner swelling theory equations [24]. Micelles were then extracted from hydrogels by incubating hydrogels in organic solvent for up to 2 weeks until there is no detectable fluorescence in the wash solution. This is to ensure that all detectable drug-loaded micelles have been removed from the hydrogel. Empty hydrogels were subsequently re-swollen in ddH₂O, weighed in butanol (to obtain swollen weight), lyophilized and then weighed in air (to obtain dried weight). This information allowed for the calculation of the mesh size of hydrogels with and without micelles.

2.2.4 Drug-loaded Micelle Release from Different Molecular Weight Hydrogel

Micelle and drug release from hydrogels were examined by incubating Dox-loaded Pluronic® F-127 hydrogels in 2 mL of phosphate-buffered saline at 37°C. At set time points, hydrogel releasate was collected, diluted by adding 3 parts DMSO (in order to disrupt the micelles), measured at 470/570 nm excitation/emission wavelength using a spectrophotometer (RF 1501, Shimadzu, Japan), and then compared to a pre-prepared Dox standard curve. In order to maintain sink conditions, fresh saline was added after each subsequent removal of the old saline at each time point. Dox release was fitted to 0, 1st, 2nd order, Higuchi, and Peppas models in order to better understand the mechanism of drug release [25-27].

2.2.5 Varying Hydrogel Initiators

In order to study the effect of using either initiators TEMED and APS or Irgacure 2959 on hydrogel properties, hydrogels were polymerized using either method (see hydrogel formation
section). Mesh size and Dox-loaded Pluronic® F-127 release was then studied in both hydrogel groups using the methods as outlined above. In order to study cytotoxicity of these initiators, U87-MG glioblastoma cells were incubated with the releasate of hydrogels polymerized using TEMED/APS or Irgacure® 2959 for 24 hours and then a MTT assay [28] conducted to determine cell viability.

2.2.6 Therapeutic Efficacy of Released Micelles from Different Molecular Weight Hydrogels

Hydrogels, 3 mm diameter, containing no micelles, empty Pluronic® F-127 micelles, Dox HCl, or Dox loaded Pluronic® F-127 micelles were preswollen in cell media for a day and then incubated in 100 μL of fresh media for 24, 48, 72, 96, 120, 144, or 168 hours. The media containing hydrogel releasate was then removed and incubated with U87-MG cells (seeded on the previous day at 7,000 per well in a 96-well plate or approximately 32,500 cells per cm²) for 24, 48, 72, 96, 120, 144, or 168 hours. The total incubation period of the specific hydrogel in cell media was equivalent to the total treatment time of its releasate with the cells; for instance, the releasate from a hydrogel incubated for 24 hours was used to treat cells for 24 hours, the releasate from a hydrogel incubated for 48 hours was used to treat cells for 48 hours, releasate from 72 hour incubated hydrogel was used to treat cells for 72 hours, and so forth. An MTT assay was then conducted to examine cell viability as according to MTT Cell Proliferation Assay protocol by American Tissue Culture Collection (ATCC): after treatment, 10 μL of MTT reagent was added, cells were incubated for 4 hours at 37°C until the formation of a purple formazan crystal, 100 μL of DMSO was added to dissolve the crystals, and then absorbance was read using a Labsystems Multiskan Plus Plate Reader (Fisher Scientific) at 570 nm wavelength absorbance.
2.3 Results and Discussion

2.3.1 Hydrogel Mesh Size

Increasing the molecular weight of PEGDA used to form our micelle-encapsulated hydrogels significantly increased the mesh size of the reservoir. Indeed, crosslinking with larger molecular weight PEGDAs resulted in larger overall mesh sizes. This trend was not disturbed with the addition and then removal of drug loaded Pluronic® F-127 micelles. The mesh sizes were 5.0 nm, 10.9 nm, and 19.0 nm, respectively for PEGDA 3.4, 10, and 20 kDa hydrogels. These results corresponded with previously published data from our lab [29]. After doxorubicin, indomethacin, or curcumin Pluronic® F-127 micelle addition and then subsequent removal, there was no significant change in mesh size of these same hydrogels (Figure 2.3). Removal of micelles results in hydrogel mesh sizes reported previously [29]. Our results suggest that addition of drug-loaded Pluronic® F-127 micelles has no effect on hydrogel mesh size.

![Figure 2.3. Addition of Pluronic® F-127 drug loaded micelles does not affect hydrogel mesh size.](image)

Hydrogels crosslinked with PEGDA of varying molecular weights (3.4, 10, and 20 kDa) with no micelles and after removal of
indomethacin-loaded, doxorubicin-loaded, and curcumin-loaded micelles show no significant difference in mesh size. Each bar represents the average plus or minus (±) the standard error of the mean of three independent experiments. Asterisks indicate significant difference (p-value less than 0.5) in mesh size between different molecular weight PEGDA groups.

With an increasing concentration of surfactant, micelle size will increase or micellar aggregation may occur [30]. There is less space between individual micelles in solution which may lead to general instability [31, 32]. In other words, by increasing the concentration of micelle inside the hydrogel, we expect the presence of larger micelles or micellar aggregates which in turn will affect hydrogel mesh size. We selected to increase concentration of micelle in the hydrogel by two-fold as a starting point, the amount is still several times past the CMC of Pluronic® F-127, and further loaded micelle amount (3x or 4x-fold) will create a hydrogel that is too soft to easily handle. Increasing the amount of curcumin loaded Pluronic® F-127 micelles in the hydrogel significantly increased hydrogel mesh size for all molecular weight PEGDA used (Figure 2.4). This was expected as the more micelles that are loaded into the hydrogel, the higher the probability of a micelle of some size will interfere with a crosslinking point and thereby increase mesh size. Taking Figures 2.3 and 2.4 together, it can be suggested that embedded micelles will not significantly affect hydrogel mesh size up to a certain threshold concentration. This threshold is based on the few smaller micelles’ ability to fit in between the hydrogel mesh and not interfere with polymer crosslinking. However, increasing polymer concentration past that threshold level will lead to a higher number of micelles and possibly larger micelle aggregates which increases probability of crosslinking interference.
Figure 2.4. Addition of Pluronic® F-127 drug loaded micelles affected hydrogel mesh size. Hydrogels cross-linked with PEGDA of varying molecular weights (3.4, 10, and 20 kDa) with no micelles added or after addition and then removal of 25 mg/mL of curcumin loaded Pluronic® F-127 micelles and 50 mg/mL (2x-fold) of curcumin loaded Pluronic® F-127 micelles show a significant increase in mesh size for the 50 mg/mL group compared to the 0 mg/mL and 25 mg/mL of added micelles groups (as represented by asterisks where p-value <0.05). Each bar represents the average plus or minus (±) the standard error of the mean of three independent experiments.

Because Pluronic® F-127 micelles are small in size (approximately 5-40 nm in size [33]) and the typical surface of a micelle is fluid and malleable, these particles may fit in the hydrogel mesh without affecting crosslinking. Therefore, the mesh size will not change (as previously observed). We expect a larger micelle of approximately 100-150 nm in diameter and made up of mixed Pluronics® to more significantly affect hydrogel crosslinking and subsequently the mesh size. Addition of larger Pluronic® F-127/L-101 micelles at the lower concentration of 25
mg/mL increased hydrogel mesh size by about 1.5 fold (Figure 2.5). It could be explained by the ideas that since these micelles are much larger than native hydrogel mesh size, micelles cannot fit in the spaces. During hydrogel formation, micelle interactions with polymer chains block some polymerization sites available to initiators. This significantly increases the mesh size.

**Figure 2.5. Addition of larger micelles affected hydrogel mesh size.** Hydrogels cross-composed of PEGDA of varying molecular weights (3.4, 10, and 20 kDa) containing no micelles or after addition and then removal of 25 mg/mL of pyrene-loaded Pluronic® F-127 micelles and 25 mg/mL of pyrene-loaded Pluronic® F-127/L101 micelles show a significant increase in mesh size for the larger Pluronic® F-127/L-101 micelle group. Each bar represents the average plus or minus (±) the standard error of the mean of three independent experiments. Asterisks show a significant difference in mesh size in the Pluronic® F-127/L-101 group only in comparison to no micelles or Pluronic® F-127 groups (p<0.05).
2.3.2 Rate of Drug-loaded Micelle Release from Hydrogels is affected by PEGDA Molecular Weight

We then tested the hypothesis that hydrogel mesh size would affect drug-loaded micelle release. Hydrogels made up of higher molecular weight PEGDA, i.e. 20 kDa, were expected to release drug-loaded micelles at a faster rate than hydrogels of lower molecular weight PEGDA. Theoretically, the larger mesh size would allow for easier escape of micelles out of the matrix through simple diffusion.

Cumulative release of doxorubicin, curcumin, and indomethacin loaded micelles from 3.4 kDa, 10 kDa, and 20 kDa PEGDA hydrogels was rapid within the first 8 hours of incubation with a slower release over the next 10 days (Figure 2.6). Increasing micelle release was observed in hydrogels made up of higher molecular weight PEGDA, since the barrier to diffusion was much smaller. This is expected as the hydrogel acts as a matrix, limiting the diffusion of micelles based on their partitioning between the hydrogel and surrounding environment. After 10 days, less than fifty percent of doxorubicin or indomethacin loaded micelles were released and less than forty percent for curcumin loaded micelles. Such release suggests that long-term and constant micelle and subsequent drug release is obtainable for all molecular weight PEGDA hydrogels tested.
Figure 2.6. Cumulative release of drug-loaded Pluronic® F-127 micelles from hydrogels composed of 3.4 kDa, 10 kDa, or 20 kDa PEGDA over 10 days.

DOX-loaded Pluronic® F-127 release (A) showed a strong positive linear correlation between rate of micelle release and hydrogel mesh size. This holds true for indomethacin-loaded Pluronic® F-127 release as well (B) as well as for curcumin-loaded Pluronic® F-127 micelles (C). Data was fitted once steady state was achieved (approximately after 24 hours of drug release). Points represent the average of three independent experiment ± the standard error of the mean (error bars).

2.3.3 Mechanism for Long-Term Micelle Release from Hydrogel

Various models were tested to in order to explain the drug release rate kinetics of drug-loaded micelles released from hydrogel. The data was fitted to zero-order, first-order, Higuchi, and Korsmeyer-Peppas release models and the R² calculated. A R² value closest to a value of 1 for that model was assumed to demonstrate best fit.
Figure 2.7. DOX-loaded Pluronic® F-127 micelle or DOX HCl release from 3.4 kDa (diamond), 10 kDa (triangle), or 20 kDa (square) PEGDA hydrogels. Release was then fitted to Higuchi or Korsmeyer-Peppas release models with calculated $R^2$ and n-values presented as shown. Highest $R^2$-values are highlighted in red on the chart as shown. Graph shows data plotted according to Higuchi release model (total percentage of DOX release versus the square root of time) which had best fit.

The Higuchi’s model for drug release best fits this micelle/hydrogel system (in comparison to 0, 1st, and Korsmeyer-Peppas models) (Figure 2.7). The Higuchi model is based off of a number of important assumptions including i) the concentration of the drug in the system is much greater than its solubility ii) edge effects are negligible iii) drug particles are very small and are therefore much smaller than the thickness of the system iv) perfect sink conditions are maintained and v) drug diffusion is constant. Higuchi’s model is often applied to matrix systems including porous structures (such as a hydrogel in this case) and as can be noted, is a more specific case of the Korsmeyer-Peppas model where the variable n is equal to 0.5 [25]. The basic equation of the Higuchi model is shown below:

$$\frac{M_t}{M_\infty} = kt^{0.5} \quad \text{Equation 2.5}$$

where $M_t$ is the cumulative amount of drug released at time $t$, $M_\infty$ is total amount of drug available in the system, $K$ is a constant, and $t$ is the time.

Therefore, the fraction of drug released is equal to the square root of time [26, 27]. In Higuchi’s model, diffusion is the dominating mechanism for drug release. Based on this model being the
best fit for our system, it can thus be concluded that drug-loaded micelles are released from the pre-swollen hydrogel matrix mainly through diffusion.

2.3.4 Type of Initiator affects Hydrogel Properties

Previously published research has shown that TEMED and APS (particularly TEMED) can be cytotoxic to cells [16, 34]. Attempts to reduce such cytotoxicity include using a different initiator to polymerize the hydrogel, using lower concentrations of initiators, or washing the hydrogel in order to flush out excess initiators. Being that our hydrogel delivery system must be implanted in the body and cannot be washed beforehand, we looked into using the photoinitiator Irgacure 2959 as a means for hydrogel polymerization. U87-MG glioblastoma cells were incubated with the releasate of 3.4 kDa, 10 kDa, or 20 kDa PEGDA hydrogels polymerized using APS and TEMED or Irgacure 2959 for 24 hours. An MTS assay showed significantly higher levels of cytotoxicity (about 20-30% more) in cells treated with the releasate of hydrogels polymerized by TEMED and APS (Figure 2.8).

![Figure 2.8. Use of different initiators significantly impacts percentage cell viability.](image)

Figure 2.8. Use of different initiators significantly impacts percentage cell viability. Blank 3.4 kDa, 10 kDa, or 20 kDa PEGDA hydrogels which were polymerized using APS and TEMED or Irgacure 2959 were incubated in DMEM
for 24 hours. U87-MG glioblastoma cells were treated with this hydrogel releasate for 24 hours. An MTT assay was then conducted in order to determine cellular viability. Points represent the average of three independent experiment ± the standard error of the mean (error bars). Asterisks represent a significant difference between initiator groups tested (p>0.05).

It is also important (especially as previous hydrogel release experiments were conducted using APS and TEMED) to examine the effects if any of using a different initiator or hydrogel mesh size and therefore drug-loaded micelle release. The mesh sizes of 3.4 kDa, 10 kDa, or 20 kDa PEGDA hydrogels polymerized using 0.15% w/v APS and TEMED and 0.5% w/v Irgacure 2959 (Figure 2.9) were determined. TEMED and APS polymerization produced hydrogels of significantly larger mesh sizes for all molecular weight PEGDA tested as compared to Irgacure 2959 polymerization. In order to determine if this increase in mesh size is sufficient to change rate of drug-loaded micelle release, DOX-loaded Pluronic® F-127 micelles were encapsulated and then released from 3.4 kDa, 10 kDa, or 20 kDa PEGDA hydrogels polymerized by the two different methods. The rate of drug-loaded micelle did not significantly change leading to the observation that the slight but significant change in hydrogel mesh size was not enough to impact micelle release (Figure 2.10).
Figure 2.9. Use of different polymerization initiators effects hydrogel mesh size. 3.4 kDa, 10 kDa, and 20 kDa PEGDA hydrogels were polymerized using 0.15% w/v TEMED and APS or 0.5% w/v Irgacure 2959 and mesh sizes were then measured for comparison. Points represent the average of three independent experiment ± the standard error of the mean (error bars). Asterisks represent a significant difference between initiator groups tested for 10 kDa and 20 kDa but not for 3.4 kDa (p>0.05).

Figure 2.10. Use of different initiators does not significantly affect rate of drug-loaded micelle release. DOX-loaded Pluronic® F-127 were encapsulated
and then released from 3.4 kDa, 10 kDa, or 20 kDa PEGDA hydrogels over a period of 5 days. Left hand graph shows the results of hydrogels polymerized using 0.15% w/v APS and TEMED while the right hand graph shows the results of hydrogels polymerized using 0.5% w/v Irgacure 2959. Points represent the average of three independent experiment ± the standard error of the mean (error bars).

2.3.5 Activity of Released DOX-loaded Micelles using U87-MG Glioblastoma Cells

It was observed that a higher molecular weight PEGDA hydrogel (20 kDa) will release more micelles over a short period of time compared to a lower molecular weight PEGDA hydrogel (3.4 kDa). More micelles should show more cytotoxicity towards tumor cells. Thus, a 20 kDa PEGDA hydrogel is hypothesized to be more effective in killing tumor cells than a 3.4 kDa PEGDA hydrogel. In order to show that DOX-loaded Pluronic® micelles released from hydrogels have therapeutic efficacy and that hydrogels made up of different molecular weight PEGDA may have different levels of effectiveness, U87-MG glioblastoma cells were treated with hydrogel releasates for up to a period of 7 days. DOX-loaded Pluronic® F-127 micelles, DOX HCl, empty Pluronic® F-127 micelles, or no micelles were encapsulated in 3 mm diameter 1 mm height 3.4 kDa, 10 kDa, or 20 kDa PEGDA hydrogels. Hydrogel releasates were incubated with the cells for up to 7 days and then an MTT assay conducted to test for drug activity.

The releasate from hydrogels containing no micelles or empty Pluronic® F-127 micelles showed negligible toxicity towards cells up to 7 days of treatment (Figure 2.11). Hydrogel releasate containing DOX HCl or DOX loaded Pluronic® F-127 micelles (scaled to same amount
of drug) showed similar cytotoxicity levels. This demonstrates that Pluronic® micelles are just as cytotoxic as the pure drug itself at the same drug loaded concentration. IC_{50} was reached around day 2 of treatment for both DOX HCl and DOX-loaded micelle treatment groups. When comparing cell survival among the 3.4 kDa, 10 kDa, and 20 kDa PEGDA hydrogels groups, there was a twofold difference after day 7 when comparing 3.4 kDa and 10 kDa or 20 kDa (20% compared to 10%). This was assumed to be due to the larger mesh sizes of the 10 kDa and 20 kDa PEGDA hydrogels which allows for the release of more drug-loaded micelles.

Figure 2.11. U87-MG glioblastoma cells treated with releasate from blank hydrogels or hydrogels containing either empty Pluronic® F-127 micelles, DOX HCl, or DOX-loaded Pluronic® F-127 micelles. (A) MTS assay results
using 3.4 kDa PEGDA hydrogels, (B) 10 kDa PEGDA hydrogels, and (C) 20 kDa PEGDA hydrogels is presented. (D) Data regraphed with only 7 day time points to better show statistical significance between different molecular weight PEGDA hydrogels. Points represent the average of three independent experiment ± the standard error of the mean (error bars).
2.4 Conclusion

Taken together, our results both confirmed as well as refuted our original hypotheses. Increasing polymer molecular weight will increase hydrogel mesh size due to a higher number of PEGDA repeats in between crosslinking sites. Addition of smaller micelles did not alter native hydrogel mesh size but addition of larger mixed Pluronic® micelles did. Increasing the amount of smaller encapsulated micelles by twofold in the hydrogel saw a significant increase in hydrogel mesh size. This data suggests that micelles or aggregates of smaller micelles which are larger than native mesh size will interfere with normal hydrogel crosslinking and increase the mesh size.

As was also expected, we observed a higher rate of drug-loaded micelle release from higher molecular weight PEGDA hydrogels. This could be due to larger spaces in the hydrogel which enable for faster micelle escape in an aqueous environment. A recent study by Kirchhof et al using PEG of different molecular weight based hydrogels for antibody release confirms that rate of release is based largely on the shape and size of the antibody as well as hydrogel mesh size. The authors also discovered that linear flexible proteins can pass through a smaller than its hydrodynamic radius hydrogel mesh size. However, a too-small hydrogel mesh size will trap large globular proteins such as bevacizumab and prevent release [35]. Similarly, Tong et al observed less burst release and a slower rate of release of bovine serum albumin (BSA) using PEG-based hydrogels of a mesh size that is smaller than protein hydrodynamic radius [36]. This same phenomenon was observed with our own micelle in hydrogel system and predicts that larger than mesh size micelles should still be able to be released from the hydrogel because the polymers making up the micelle are very dynamic in solution.
In the modeling on this system, we observed our device most closely coincided with the Higuchi model for drug release where an encapsulated substance (in this case, the micelle) follows the laws of diffusion from an area of high concentration (the hydrogel) to an area of low concentration (in this case, an outside aqueous environment). The amount of drug released at a specific time equals a generalized diffusion constant (K) multiplied by the square of time. A recent publication by Gao et al using free lipsomes encapsulated in a hydrogel found that rate of liposome release also corresponded most closely to Higuchi’s model [37]. Although the authors used liposomes compared to micelles in their system, their model was very comparable to ours as the drug-loaded liposomes were not conjugated but were allowed to leave the hydrogel mainly by diffusion. We can change the hydrogel mesh size by not only using different molecular weight polymers but also by altering the polymerization initiators used. Irgacure 2959 followed by 24 hours exposure to UV light created hydrogels of significantly smaller mesh size and lower cytotoxicity than those polymerized using TEMED/APS. However, this small difference in mesh size did not significantly alter rate of drug-loaded micelle release. A previously published study in our lab has established that TEMED/APS has cytotoxic effects against cells so these results were not very surprising [16]. Moreover, it is known that changing the concentration of initiators or mode of polymerization will result in different levels of hydrogel crosslinking efficiency and therefore alter the crosslinking density [38-40]. In our case, UV polymerization using Irgacure 2959 is more efficient and created a smaller hydrogel mesh size compared to TEMED/APS. However, this change in mesh size was below the threshold level for effecting rate of drug-loaded micelle release as we did not observe a difference.

The results from in-vitro experiments showed a slight but significant cytotoxicity level for U87-MG cells treated with the releasate of higher molecular weight PEGDA hydrogels
compared to lower molecular weight PEGDA hydrogels. We further believe that a more significant cellular response might be observed using a more cytotoxic or fast-acting drug compared to Dox [41], loading more drug into the hydrogels, or even by using bigger-sized hydrogels with larger welled plates containing more cells per well. Nair et al showed that U87-MG cells typically take up to 72 hours to reach IC\textsubscript{50} when treated with <10 μM Dox [42]. When treated with a very high concentration of 100 μM Dox, IC\textsubscript{50} was reached after 24 hours [42]. Observations during our experiments showed that by the 4\textsuperscript{th} day, most of the U87-MG cells have detached from the plate surface and are apoptotic. This leaves a very narrow window by which to detect any significance between treatment groups, particularly hydrogel mesh size where the difference of released Dox varies by less than a few micrograms over a 5 day period between 3.4 kDa and 20 kDa PEGDA gels.

All in all, our results show the highly tune-ability of hydrogels in this system and its properties which effect rate of drug release. Each component (be it drug, micelle, or hydrogel) in the micelle/hydrogel device is interrelated and it’s critical to elucidate how one may alter the other. Furthermore, we highlighted the therapeutic potential of using drug-loaded micelles in hydrogels in cancer treatment and showed that released drug-loaded micelles are just as toxic to tumor cells as the hydrophilic form of the drug itself. This is very important as it suggests cells are able to take up these Pluronic® F-127 micelles and that the micelles are able to release its drug-loaded contents once inside the cell [43, 44].
2.5 References


CHAPTER 3
UNDERSTANDING MICELLE PROPERTIES AND EFFECTS ON RELEASE FROM THE HYDROGEL

3.1 Introduction

In the previous chapter, we studied the effect of changing a hydrogel property, mesh size, on overall drug release. We discovered that a larger mesh size will enable faster release of the drug and that the presence of drug-loaded micelles embedded in the hydrogel or use of different initiators significantly alters hydrogel mesh size. Conversely, it can be deduced that certain micelle properties such as size and certain drug properties such as hydrophobicity or molecular weight which may or may not impact micelle size should also affect drug-loaded micelle release from the hydrogel [1]. It has been reported in literature that more hydrophobic moieties on a polymer will allow for formation of smaller micelles due to increased hydrophobic interactions [2]. Similarly, if two polymers are of similar solubility and composition, the higher molecular weight polymer will have a lower critical micelle concentration and have the ability to form smaller micelles [3]. For example, it is generally known that PLA-PEG block copolymer micelles depend on the ratio of hydrophobic to hydrophilic portions as well as overall polymer length [4, 5]. Somekawa et al showed that the multiblock index (or block number of copolymers) increases polymer molecular weight but decreases micelle size. This multiblock index played a greater role in determining micelle size than hydrophobic to hydrophilic composition ratios or polymer molecular weight [4].

Other factors which may affect micelle size include charge density of the environment [6], micelle core crystallinity [7, 8], and surfactant concentration and overall structure [9, 10]. The majority of work focuses on polymer properties and its effect on micelle size. However to
our knowledge, not much has been shown relating loaded drug properties to micelle size. Micelle size not only effects general rate of release but impacts everything from cell uptake [11] to tumor penetration and retention [12-14]. Consequently, it is a critical component to understand in our micelle/hydrogel delivery system. In this chapter, we plan to test two main hypotheses: 1) general hydrophobicity of the drug or Pluronic® polymer will impact micelle size; and 2) a smaller micelle will be released at a faster rate from the hydrogel than a larger micelle. By altering micelle size through drug or polymer modifications, we hope to achieve control over micelle from hydrogel release.
3.2 Methods

3.2.1 Materials

Doxorubicin hydrochloride was purchased from AK Scientific (Union City, CA, USA), Indomethacin from Sigma (St Louis, MO, USA), and Curcumin was obtained courtesy of Dr. Xiaolong He previously at the University of Illinois, department of Biopharmaceutical Sciences. Pyrene was purchased from Sigma Aldrich (St. Louis, MO, USA). 3,3'-Dioctadecyloxacarbocyanine Perchlorate (DiO) and 1,1'-Dioctadecyl-3,3',3',3'-Tetramethylindocarbocyanine Perchlorate (DiI) were purchased from Fisher Scientific. PEGDA molecular weight 3.4 kDa, 10 kDa, and 20 kDa were purchased from Laysan Bio Inc (Arab, AL, USA). Pluronic® F-127, L-121, L-101, L-61, and P-105 were generous donations from BASF (Chicago, IL, USA). Triethylamine was from Sigma (Milwaukee, WI), 0.22 μm syringe filters were purchased from GE Healthcare Life Sciences (Buckinghamshire, UK).

3.2.2 Micelle Formation

The critical micelle concentrations of Pluronic® F-127 and Pluronic® F-127/L-101 mixed micelle were determined using pyrene fluorescent shift on SpectraMAX GeminiXS microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA) [15]. Pluronic® F-127 micelles encapsulating Dox, pyrene, or the fluorescence resonance energy transfer (FRET) pair Dil and DiO were made through the oil-in-water emulsion method (Figure 3.1) [16, 17]. Briefly, 6 mg of Dox HCl was combined with 0.5 mL of chloroform containing 20 μL of triethylamine to form doxorubicin free base. The drug solution was then added drop wise to a solution of 60 mg Pluronic® F-127 in 120 mL of deionized water under rapid stirring. Rapid stirring and solvent evaporation overnight in the dark allowed for micelle formation. Unincorporated drug was
removed through centrifugation at 4,000 rpm for 15 min followed by filtration through a Medical Millex-GV Syringe Filter 0.22 μm, PVDF, 33 mm, gamma sterilized. Micelle solutions were frozen overnight in the -20°C freezer and then lyophilized using a Labconco FreeZone® 6 Litre FreezeDry System (Labconco, Kansas, MO). Lyophilized micelles were stored in 50 mL conical vials in the -20°C freezer until use.

Figure 3.1. Schematic overview of drug-loaded Pluronic® micelle formation.

All micelles are made using the oil-in-water emulsion method.

Mixed Pluronic® F-127 and L-101, L-121, or L-61 micelles were also made using the oil-in-water emulsion method. 0.1 g of Pluronic® F-127 and 0.1 g of the hydrophobic Pluronic® was mixed with 100 mL of water and left to stir in the fridge overnight until fully dissolved. For a typical drug to polymer ratio of 1:10, 20 mg of Dox, Indo, or Curcumin was dissolved in chloroform and added drop-wise to the polymer solution. Solution was left to stir in the fridge in the dark overnight in order to allow for micelle formation. Micelle solution was then heated for 1 hour at 70°C to form smaller, more stable micelles. Unincorporated drug was removed through centrifugation at 4,000 rpm for 15 min followed by filtration through a 0.45 μm filter and then stored as a lyophilized powder in the freezer.
3.2.3 Micelle Characterization

Micelle diameter and ζ-potential were determined by Nicomp 380 Zeta Potential/Particle Sizer. Dox encapsulation efficiency was determined by dissolving a known amount of micelles in 1:4 H₂O:DMSO and then comparing to a prepared Dox standard curve. Encapsulation efficiency was then calculated as the ratio of actual drug loaded into micelles over theoretical loading x 100%. To test for micelle stability, solutions of 1 mg/mL micelles were left at room temperature and then periodically observed for any precipitations for up to a period of 7 days. In order to test for micelle activity compared to pure drug, U87-MG cells were incubated with different concentrations of Dox HCl or Dox-loaded Pluronic® F-127 micelles for 24 hours and then an MTT assay conducted [18].

3.2.4 Drug-loaded Micelle Release from Hydrogel

The effect of using a drug with differing hydrophobicities on overall micelle release from hydrogels were examined by incubating Dox, indo, curcumin-loaded Pluronic® F-127 hydrogels in 2 mL of phosphate-buffered saline at 37 C. At set time points, hydrogel releasate was collected, diluted by adding 3 parts DMSO (in order to disrupt the micelles), measured at 470/570 nm ex/em using a spectrophotometer (RF 1501, Shimadzu, Japan), and then compared to a prepared drug standard curve. In order to maintain sink conditions, fresh saline was added after the removal of the old after each time point. Dox and curcumin release was fitted to 0, 1st, 2nd order, Higuchi, and Peppas models for comparison. The experiment was stopped when more than 90% of loaded drug was released from the hydrogel (in this case, approximately 30 days for Dox).
3.3 Results and Discussion

3.3.1 Micelle Formation and Characterization

We have chosen to use the Pluronic® block copolymers (PBC) as a platform for our micelles. These block copolymers consist of ethylene oxide (EO) and propylene oxide (PO) blocks, which can spontaneously self-assemble into micelle structures of approximately 5-200 nm in size in aqueous solutions [19]. Pluronics® can be easily synthesized by sequential polymerization of PO and EO blocks in the presence of an alkaline catalyst [20]. Typically, this results in polymers with a low polydispersity index but chromatographic fractionation may be employed to remove impurities [21]. A summary of all Pluronics® that have been characterized is presented in Table 3.1. We ultimately decided to select Pluronic® F-127 and Pluronic® F-127/L-101 micelles for future studies for a number of reasons but mainly due to a large size difference. Pluronic® F-127 was shown to be the most nontoxic of all the poloxamers and was able to form micelles at low polymer concentrations (low CMC), and more importantly formed micelles of approximately 10-50 nm (Table 3.1) [22]. Structurally, Pluronic® F-127 has an average molecular mass of 13,000 Da and contains approximately 70% ethylene oxide and 30% propylene oxide which accounts for its hydrophilicity [21]. Pluronic® F-127 was also shown to have the highest DOX drug encapsulation efficiency in comparison to other Pluronics® or mixed Pluronics®. This is critical because all activity studies using this drug delivery platform will use DOX as a model API. Drug-loaded micelles formed from Pluronic® F-127 were also shown to be stable for up to 7 days in solution with no drug precipitation. In contrast, Pluronic® F-127/L-101 micelles are approximately 150 nm in size, are shown to be stable in solution for up to 5 days, and from our study using U87-MG cells, are found to be nontoxic. Thus, the 3-15 fold size
The difference between Pluronic® F-127 micelles and Pluronic® F-127/L-101 micelles would enable us to further study the difference in release from a hydrogel with a smaller versus larger micelle.

Table 3.1. Summary of various Pluronics® tested comparing overall micelle stability in solution over the course of 7 days at room temperature, CMC, particle size, DOX and indomethacin encapsulation efficiency, and IC50 using U87-MG glioblastoma cells.

<table>
<thead>
<tr>
<th>Pluronic®</th>
<th>Stability</th>
<th>CMC (M)</th>
<th>Size (nm)</th>
<th>DOX Encapsulation Efficiency (%)</th>
<th>Indo Encapsulation Efficiency (%)</th>
<th>IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-127</td>
<td>Stable for &gt;7 days at room temperature</td>
<td>2.48 x 10^-5</td>
<td>~9</td>
<td>30.8±7.4</td>
<td>47.4 ± 6.96</td>
<td>&gt;1379 μM</td>
</tr>
<tr>
<td>P-105</td>
<td>Stable for &gt;5 days at room temperature</td>
<td>3.84 x 10^-3</td>
<td>~7</td>
<td>22.3 ± 3.43</td>
<td>70.3 ± 5.26</td>
<td>1379 μM</td>
</tr>
<tr>
<td>L-61</td>
<td>Stable only at 4°C</td>
<td>1.25 x 10^-3</td>
<td>&gt;1000</td>
<td>21.7 ± 5.77</td>
<td>64.7 ± 9.49</td>
<td>86 μM</td>
</tr>
<tr>
<td>F-127/L121</td>
<td>Stable for &gt;7 days at room temperature</td>
<td>7.73 x 10^-4</td>
<td>&gt;200</td>
<td>9.15±0.37</td>
<td>15.1 ± 0.64</td>
<td>&gt;1379 μM</td>
</tr>
<tr>
<td>F-127/L101</td>
<td>Stable for &gt;5 days at room temperature</td>
<td>8.93 x 10^-4</td>
<td>&gt;150</td>
<td>9.24±0.21</td>
<td>-</td>
<td>1379 μM</td>
</tr>
<tr>
<td>L61/P-105</td>
<td>Stable for &gt;7 days at room temperature</td>
<td>8.93 x 10^-5</td>
<td>~7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

All Pluronics® are, as denoted by the first letter followed by a dash for each named type, available in various physical states at room temperature. For example, Pluronic® L-101 or L-61 contain the letter “L” which signifies that these Pluronics® are liquids. Similarly Pluronic® F-127 has an “F” for flake and Pluronic® P-105 has a “P” for paste [23]. Loading drug into these micelles and then lyophilizing the micelle solutions does not alter the physical state of the Pluronic® (Figure 3.2).
Figure 3.2. From left to right: DOX loaded Pluronic® L-61 micelles (shown as a liquid), DOX loaded Pluronic® P-105 micelles (shown as a paste), and DOX loaded Pluronic® F-127 micelles (shown as a flake). All samples were lyophilized overnight using a Labconco FreeZone® 6 Litre FreezeDry System.

Pluronic® F-127 combined with Pluronic® L-101 will form large mixed Pluronic® micelles of approximately 100-150 nm in diameter. Hydrogels have a mesh-like structure where particles of a certain size may not be able to pass through. Therefore in these two micelle populations of varying size, it was assumed that the larger mixed micelles will be unable to fit through the hydrogel mesh and be trapped inside while the smaller Pluronic® F-127 micelles will be able to easily pass through.

Just as we can alter the micelle size by changing the type of Pluronic® used, we have attempted to alter micelle properties by changing the type of drug encapsulated. We chose to load three different APIs into our micelles, doxorubicin, indomethacin, and curcumin because they vary greatly in hydrophobicity [24]. These drugs differ in their octanol/water partition coefficient by several orders of magnitude (logP of indomethacin is 4.25, logP of curcumin is
3.28, and logP of doxorubicin is 1.27) [24], allowing us to also understand the effect that lipophilicity of the drugs has on release of micelles from a hydrogel system.

Comparable to what has been cited in the literature [25], the critical micelle concentration of Pluronic® F-127 was determined to be $2.48 \times 10^{-5}$ M. When loaded with doxorubicin, micelles were found to be $41.2 \pm 2.7$ nm in diameter compared to the smaller empty Pluronic® F-127 micelles which were $8.48 \pm 2.35$ nm in diameter (Figure 3.3). Micelles loaded with indomethacin were smallest in size, approximately $5.9 \pm 2.0$ nm in diameter (Figure 2). Micelles encapsulating curcumin were $21.6 \pm 7.23$ nm and in between the sizes for Dox-loaded and curcumin-loaded micelles. This shift in size of micelle is expected based on the relative hydrophobicity of each drug. A general observation of drug-loaded micelles is that their size is dependent on the hydrophobicity of loaded drug, with highly hydrophobic drugs resulting in tighter cores and smaller sizes of micelles [26].
Figure 3.3. Micelle size is related to drug properties. (A) Pluronic® F-127 micelles loaded with doxorubicin, indomethacin, curcumin or no drug exhibit mono-modal distribution and micelle size increases with increasing drug hydrophilicity. (B) Graph relating micelle size to logP of the drug encapsulated. Average sizes of empty, doxorubicin, curcumin, and indomethacin loaded micelles based on three independent experiments.

Drug loading or encapsulation efficiency of the micelles was determined in order to have an understanding of the approximate percentage of micelles released over time. Additionally,
encapsulation efficiency of our micelles is important to rule out potential drug crystallization or precipitation when micelles are diluted in an aqueous environment *in vivo*. The encapsulation efficiencies we report are on the order of what has been reported in the literature [1, 27, 28]. Doxorubicin encapsulation efficiency was most effective, 30.8±7.4 %, at a drug to polymer ratio of 1 to 10 by weight. Similarly, curcumin and indomethacin showed the greatest encapsulation efficiency at 39.7±1.4% and 47.4±6.96% (respectively) with a 1 to 10 drug to polymer ratio (Figure 3.4). Encapsulation efficiency decreased with increasing drug to polymer ratio. This could be due to increasing hydrophobic interactions between drug particles compared to drug and polymer leading to more drug aggregation. Consequently, micelles used in the remainder of these experiments were made using the 1 to 10 drug to polymer ratio. We also observed that the more hydrophobic the drug, the higher the encapsulation efficiency in the micelle at a drug to polymer ratio of 1 to 10. Similarly, this may be due to increased intermolecular interactions between the more hydrophobic drug and polymer compared to the drug and water.

![Graph showing encapsulation efficiency](image)

**Figure 3.4. Drug encapsulation efficiency in micelles.** Encapsulation of drug into micelles was highest at a drug to polymer weight ratio of 1:10 for doxorubicin as well as for curcumin and indomethacin. Points represent average
plus or minus (±) the standard error of the mean based on three independent experiments.

3.3.2 Rate of Drug-loaded Micelle Release from Hydrogels is also affected by Drug Hydrophobicity (A Short-Term Release Study)

Although smaller micelles would be expected to release faster out of the hydrogel, this was not the case. As was observed, a lower percentage of indomethacin-loaded micelles were released over time compared to the larger curcumin or doxorubicin-loaded micelles (Figure 4). In fact, we found that the more hydrophilic the drug loaded into the micelle, the faster the rate of drug release from the hydrogel/micelle system (Figure 3.5). The overall higher hydrophobicity of these micelles due to drug loaded may have had significant effect on the diffusion and partitioning of the micelles between the aqueous and polymer phases. On the other hand, if we are to assume some of the drug must escape out of the core of more centrally bound micelles in order to diffuse out of the hydrogel system, a more hydrophilic drug is typically held less tightly, can better dissolve in the surrounding aqueous environment, and therefore would be more able to easily diffuse out of the micelle.
Figure 3.5. Cumulative release of different drug-loaded Pluronic® F-127 micelles from hydrogels composed of (A) 3.4 kDa, (B) 10 kDa, or (C) 20 kDa PEGDA over 10 days. DOX-loaded Pluronic® F-127 release showed a strong positive linear correlation between rate of micelle release and drug hydrophilicity. Rate was calculated once steady state has been achieved—usually after 24 hours. This holds true for indomethacin-loaded Pluronic® F-127 release as well as for curcumin-loaded Pluronic® F-127 release. Points represent the average of three independent experiment ± the standard error of the mean (error bars).
Therefore, our results suggest that altering the hydrogel mesh size or the micelle cargo can vary amount and duration of micelle release from the hydrogel matrix. Diffusion through hydrogels with larger mesh is likely to be more efficient and faster than those with smaller mesh sizes. Modulation of mesh size of our hydrogel matrix can limit or extend amount of release over time. Having continuous release from the hydrogel is advantageous for our micelles because it allows for long-term administration of toxic drugs in a localized manner. Moreover, release can be tailored specifically and modified via polymer choice and crosslinking of the hydrogel, allowing for a fine-tunable system [29].

3.3.3 Mechanism for Long-Term Dox-loaded Micelle compared to Curcumin-loaded Micelle Release from Hydrogel

We looked at the release kinetics for a slightly hydrophilic drug (Dox) compared to a more hydrophobic drug (curcumin). Both these drugs are extremely fluorescent and easily detectable at even minute quantities when released from the hydrogel using a basic spectrophotometer (unlike indo which relies on absorbance). Dox or curcumin were loaded into Pluronic® F-127 micelles and then released from 3.4 kDa, 10 kDa, and 20 kDa PEGDA hydrogels for over 30 days (until more than 90% of drug loaded was released). Again, the data was fitted to zero-order, first-order, Higuchi, and Korsmeyer-Peppas release models and the $R^2$ calculated [30-32]. An $R^2$ closest to a value of 1 for that model showed best fit. Rate of release is faster for the Dox-loaded Pluronic® F-127 micelles for all molecular weight PEGDA hydrogels compared to curcumin-loaded Pluronic® F-127 micelles (Figure 5). This was unexpected as we have shown that curcumin-loaded Pluronic® F-127 micelles are statistically
smaller than Dox-loaded Pluronic® F-127 micelles. Hence, rate of micelle release is not governed by size alone and must be attributed to other factors.

Another interesting observation is that the release of hydrophilic drug from the hydrogel is faster than hydrophobic drug from the hydrogel/micelle system (Figure 3.6A). Similarly, the release of pure hydrophobic drug from the hydrogel is slower than the same hydrophobic drug release from the hydrogel/micelle release (Figure 3.6B). We can infer from this observation that having drug-loaded micelles present in the hydrogel will slow rate of drug release. This could be due to either the drug having to leave the micelle embedded in the hydrogel first or the entire drug-loaded micelle (which is significantly larger than the pure drug molecule) being released [33]. Extending rate of drug release would be beneficial in a number of circumstances (as mentioned in chapter 1) where we might prolong absorption of drugs with shorter half-lives, improve tolerability of the drug to the patient and thereby reduce side effects, and reduce the dosing intervals [34-36]
Figure 3.6. (A) **DOX-loaded Pluronic® F-127 micelle or DOX HCl release** from 3.4 kDa (diamond), 10 kDa (triangle), or 20 kDa (square) PEGDA hydrogels. Release was then fitted to Higuchi or Korsmeyer-Peppas release models with calculated R² and n-values presented as shown. Highest R²-values are highlighted in red on the chart as shown. Graph shows data plotted according to Higuchi release model (total percentage of DOX release versus the square root of time) which had best fit. (B) **Curcumin-loaded Pluronic® F-127 micelle or curcumin release** from 3.4 kDa (diamond), 10 kDa (triangle), or 20 kDa (square) PEGDA hydrogels. Points represent the average of three independent experiment ± the standard error of the mean (error bars).
3.4 Conclusion

In this chapter, we have shown that we can easily encapsulate indomethacin, doxorubicin, and curcumin inside Pluronic® micelles. These micelles depending on the type of Pluronic® used have demonstrated low CMC, are stable in solution and empty micelles are considered non-cytotoxic. Drug loading capacity for these polymers is also good and is shown to be highest when at a ratio of 1:10 weight percentage drug to polymer. Most importantly, micelle size can be easily manipulated by changing the type of Pluronic® or by mixing two types (which also was shown to increase overall stability compared to using one Pluronic® alone) and by changing the type of drug loaded. Micelle formation results from a reduction in free energy from the removal of hydrophobic blocks from the aqueous environment. These hydrophobic blocks then rearrange themselves in the micelle corona and are typically held together by van der waal interactions [37, 38]. Typically, a very hydrophilic polymer has a high critical micelle concentration and will tend to exist in solution as a unimer while a very hydrophobic polymer will form nonmicellar structures such as lamellae and rods [39]. This explains why hydrophobic Pluronics® such as L-61 or L-101 will form large aggregates of greater than 1 µm while Pluronics® with higher hydrophilic content will micelles of approximately 10 nm in size. Similarly, a more hydrophobic drug such as Indo was found to form smaller micelles compared to Dox. This was assumed to be due to increased van der waal forces as well as other intramolecular interactions between the hydrophobic portions of the polymer and drug and thus pulling the polymer chains closer towards the corona.

Initially, we had assumed a more hydrophobic drug will form smaller micelles which will be released faster from the hydrogel network. Thus rate of release would be partly contingent on micelle size as well as hydrogel mesh size. Surprisingly, our experiments showed
that there was no trend between micelle size and rate of release-Dox loaded micelles which are significantly larger than curcumin loaded micelles are still released faster. However, there was a relationship between rate of drug release and drug solubility in water. We have observed that with increasing drug solubility in an aqueous environment: Indo with a solubility of 0.009 mg/mL [40], curcumin at <0.1 mg/mL [41], and Dox at 7 mg/mL [42], there was a higher rate of drug release from the hydrogel. This could be due to mainly a very hydrophobic drug being more tightly bound to the micelle core. In such a situation, the majority of the drug molecules cannot easily escape out of the micelle corona in order to diffuse out of the hydrogel. If the micelle is larger than the hydrogel mesh size, the drug is essentially “stuck” along with the micelle. Kataoka et al has shown that using triethylamine to make a drug (Dox) more hydrophobic allowed for the formation of micelles with significantly higher drug loading [43]. Thus, a more hydrophobic drug is more likely to remain inside the micelle. On the other hand, a more hydrophilic drug can readily leave the micelle into the aqueous surroundings. This phenomenon was reported in several published studies where the authors reported drug diffusion out of the micelle/hydrogel [44] or liposome/hydrogel system [45]. However, little research has been done for whole intact drug-loaded micelle release from the hydrogel system. This serves the basis for chapter 4 of this thesis work.
3.5 References


CHAPTER 4

HYDROGEL/MICELLE MULTISCALE DRUG DELIVERY SYSTEM WILL RELEASE WHOLE INTACT MICELLES

4.1 Introduction

Previously published literature has tended to focus on the therapeutic aspects of nanoparticle encapsulated in hydrogel systems [1-3] versus the mechanism of nanoparticle release. In these published works, the authors had assumed the drug must first escape the hydrogel encapsulated micelle. Other works attempting to elucidate micelle release from hydrogels tended to focus on reversible gels where at a certain temperature or pH, the hydrogel will break down into micelles [4-6]. These systems are often injectable, will polymerize in-situ, will release drug at a 0th order, and will respond to stimulation at physiological conditions [5, 7, 8]. In this chapter is presented the first time a micelle made up of a different material from the hydrogel was shown to be released intact.

There are multiple advantages for whole micelle compared to naked drug diffusion out of the hydrogel system as have been mentioned in chapter 3. Encapsulation of the drug within a polymeric micelle will better solubilize and protect the drug against recognition by the RES system [9]. Hence, an effective micelle system should increase circulation time of the drug, be an appropriate size for tumor penetration and retention, show minimum cargo leakage, and reduce systemic toxicity [9, 10]. A major limitation, however, is that the fluid volume of the body is very large (approximately 40 litres, 25 litres of which is intracellular [11]) and assuming a micelle disassembles upon release from the hydrogel, it would be extremely difficult for reassembly again. Polymer concentration would be diluted almost instantaneously to below critical micelle concentration [12, 13]. Use of higher concentrations of polymer in order to
ensure partial reassembly of some micelles is oftentimes not feasible due to toxicity related dose limitations [14, 15]. Release of disassembled micelles result in more rapid release of drugs from the hydrogel i.e. dose dumping which is quickly eliminated from the body before reaching the target site [16-18]. This is currently a major limitation using direct intravenous injection of micelles [14, 19]. Meanwhile, much research has been done in attempts to stabilize drug encapsulation by micelle using either chemical crosslinking or direct conjugation of drug to polymer [15, 18, 20]. These micelles were indeed able to achieve greater stability despite extensive dilutions past CMC. However, a higher dosage of these micelles must be administered in order to achieve therapeutic effect in vivo due to slow degradation and drug release [21, 22]. We have shown in chapter 3 that Dox-loaded Pluronic® F-127 micelles are just effective therapeutically as pure Dox HCl itself towards U87-MG glioblastoma cells. In this chapter, we hope to understand 4 main hypotheses: 1) micelles remain intact inside of the hydrogel post-encapsulation 2) that whole intact micelles are able to be released from our hydrogel system 3) that polymer concentration plays a major role in release of intact micelles and finally 4) that smaller unencapsulated micelles are able to pass through a hydrogel proving that particle size and mesh size play key roles in intact micelle release. Only by uncovering the answers to these questions can we design an effective micelle/hydrogel system that can truly utilize the advantages of both the hydrogel and micelle.
4.2 Methods

4.2.1 Materials

Pyrene was purchased from Sigma Aldrich (St. Louis, MO, USA) and the FRET pair DiO and DiI was purchased from Fisher Scientific (Hanover Park, IL). PEGDA molecular weight 3.4, 10, and 20 kDa were purchased from Laysan Bio Inc (Arab, AL, USA). Pluronic® F-127 and Pluronic® L-101 were generous donations from BASF (Chicago, IL). All other chemicals and reagents were received from Thermo-Fisher (Waltham, MA) and used without further purification.

4.2.2 Micelle Preparation and Characterization

All pyrene and FRET loaded micelles were made using the oil-in-water emulsion method as described previously in chapter 3. In order to encapsulate pyrene in Pluronic® F-127 micelles, 10 mg of pyrene was solubilized in 160 μL of chloroform. Meanwhile, Pluronic® F-127 was dissolved in 100 mL of water. The pyrene in chloroform was then added using a needle and syringe to the rapidly stirring polymer solution. The entire mixture was left to stir overnight in the dark until organic solvent evaporation and subsequent micelle formation. Unincorporated pyrene was removed by filtering the micelle solution through a 0.45 μm syringe filter. Micelles were then lyophilized and stored at -20°C for future use. FRET-loaded micelles are made the same way with one notable exception: instead of 10 mg of pyrene, 1 mg of DiO and 1 mg of DiI was dissolved in chloroform and then added dropwise to 100 mg of Pluronic® F-127 dissolved in 100 mL of water.

Pluronic® F-127/L-101 micelles are made using a few extra steps based on a method established by Kabanov et al [23]. 0.1 g of Pluronic® F-127 and 0.1 g of Pluronic® L-101 was
added to 100 mL of water. The mixture was left to stir overnight in a 4°C refrigerator until both Pluronics® are fully dissolved. Either FRET or pyrene in chloroform solution was then added to the polymer solution using a needle and syringe the next morning. Solution was again allowed to stir overnight to allow for micelle formation and chloroform evaporation. The next day, micelle solution was heated in a water bath at 70°C for 30 min, filtered through a 0.45 μm syringe filter in order to remove unincorporated dye, lyophilized, and then stored at -20°C for future use. Particle size and size distribution was measured for all purified micelles using dynamic light scattering (DLS) with a Nicomp 380 Particle Sizer (Particle Sizing Systems. Santa Barbara, CA).

4.2.3 Pyrene or FRET-loaded Micelle Release from Hydrogel

Pyrene or a FRET pair (DiO and Dil) was loaded into Pluronic® F-127 and Pluronic® F-127/L-101 micelles and then released from hydrogels of molecular weight 3.4 kDa, 10 kDa, or 20 kDa. Hydrogels were incubated in DPBS at 37°C for set time points. Pyrene loaded micelle release was determined via fluorescence measurements of the hydrogel releasate after 4, 24, 48, 72, 96, and 120 hours at 390 nm emission and then scanning for 333/335 nm excitation RFU with a range set to between 300-400 nm using a spectrophotometer (RF 1501, Shimadzu, Japan). FRET loaded micelle release was determined using the same time points with the spectrophotometer light filter set to 484 nm excitation and then finding 565/501 nm emission RFU from a range of 490-600 nm.
Micelle Release through Hydrogel using Franz Cells

A Franz cell apparatus was used to test the micelle’s ability to pass intact through a hydrogel (Figure 4.1). A 3.4k kDa, 10 kDa, or 20 kDa molecular weight PEGDA hydrogel was pre-swollen in DPBS for 24 hours before it is placed in between the donor and acceptor chambers of the Franz cell (making sure that the opening was completely covered by hydrogel). Silicon glue and pieces of parafilm were then used to create an airtight seal between the two chambers. DiI or FRET loaded into Pluronic® F-127 or Pluronic® F-127/L-101 micelles were applied to the donor chamber while the receiver chamber was filled with fresh DPBS. Temperature of the entire system was maintained at 37°C by circulating heated water through the outer jacket. Franz cells were kept covered with aluminum foil to prevent photobleaching for the entirety of the experiment. Parafilm was added to the top of the donor cell to prevent sample evaporation. At set time points, a sample was removed from the donor chamber, fluorescence was measured using a spectrophotometer (RF 1501, Shimadzu, Japan), and fresh DPBS was added as replacement.

Figure 4.1. Schematic overview of Franz Cell System
4.2.5 Hydrogel Imaging

FRET, DiO, or DiI alone or loaded into Pluronic® F-127 micelles are encapsulated inside of 20 kDa PEGDA hydrogels. A cryostat (courtesy of Dr. Jim Wang’s lab at UIC Department of Biopharmaceutical Sciences) was first used to cut hydrogels into 50 µm thickness slices. Hydrogel slices were then imaged using an Olympus IX70 inverted microscope equipped with a fluorescence illuminator (IX70-S1F2, Olympus America, Inc., Center Valley, PA) using a 10x objective, a CCD camera (QImaging Retiga 1300B, Olympus America, Inc.) and filter for FITC (450 nm excitation and 535 nm emission).
4.3 Results

4.3.1 Understanding Intact Micelles Release from the Hydrogel using Pyrene

One of the biggest limitations in micelle delivery is the micelle’s rapid dissolution [24-26]. This limitation warranted investigation on the release stability of our drug-loaded micelles from the hydrogel matrix. Pyrene-loaded micelles were used to determine the stability of micelles upon release due to the unique fluorescence shift of pyrene dependent on hydrophobicity of solvent [27-29]. Pyrene loaded Pluronic® F-127 micelles or naked pyrene molecules were encapsulated and released from PEGDA hydrogels made up from PEGDA 3.4, 10, and 20kDa. Fluorescence of the hydrogel releasate was then measured at 335 and 333 nm excitation wavelength and 390 nm emission wavelength. If a higher fluorescence peak was observed at 335 nm versus 333 nm, it is assumed that the pyrene is being released in a micellar state. Hence the ratio would be above the value of 1. The 335/333 nm ratio is above the threshold value of 1 for intact micelle release (Figure 4.2). Continuous dilutions where fresh buffered saline is added to replace what was removed (in order to maintain sink conditions), showed the 335/333 intensity ratio value dropping over time to below 1. This can be due to micelle concentration in the hydrogel releasate falling to below CMC over time. Hence over time, the micelles that are freed from the hydrogel and which are below CMC in the releasate will rapidly disassociate and release their pyrene loaded contents.

Hydrogels composed of the highest molecular weight PEGDA (20kDa) showed the highest 335/333 nm intensity ratio in the releasate (Figure 4.3), which likely corresponds to a greater proportion of micelles released in 5 days. Conversely, hydrogels crosslinked using the lowest molecular weight PEGDA showed the lowest 335/333 nm fluorescence ratio of releasate,
which corresponds to slower or less micelle release over 5 days. Pyrene-loaded micelle release from the hydrogels suggests that micelle stability is retained after release from the hydrogel.

**Figure 4.2. Representative image of pyrene maximum wavelength shift.** At a set emission wavelength of 390 nm, peak excitation for pyrene alone is at 333 nm while peak excitation for pyrene in micelle is at 335 nm. Each peak represents the average plus or minus (±) the standard error of the mean of three independent samples.
Figure 4.3. Fluorescent measurement of pyrene-loaded Pluronic® F-127 micelles released from hydrogels composed of 3.4 kDa, 10 kDa, or 20 kDa PEGDA hydrogels. Points represent the average of three independent experiment ± the standard error of the mean (error bars).

Because pyrene loaded Pluronic® F-127 micelles are approximately 10 nm in diameter and therefore are very small (Figure 4.4A), these micelles should be able to very easily travel out of the hydrogel mesh.

![Graph A](image1)

![Graph B](image2)

Figure 4.4. (A) Pyrene loaded Pluronic® F-127 micelles are approximately 10 nm in diameter and (B) pyrene loaded Pluronic® F-127/L-101 micelles are approximately 150 nm in diameter. Loading of pyrene into micelles did not significantly affect micelle size. Each peak represents the average plus or minus (±) the standard error of the mean of three independent samples.

We repeated the previous experiment using pyrene loaded Pluronic® F-127/L-101 micelles which are about 150 nm in diameter (Figure 4.4B). As has already been shown
in chapter 2, larger micelles disrupted hydrogel crosslinking and therefore increased mesh size. We would expect this larger mesh size to allow for intact Pluronic® F-127/L-101 micelle escape. Indeed, the 335/333 nm ratio is above the threshold value of 1 for intact micelle release for 10 kDa and 20 kDa PEGDA hydrogels (Figure 4.5). However, the 335/333 nm ratio remained below 1 for 3.4 kDa PEGDA hydrogel showing no presence of intact micelles in the hydrogel releasate. Again, continuous dilutions where fresh buffered saline was added to replace what was removed (in order to maintain sink conditions), showed the 335/333 ratio value dropping over time to below 1.

![Figure 4.5. Fluorescent measurement of pyrene-loaded Pluronic® F-127/L-101 micelles released from hydrogels composed of 3.4 kDa, 10 kDa, or 20 kDa PEGDA hydrogels. Points represent the average of three independent experiment ± the standard error of the mean (error bars).](image)

Figure 4.5. Fluorescent measurement of pyrene-loaded Pluronic® F-127/L-101 micelles released from hydrogels composed of 3.4 kDa, 10 kDa, or 20 kDa PEGDA hydrogels. Points represent the average of three independent experiment ± the standard error of the mean (error bars).
4.3.2 Understanding Intact Micelles Release from the Hydrogel using FRET

One of the biggest limitations to using pyrene loaded micelles to show release of intact micelles from hydrogels is that micelles are capable of disassembling and then reassembling outside the hydrogel. These reassembled micelles in the hydrogel releasate will still contain a pyrene molecule and fluoresce at the same 335 nm wavelength. To resolve this issue, we utilized a method wherein the reassembled micelle loses its inherent fluorescent properties compared to if it is released intact from the hydrogel.

We chose to load the FRET pair DiO and DiI into Pluronic® F-127 or Pluronic® F-127/L-101 micelles. FRET-loaded Pluronic® F-127 micelles are approximately 5 nm in diameter and FRET-loaded Pluronic® F-127/L-101 micelles are about 80 nm in diameter (Figure 4.6).

Figure 4.6. Particle sizes of FRET loaded into Pluronic® F-127 micelles or Pluronic® F-127/L-101 micelles. Each peak is the represents the average plus or minus (±) the standard error of the mean of three independent samples.

Micelles which are released from the hydrogel intact (with DiO and DiI in contact so energy transfer can occur) will fluoresce at 565 nm wavelength. Micelles which are released
disassembled will only fluoresce at the donor maximum emission wavelength of 501 nm (Figure 4.7). Most importantly, disassembled micelles should not be able to easily reassemble again in the hydrogel releasate without losing previously encapsulated FRET pairs.

**Figure 4.7. Representative image of FRET maximum wavelength shift.** At an excitation of 490 nm, peak excitation for DiO and DiI unencapsulated is at 501 nm (left panel) while peak excitation for DiO and DiI in micelle is at 565 nm (right panel). Each peak represents the average plus or minus (±) the standard error of the mean of three independent samples.

Fluorescence of the hydrogel releasate using encapsulated FRET-loaded Pluronic® F-127 micelles was measured at 490 nm excitation and 565/501 nm emission. If a higher fluorescence peak was observed at 565 nm versus 501 nm, it is assumed that the FRET pair is being released from the hydrogel while inside of an intact micelle. Hence, the measured RFU ratio of 565/(565 + 501) would be above a value of 0.5 (Figure 8). Similarly, as with previous pyrene experiments, subsequent dilutions where fresh buffered saline is added to replace what was removed (in order to maintain sink conditions) showed the 565/(565 + 501) fluorescence intensity ratio (as will be defined as F_{565/(565+501)}) value dropping over time to below 0.5 (Figure
8). This again is assumed to be the Pluronic® concentration in the hydrogel releasate having fallen to below CMC. Based on this idea, the micelles that are freed from the hydrogel and which are below CMC in the releasate will rapidly disassociate and release their contents. The FRET pair then separates (as it is no longer held in close proximity within the same micelle) and will fluoresce at the donor maximum wavelength of 501 nm. Intact micelles were released from 20 kDa and 10 kDa but not 3.4 kDa PEGDA hydrogels (Figure 4.8) demonstrating that the higher molecular weight PEGDA produced a suitable mesh size which allowed a high enough concentration of Pluronic® (past CMC) to pass through to remain as intact micelles.

![Figure 4.8. F565/(565+501) of 25 mg/mL FRET-loaded Pluronic® F-127 micelles released from hydrogels composed of 3.4 kDa, 10 kDa, or 20 kDa PEGDA hydrogels. Each peak represents the average plus or minus (±) the standard error of the mean of three independent samples.]

To understand if Pluronic® concentration in the hydrogel releasate affects the number of intact micelles which are released, the amount of FRET loaded micelles originally loaded into the...
hydrogel was decreased by half (from 25 mg/mL to 12.5 mg/mL). The presence of intact micelles was detected in the releasate of only 20 kDa PEGDA hydrogels but not 3.4 kDa or even 10 kDa PEGDA hydrogels (Figure 4.9). This is an important change from the previous experiment using 25 mg/mL of loaded micelles where the 10 kDa PEGDA hydrogel showed intact micelle release as well and demonstrates that loaded micelle amount does effect intact micelle release.

Figure 4.9. F565/(565 + 501) measurement of 12.5 mg/mL FRET-loaded Pluronic® F-127 released from hydrogels composed of 3.4 kDa, 10 kDa, or 20 kDa PEGDA hydrogels. Each peak represents the average plus or minus (±) the standard error of the mean of three independent samples.

Hydrogels composed of the highest molecular weight PEGDA (20 kDa) showed the highest F565/(565 + 501) of releasate (Figures 10 and 11), which corresponds to the highest amount of intact micelles released in 5 days. Conversely, hydrogels crosslinked using the lowest molecular weight PEGDA showed the lowest F565/(565 + 501) of releasate, which corresponds to slower or less
micelle. Repetition of the hydrogel release experiments using 25 mg/mL of the larger FRET-loaded Pluronic® F-127/L-101 again showed release of intact micelles from 20 kDa and 10 kDa but not from the 3.4 kDa PEGDA hydrogels (Figure 4.10).

Figure 4.10. $F_{565/(565+501)}$ of 25 mg/mL of FRET-loaded Pluronic® F-127/L101 micelles released from hydrogels composed of 3.4 kDa, 10 kDa, or 20 kDa PEGDA hydrogels. Each peak represents the average plus or minus (±) the standard error of the mean of three independent samples.

Reducing the amount of loaded micelles in the hydrogel by half (25 mg/mL to 12.5 mg/mL) showed release of intact micelles by the 20 kDa but not the 10 kDa and 3.4 kDa PEGDA hydrogel (Figure 4.11).
Figure 4.11. $F_{565/(565+501)}$ of 12.5 mg/mL of FRET-loaded Pluronic® F-127/L101 micelles released from hydrogels composed of 3.4 kDa, 10 kDa, or 20 kDa PEGDA hydrogels. Each peak represents the average plus or minus (±) the standard error of the mean of three independent samples.

These results showing release of the larger Pluronic® F-127/L101 micelles is consistent to the previous experiments using Pluronic® F-127 micelles. Pluronic® F-127/L-101 micelles have already been shown in chapter 2 to expand normal hydrogel mesh size by interfering with crosslinking. This allows for the release of intact micelles which otherwise would have been large to fit. Initial micelle amount loaded into the hydrogel again pushes the concentration of Pluronic® in the releasate to above or below CMC thereby providing stability to released micelles.

4.3.3 Intact Micelle Release from Hydrogel is Polymer Concentration Dependent

Results from experiment section 4.3.2 show that the concentration of polymer inside the hydrogel is important for release of intact micelles. In this section, we want to prove that
polymer concentration outside the hydrogel is also critical to determining release of intact micelles. To accomplish this task, old hydrogel releasate will not be removed after each time point but will be left to incubate with the hydrogel for the duration of the experiment (5 days). Thus, the polymer concentration surrounding the hydrogel will not be diluted over time as before. 25 mg/mL or 12.5 mg/mL of FRET-loaded Pluronic® F-127 micelles are loaded into 3.4 kDa, 10 kDa, or 20 kDa PEGDA hydrogels and released over time as shown previously. Fluorescent measurements are taken at 4, 24, 48, 72, 96, and 120 hours and the *old medium is then put back* with the hydrogels. The presence of intact micelles was detected in the releasates of 3.4 kDa, 10 kDa, and 20 kDa PEGDA hydrogels after just 4 hours (Figure 4.12). These micelles seem to be stable for up to 2 days in solution as the $F_{565/(565+501)}$ did not significantly decrease over time. After 2 days, the $F_{565/(565+501)}$ for micelles released from the 10 kDa hydrogel significantly decreased showing there is some possible micelle instability and drug precipitation. The 20 kDa hydrogels showed the highest $F_{565/(565+501)}$ followed by 10 kDa and 3.4 kDa demonstrating that higher molecular weight PEGDA resulted in increased rate of intact micelle release.
We increased (instead of decreased as previously in section 4.3.3) the amount of micelles added to each hydrogel by 25 mg/mL (2x fold) in order to confirm our previous observations with a different concentration of loaded micelles. Increasing the amount of FRET-loaded micelles in the hydrogel from 25 mg/mL to 50 mg/mL again showed presence of intact micelles for all molecular weight PEGDA hydrogels used (Figure 4.13). However, this time the $F_{565/(565+501)}$ does not substantially change over time and therefore showed better stability than in previous experiments using 25 mg/mL of loaded micelles. We can consequently presume that an increased concentration of intact micelles in the hydrogel leads to higher micelle stability. The 20 kDa followed by 10 kDa and then 3.4 kDa PEGDA hydrogels showed a decreasing rate of intact micelle release due to decreasing hydrogel mesh size. These results prove that in addition to mesh size, micelle polymer concentration outside the hydrogel governs whether intact micelles can be released. Experiments also show that released micelles are stable in solution and that burst release of intact micelles occurred within the first 4 hours.
Figure 4.13. $F_{565/(565+501)}$ of 50 mg/mL of FRET-loaded Pluronic® F-127 micelles released from hydrogels composed of 3.4 kDa, 10 kDa, or 20 kDa PEGDA hydrogels without removing old DPBS. Each peak represents the average plus or minus (±) the standard error of the mean of three independent samples.

The same experiment was again repeated using either 25 mg/mL or 50 mg/mL of larger FRET-loaded Pluronic® F-127/L-101 in 3.4 kDa, 10 kDa, and 10 kDa PEGDA hydrogels. This is to understand whether changing micelle size will affect intact micelle release under these specific non-dilution conditions. A $F_{565/(565+501)}$ value above 0.5 was obtained for all molecular weight PEGDA hydrogels and suggested that there was intact micelle release after 24 hours (Figure 4.14). $F_{565/(565+501)}$ values did not decrease over time unlike what was observed for the smaller Pluronic® F-127 micelles. This might be due to the Pluronic® F-127/L-101 micelles being more stable in solution in solution than Pluronic® F-127 micelles due to extra polymer layers of the micelle which provide more shielding to the loaded FRET pair. Increasing the amount of Pluronic® F-127/L-101 micelles loaded into the hydrogel by twofold showed
presence of intact micelles in the releasate for all molecular weight PEGDA hydrogels over the span of 5 days as expected.

Figure 4.14. $F_{565/(565+501)}$ of 25 mg/mL of FRET-loaded Pluronic® F-127/L-101 micelles released from hydrogels composed of 3.4 kDa, 10 kDa, or 20 kDa PEGDA hydrogels. Each peak represents the average plus or minus (±) the standard error of the mean of three independent samples.

$F_{565/(565+501)}$ values significantly increased for 3.4 kDa PEGDA hydrogels using 50 mg/mL of loaded micelles compared to 25 mg/mL (Figure 4.15). Micelles released demonstrated good stability for up to 5 days in solution as $F_{565/(565+501)}$ values remained steady during that time frame. 20 kDa and 10 kDa PEGDA hydrogels showed the same rate of intact micelle release while 3.4 kDa PEGDA hydrogels showed a slightly slower rate. Pluronic® F-127/L-101 micelles, as has already been established, will increase the hydrogel mesh size presumably by interfering with polymer crosslinking, thereby allowing for intact micelle escape. The $F_{565/(565+501)}$ value approached the maximum at 1 for 10 kDa and 20 kDa PEGDA hydrogels within the first 24 hours for both 25 mg/mL and 50 mg/mL loaded micelle concentrations.
(Figure 4.14 and 4.15). This demonstrates very fast release or burst release of almost purely intact micelles.

**Figure 4.15.** $F_{565/(565+501)}$ of 50 mg/mL of FRET-loaded Pluronic® F-127/L-101 released from hydrogels composed of 3.4 kDa, 10 kDa, or 20 kDa PEGDA hydrogels. Each peak represents the average plus or minus (±) the standard error of the mean of three independent samples.

One of the factors in modeling drug release from hydrogels is maintaining sink conditions where the amount of dissolution medium is enough to dissolve the expected amount of drug released the device [30, 31]. “Old” DPBS which the hydrogel has been incubating in at each set time point must be removed and then replaced with fresh buffer to prevent solute saturation. This likewise mimics physiologic conditions where a very large body fluid volume is continuously in contact with the hydrogel implant. However, a major limitation to maintaining sink conditions is that the micelle polymer concentration in the hydrogel is constantly being diluted at each time point. At some point, the Pluronic® concentration being released becomes
lower than CMC and the micelle falls apart as soon as it is reaches the outside of the hydrogel. Thus it will be difficult to accurately measure whether intact micelles are coming out of the hydrogel or not at later time points. The above set of experiments provided evidence that intact micelles are still being released by the hydrogel but fall apart due to dilutions. Likewise, our original question of whether polymer concentration outside of the hydrogel which effect release of intact micelles may be answered. Indeed, the concentration of polymer both outside and inside the hydrogel will both affect release of intact micelles.

Overall, the pyrene and FRET experiments suggest the release of intact micelles from hydrogels provided that these micelles are encapsulated within the mesh. Figure 4.16 provides a summary of this mechanism. Smaller encapsulated Pluronic® F-127 micelles can easily diffuse out of the hydrogel. Larger encapsulated Pluronic® F-127/L-101 micelles increase hydrogel mesh size and are also released as intact.

Figure 4.16. Schematic for intact micelle release from hydrogel. Smaller Pluronic® F-127 micelles (left) and larger Pluronic® F-127/L-101 micelles (right) can both diffuse intact from hydrogels.
4.3.4 Assessing Drug’s Ability to Pass through a Hydrogel Using Franz Cell

The data above showed that micelles when encapsulated in a hydrogel can increase the mesh size and thereby allow for release. In this section, we wanted to understand whether micelles that are not encapsulated are able to move intact through the hydrogel mesh. Therefore for the following experiments, micelles were not directly embedded in the gel but instead forced to pass through the mesh as is. A Franz cell device (Figure 4.1) [32, 33] was used to experimentally measure the diffusion coefficients of Fluorescein, DiI-loaded Pluronic® F-127, and DiI-loaded Pluronic® F-127/L-101 micelles through a 3.4 kDa, 10 kDa, or 20 kDa PEGDA hydrogel. Diffusion coefficients will be calculated from the lag time using the following equation (4.1) [34, 35]:

\[ D = \frac{h^2}{6T_L} \quad \text{Equation 4.1} \]

Where \( h^2 \) is the thickness of the hydrogel, \( t_L \) is the lag time, and \( D \) is the diffusion coefficient. Lag time is defined as the amount of time needed to establish a constant gradient across the hydrogel. This value can be extrapolated by plotting the total amount of solvent that has traveled across the hydrogel barrier versus time \( t \) and then finding the x-intercept of the linear portion of the graph. We can further calculate the theoretical diffusion coefficient of the solute using Stokes-Einstein Equation (4.2):

\[ D = \frac{kT}{6\pi\eta r} \quad \text{Equation 4.2} \]

\( D \) is the diffusion coefficient, \( k \) is Boltzmann’s constant, \( T \) is the temperature, \( r \) is the radius of the solute molecule, and \( \eta \) is the solvent viscosity. This equation assumes that the solute
molecule has a hydrodynamic radius which is perfectly spherical, is in constant Brownian motion, and is very large compared to the diameter of the solvent molecule.

To test the validity of our Franz Cell system set-up, a solution of 2 mg/mL fluorescein was added to the donor chamber and passed through a preswollen disk of 3.4 kDa, 10 kDa, or 20 kDa PEGDA hydrogel. Fluorescent measurements at 494 nm excitation and 521 nm emission were taken every 2 minutes for the first 10 minutes and then every hour for 8 hours total. Jacket temperature was set to 37°C to simulate physiologic conditions. Lag time was found to be between 2-4 minutes for all molecular weight PEGDA hydrogel. Diffusion coefficients are presented in Table 4.1 and expectedly show that with higher mesh size, the fluorescein dye was able to move through the hydrogel faster. A visual image of fluorescein moving through the hydrogel and accumulating in the acceptor chamber is presented (Figure 4.17). An increase in yellow coloring can be seen from 5 minutes to 8 hours. Our data corresponds to reported literature values for diffusion coefficients of fluorescein in pure water which are always in the $10^{-5}$ or $10^{-6}$ cm$^2$/s magnitude [36-38].

Table 4.1. Diffusion coefficients of fluorescein through 3.4 kDa, 10 kDa, or 20 kDa PEGDA hydrogel.

<table>
<thead>
<tr>
<th>PEGDA (kDa)</th>
<th>D (cm$^2$/s)</th>
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<tbody>
<tr>
<td>3.4</td>
<td>2.04x10^{-6}</td>
</tr>
<tr>
<td>10</td>
<td>1.33x10^{-5}</td>
</tr>
<tr>
<td>20</td>
<td>5.95x10^{-5}</td>
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</table>
Figure 4.17. Fluorescein passing through a 20 kDa PEGDA hydrogel. At 10 min, a very faint amount of color can be seen in the donor chamber of the Franz Cell signifying a small amount of fluorescein has passed through the hydrogel (left panel). After 8 hours, a bright yellowish green color can be clearly observed (right panel) as fluorescein concentrations in both chambers have reached equilibrium.

Next, the fluorescent dye DiI was loaded into Pluronic® F-127 or Pluronic® F-127/L-101 micelles. DiI was selected as it is a lipophilic dye with a bright fluorescent color that can be easily observed by the eye as micelles move through the Franz cell [39, 40]. Sizes of the micelles are approximately 5 nm for DiI-loaded Pluronic® F-127 and 100 nm for DiI-loaded Pluronic® F-127/L-101 (Figure 4.18).
Figure 18. Particle sizes of Dil loaded into Pluronic® F-127 micelles or
Pluronic® F-127/L-101 micelles. Micelle sizes remain consistent with previous
data. Each peak is the represents the average plus or minus (±) the standard error
of the mean of three independent samples.

The lag times for Dil-loaded Pluronic® F-127 micelles passed through a 20 kDa PEGDA
hydrogel was about 2 minutes while the lag times for 10 kDa and 3.4 kDa PEGDA hydrogels
were 5 and 8 minutes respectively (Figure 4.19A). These corresponded to higher diffusion
coefficients with increasing molecular weight PEGDA (Figure 4.19B). For comparison, the
diffusion coefficient of a 10 nm radius sphere (representing a typical Pluronic® F-127 micelle)
was calculated using the Stokes-Einstein equation (Figure 4.19B) [41]. Theoretically, a micelle
of that size should move much slower in water than the particles being released from the
hydrogel. This leads to the hypothesis that the entire Dil-loaded micelle is not capable of
passing through the hydrogel but instead only the dye itself.
Figure 4.19. Dil loaded into Pluronic® F-127 micelles passing through a 3.4 kDa, 10 kDa, or 20 kDa hydrogel using a Franz Cell device (top panel). Each point represents the average plus or minus (±) the standard error of the mean of three independent samples. Diffusion coefficients of Dil loaded into Pluronic® F-127 micelles passing through a hydrogel of noted molecular weight PEGDA (Bottom panel). Stokes-Einstein equation was used to calculate the diffusion coefficient of a 10 nm micelle for comparison.

<table>
<thead>
<tr>
<th>PEGDA (kDa)</th>
<th>D (cm$^2$/s)</th>
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<tbody>
<tr>
<td>3.4</td>
<td>2.76x10^{-6}±6.26x10^{-7}</td>
</tr>
<tr>
<td>10</td>
<td>5.33x10^{-6}±2.70x10^{-6}</td>
</tr>
<tr>
<td>20</td>
<td>6.33x10^{-5}±3.86x10^{-6}</td>
</tr>
<tr>
<td>Water (calculated)</td>
<td>3.05x10^{-7}</td>
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To better study this assumption, a 5 mg/mL solution of Dil-loaded Pluronic® F-127/L-101 was allowed to diffuse through a hydrogel of molecular weight 3.4 kDa, 10 kDa, or 20 kDa.
PEGDA. The measured diffusion coefficients for the larger Pluronic® F-127/L101 micelles are 10x-order of magnitude slower than using the Pluronic® F-127 micelles (Figure 4.20A and 4.20B). As expected, with greater molecular weight PEGDA and therefore larger mesh size, a higher diffusion coefficient was obtained. Similarly as before, the Stokes-Einstein equation was used to calculate the diffusion coefficient of a 100 nm micelle in water for comparison. The theoretical diffusion coefficient (in water) was found to be on a magnitude of 10x slower than what was observed (Figure 4.20B). This supported the previous idea that when entire micelles cannot move through the hydrogel mesh size due to size incompatibility, the encapsulated dye can escape from the micelle and pass to the other side. We observed that the diffusion coefficients for each of the different molecular weight PEGDA hydrogels were substantially slower for the Pluronic® F-127/L-101 micelles than for the Pluronic® F-127 micelles. Because Pluronic® F-127/L-101 micelles are bigger and more stable than Pluronic® F-127 micelles (Figure 4.18), the encapsulated dye must move through more layers of polymer before being able to pass out of the micelle and across the hydrogel. Hence, this would take up more time. Another possibility could be that indeed some but not all micelles are able to pass intact through the hydrogel if there is compatibility between both mesh and particle size. The Pluronic® F-127 micelles are on average about 10 nm in size and can theoretically pass intact through the 10 kDa and 20 kDa PEGDA hydrogel mesh. In a mixed population of Pluronic® F-127 micelles, the smaller micelles may pass through while larger ones would get trapped and take time to release their encapsulated dye. Meanwhile, all of the Pluronic® F-127/L-101 micelles would be retained on the surface of the hydrogel until they are able to break apart and diffuse through the mesh. In both cases, the diffusion coefficients for Pluronic® F-127 micelles will always be larger than for Pluronic® F-127/L-101 micelles and increase with increasing molecular weight PEGDA.
Figure 4.20. DiI loaded into Pluronic® F-127 L-101 micelles passing through a 3.4 kDa, 10 kDa, or 20 kDa hydrogel using a Franz Cell device (top panel).

Each point represents the average plus or minus (±) the standard error of the mean of three independent samples. Diffusion coefficients of DiI loaded into Pluronic® F-127L-101 micelles passing through a hydrogel of noted molecular weight PEGDA (bottom panel). Stokes-Einstein equation was used to calculate the diffusion coefficient of a 100 nm micelle for comparison.

<table>
<thead>
<tr>
<th>PEGDA (kDa)</th>
<th>D (cm²/s)</th>
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<tbody>
<tr>
<td>3.4</td>
<td>$3.79 \times 10^{-7} \pm 2.15 \times 10^{-7}$</td>
</tr>
<tr>
<td>10</td>
<td>$5.88 \times 10^{-7} \pm 1.53 \times 10^{-8}$</td>
</tr>
<tr>
<td>20</td>
<td>$4.14 \times 10^{-6} \pm 2.02 \times 10^{-7}$</td>
</tr>
<tr>
<td>Water (calculated)</td>
<td>$2.27 \times 10^{-8}$</td>
</tr>
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To test which hypothesis was true: whether some intact dye-loaded micelles are able to travel across the hydrogel or the dye must first escape from the micelle before diffusing out of the
hydrogel, FRET molecules (DiO and DiI) were loaded into Pluronic® F-127 or Pluronic® F-127/L-101 micelles. Micelles were then allowed to diffuse through hydrogels of molecular weight PEGDA 3.4 kDa, 10 kDa, or 20 kDa using the same Franz Cell system. Fluorescent readings were taken from the donor cell at time points 4 hours, 3 days, and 5 days. Using FRET-loaded Pluronic® F-127 micelles, there is a mixture of intact as well as disassembled micelles in the donor cell after just 4 hours (Figure 4.21A). A sample (20 kDa PEGDA hydrogel) was taken from the donor cell after 5 days, plotted, and the subsequent graph presented below to demonstrate that the maximum emission wavelength was at 565 showing presence of intact micelles (Figure 4.21B). This proves crossing of intact FRET-loaded Pluronic® F-127 micelles across the hydrogel barrier. Surprisingly, intact micelle concentration did not significantly increase with increasing PEGDA molecular weight or with more time. This might be due to micelle instability in solution at 37°C where although initially intact micelles are passing through the hydrogel, over time these same micelles gradually fall apart in the donor cell therefore driving the $F_{565/(565+501)}$ value back down to below 0.5. As expected, Pluronic® F-127/L-101 micelles were not able to pass intact through the hydrogel (Figure 4.21C). Average 565/565+501 ratio value remained at 0.2. Similarly, a sample (20 kDa PEGDA hydrogel) taken from the donor cell after 5 days showed a maximum emission wavelength at 501 (Figure 4.21D). In summary, these results suggest that intact micelles are able to pass through a hydrogel if mesh size and particle size are compatible without the need for the micelles to be encapsulated directly into the hydrogel (Figure 4.22).
Figure 4.21. (A) DiI loaded into Pluronic® F-127 micelles passing through a 3.4 kDa, 10 kDa, or 20 kDa hydrogel using a Franz Cell device. (B) A sample was taken from the Franz Cell at day 5 to show presence of intact micelles. (C) DiI loaded into Pluronic® F-127 micelles passing through a 3.4 kDa, 10 kDa, or 20 kDa hydrogel using a Franz Cell device. (D) A sample was taken from the Franz Cell at day 5 to show only disassembled micelles. All points and peaks represent the average plus or minus (±) the standard error of the mean of three independent samples.
Figure 4.22. Schematic of micelles passing through hydrogel. Smaller Pluronic® F-127 micelles are able to pass through intact depending on mesh size. However, larger Pluronic® F-127/L-101 micelles are retained in the mesh and break apart.

4.3.5 Micelles are Intact Inside the Hydrogel

One important piece of evidence in proving that whole micelles are released is in showing that micelles remain intact inside of the hydrogel after encapsulation. To better support this assumption, DiO + DiI, DiO alone, or DiI alone or loaded into Pluronic® F-127 micelles are encapsulated inside of 20 kDa PEGDA hydrogels. Hydrogel slices cut using a cryostat was imaged using a fluorescent microscope. As can be observed, DiI or DiO loaded into Pluronic® F-127 micelles (Figure 4.23A and 4.23B) did not show nearly as much fluorescence intensity as FRET loaded Pluronic® F-127 micelles (Figure 4.23C). This demonstrates that indeed in the FRET group, DiO and DiI are found within the same micelle, allowing for energy transfer. DiO, DiI, or FRET loaded into the hydrogel alone showed very little fluorescence compared to the
micelle groups (Figures 4.23D, 4.23E, and 4.23F respectively). A blank hydrogel containing empty Pluronic® F-127 micelles was used as a negative control (Figure 4.23G). Because DiO and DiI are very hydrophobic molecules that will crystallize in solution, incorporation into micelles greatly increases solubility and therefore fluorescence. This further proves that DiO and DiI are located inside of micelles while in the hydrogel (Figures 4.23D, 4.23E, and 4.23F).

Finally, loading DiO and DiI into separate Pluronic® F-127 micelles but then combining equal quantities of each micelle type into the same hydrogel resulted in a recovered fluorescence intensity similar to the FRET-loaded Pluronic® F-127 micelle group (Figure 4.23H). A possible explanation is that because micelles are very fluid, that two separate solutions of micelles can merge together and exchange loaded contents (especially in such close proximity as inside of the same hydrogel). Kim et al found that with two populations of micelles one containing DiI and one containing DiO, there was a gradual recovery in the FRET phenomenon as $F_{565/(565+501)}$ values rose over time (despite the micelles remaining intact) ) [42]. The authors attributed this to translocation of loaded micelle contents due to dynamic micelle instability. Such an observation was seen in this experiment as well where two types of micelles combined to form a single uniform population when forced into very close proximity.
Figure 4.23. All images taken using an Olympus IX70 fluorescent microscope equipped with a CCD camera (4x objective lens; UIC Biopharmaceutical Sciences Department, Gemeinhart Lab) using FITC filter and 3 seconds exposure time. (A) 20 kDa PEGDA hydrogel containing DiI-loaded Pluronic® F-127 micelles, (B) DiO-loaded Pluronic® F-127 micelles, (C) FRET-loaded Pluronic® F-127 micelles, (D) DiI, (E) DiO, (F) DiI and DiO, (G) blank, or (H) DiO and DiO previously loaded into separate Pluronic® F-127 micelles but then encapsulated in the same hydrogel.
4.4 Conclusion

In summary, we have presented data showing that micelles remain intact after encapsulation within the hydrogel. However, results also suggest that micelles within such close proximity within the mesh are capable of exchanging encapsulated contents with each other. Secondly, from the pyrene experiments, Pluronic® F-127 micelles which are smaller than native hydrogel mesh size are capable of being released intact. Release of intact Pluronic® F-127 micelles up to a period of 2 days was further confirmed by the FRET data. However, Pluronic® F-127/L-101 micelles which are larger than native hydrogel mesh size are also able to be released from the hydrogel intact. As mentioned in the previous chapter, this could be partly due to these micelles interfering with normal PEGDA crosslinking and therefore increasing the hydrogel mesh size. Our results comparing the pyrene and FRET groups also provided evidence of micelle disassembly and then reassembly out of the hydrogel. We showed that Pluronic® concentrations both inside and outside of the hydrogel is crucial for presence of intact micelles in the releasate. Doubling the amount of micelle encapsulated in the hydrogel led to release of more intact micelles from lower molecular weight PEGDA hydrogels. Similarly, not diluting the hydrogel releasate after each time point (previously done in order to maintain sink conditions) showed the presence of more intact micelles instead of a decrease over time.

Franz Cell experiments show that some Pluronic® F-127 micelles are able to pass through the hydrogel intact (although some of the encapsulated dye is also capable of leaving the micelle to diffuse through the gel). Alternatively, larger than mesh size Pluronic® F-127/L-101 micelles cannot pass through the hydrogel intact. In such a situation, all of the fluorescent dye will diffuse out of the trapped micelles and pass through the hydrogel anyway. Specifically, measured diffusion coefficients for Dil-encapsulated Pluronic® F-127 micelles and Dil-
encapsulated Pluronic® F-127/L-101 micelles are closer to theoretical diffusion coefficients for naked DiI dye compared to intact micelles. Lastly, both the hydrogel mesh size and micelle size are critical for release of intact micelles. As expected, a larger mesh size will release more intact micelles over a longer period of time and a smaller micelle can more easily pass through the mesh intact. Taken altogether, these results for the first time show encapsulated and unencapsulated micelles are able to be released from a hydrogel intact. Conversely, data also suggests that the encapsulated contents of a micelle is also capable of leaving the micelle in order to diffuse through the gel should the micelle be “stuck”. We highlight the importance of understanding the mechanism behind micelle release from hydrogel systems in order to provide guidelines for designing a suitable and effective drug delivery device in the future.
4.5 References


CHAPTER 5
CONCLUSIONS AND OUTLOOK

5.1 Conclusions and Outlook

The scope of this project encompassed 3 main objectives: to understand the effect of each of the components specifically drug, micelle, and hydrogel in the overall system in order to show that intact drug loaded micelles are being released from the hydrogel, and lastly to show that drug-loaded micelles released from the hydrogel has therapeutic activity. Our initial goal was essentially to create a drug delivery device that has the ability to combine the advantages of a micelle as well as a hydrogel system as well as reduce the limitations of each.

In order to understand the influence of hydrogel mesh size on micelle release and therefore design a system with better controlled release kinetics, we released DOX loaded Pluronic® F-127 micelles from hydrogels composed of 3.4 kDa, 10 kDa, and 20 kDa PEGDA. It was already established experimentally and in literature that higher molecular weight PEGDA will form hydrogels of larger mesh size [1, 2]. Results show a significantly higher rate of micelle release in hydrogels made up of higher molecular weight PEGDA which can be hypothesized to be due to larger spaces from which the micelle can escape. We found that using different initiators such as either TEMED and APS or Irgacure 2959 can alter the mesh size but does not significantly affect rate of micelle release from these same hydrogels. In order to understand the effect of drug hydrophobicity on micelle release, we loaded three drugs of different solubilities (but all basically hydrophobic) into Pluronic® F-127 micelles and then released the micelles from PEGDA hydrogels as before. We hypothesized that a more hydrophobic drug would be released faster than a more hydrophilic drug because a more hydrophilic drug will form a smaller micelle which is then better able to escape the hydrogel mesh. As shown by experimental data, a more
A hydrophobic drug will indeed form a smaller micelle as the drug is better able to pull the polymer chains closer together due to intramolecular interactions. However, our results also refuted part of our hypothesis—a more hydrophilic drug (DOX) gets released from our micelle/hydrogel system at a significantly higher rate than a more hydrophobic drug (curcumin or indomethacin). We presume the reason to be that a more hydrophobic drug is bound more tightly to the interior of a micelle and hence the entire micelle must be released from the hydrogel for drug escape. However, a more hydrophilic drug such as DOX is loosely encapsulated in the micelle core and forms a larger micelle complex (as shown in results). The drug can then more easily diffuse out of the bound micelle and subsequently out of the hydrogel.

We conducted a long term release experiment for DOX or curcumin loaded into Pluronic® F-127 micelles from 3.4 kDa, 10 kDa, and 20 kDa PEGDA hydrogels in order to study the mechanism behind release. Results were fitted to 0 order, 1st order, 2nd order, Higuchi and Peppas/Korsmeyer models for drug release and were found to fit Higuchi’s model best. This indicates that drug-loaded micelle is released from the hydrogel via a purely diffusion based mechanism and that rate is directly proportional to the square root of time.

Lastly, we attempted to alter micelle characteristics by using different types of Pluronics® in micelle formation. The goal was to study any affects of drug release from hydrogel by using either a smaller micelle (ones made of a very hydrophilic Pluronic® such as F-127) or a larger micelle made up completely or partially of a more hydrophobic Pluronic® such as L-61. We believed that a smaller micelle will be released faster from the hydrogel and that a micelle which is significantly larger than the hydrogel mesh size will either be trapped and retained in the hydrogel core or be released very slowly by gradual disassembly. The Pluronic® used should also have low CMC, high IC50 (and therefore low cytotoxicity), and a good encapsulation.
efficiency towards the drugs used in our study. Hence, we underwent an extensive characterization of a number of Pluronics® (Table 3.1). Pluronic® F-127 compared to Pluronic® P-105 and L-61 showed very low cytotoxicity towards U87-MG cells, is stable after 7 days at room temperature while in solution, the highest encapsulation efficiency for DOX, and most importantly formed small micelles of approximately 10-50 nm in diameter. Pluronic® F-127 also showed the highest fluorescence intensity at the same concentration in solution compared to Pluronic® P-105 or Pluronic® L-61 micelles. This is important for the overall detection of released micelles from the hydrogel. Pluronic® F-127/L-101 micelles was also selected for the duration of this study because these micelles showed good stability while in solution for up to 5 days and formed micelles of about 150 nm in diameter. The micelle size difference is ideal in showing whether small micelles compared to large ones can be from the hydrogel at a faster rate.

Before showing the release of intact micelles, we first confirmed that micelles are intact after encapsulation in the hydrogel. DiO, DiI, or DiO and DiI together loaded into Pluronic® F-127 micelles or alone were embedded in 20 kDa PEGDA hydrogels and then cut to a thickness of 50 uM using a cryotome for fluorescent imaging under a DAPI filter. The experimental group containing both DiO and DiI in micelles showed the brightest fluorescent intensity in comparison to DiO or DiI in micelles alone. DiO, DiI, or DiI + DiO directly embedded into the hydrogel without micelles showed very little fluorescence. This showed that there was a resonance energy transfer between FRET pairs within the embedded micelle showing that it remains intact while inside the hydrogel.

We then used pyrene loaded Pluronic® F-127 micelles to prove the release of intact micelles from hydrogels. The pyrene experiments demonstrated release of whole micelles from
10 kDa and 20 kDa PEGDA hydrogels for a period of 5 days tested but not the 3.4 kDa PEGDA hydrogels. We hypothesized this was due to the too-small mesh size of the 3.4 kDa PEGDA hydrogels which did not allow for micelle escape. Surprisingly, however, when the pyrene experiment was repeated using much larger than mesh size Pluronic® F-127/L-101 micelles, intact micelles were still found in the hydrogel releasate. We believed the reasons to be that presence of micelles in the hydrogel interfered with normal PEGDA crosslinking and therefore increased hydrogel mesh size or that the pyrene loaded micelles were reassembling outside the hydrogel. The first hypothesis was tested by measuring hydrogel mesh size before and after micelle encapsulation. Our results showed that indeed addition of larger Pluronic® F-127/L-101 micelles will increase hydrogel mesh size by up to three-fold. However, Pluronic® F-127 micelles will not affect mesh size due to their small size (which can fit in between crosslinking sites) up to a certain concentration. Increasing the amount of micelles encapsulated by two-fold (as shown in the curcumin-loaded Pluronic® F-127 micelle experiment) does significantly affect the mesh size. The reason might be that at that high of a concentration, micelles tend to aggregate, form large clusters of micelles, and therefore interfere with hydrogel crosslinking. In order to prove that intact micelles are being released and that this mechanism is not due to pyrene-loaded micelles are disassembling and then reassembling outside the hydrogel, we selected a pair of molecules that cannot reassemble as easily into the same micelle (DiO and DiI as part of FRET). FRET has been used extensively in literature to prove the stability of micelles in the blood or serum through imaging techniques [3-5]. In this case, FRET molecules were loaded into Pluronic® micelles which were then released from hydrogels-if exciting the donor molecule (DiO) leads to maximum emission wavelength at the acceptor molecule (DiI), the micelle was deemed to be intact. Indeed, results from this experiment showed intact Pluronic®
F-127 micelles releasing from 10 kDa and 20 kDa but not 3.4 kDa PEGDA hydrogels over a period of 24 hours. Intact Pluronic® F-127/L-101 micelles were released from 20 kDa and 10 kDa but not 3.4 kDa PEGDA hydrogels over a period of 8 hours. This much shorter time span for the presence of intact FRET micelles (8 or 24 hours) compared to the longer time span for intact pyrene micelles (7 days) demonstrates the ability of pyrene loaded micelles to reassemble in the hydrogel releasate over time. Interference with normal hydrogel crosslinking and hence expanding normal mesh size combined with micelle reassembly outside the hydrogel allowed larger micelles to be released from the hydrogel. More importantly, FRET results also confirmed the pyrene experiment in showing that intact micelles are indeed being released from the hydrogel.

In order for intact micelles to be detected in the hydrogel releasate, it is logical to assume that the concentration of polymer must be higher than critical micelle concentration (CMC). Otherwise, micelles will be quickly diluted and disassociate. Indeed, when the amount of FRET loaded Pluronic® F-127 or Pluronic® F-127/L101 micelles in the hydrogel was halved, less intact micelles was released over time, and only at the highest molecular weight PEGDA (20 kDa) was there observed to be intact micelles. All pyrene and FRET experiments were conducted under a similar procedure where fresh saline was added to the hydrogels after each incubation time point in order to maintain sink conditions. However, this also dilutes micelle concentration and pushes the amount to below CMC. Hence over time, the amount of intact micelles present in the hydrogel releasate decreases. We reversed this method by leaving the hydrogel in the same saline for the duration of the study (5 days) and observed the presence of intact micelles after 4 hours and up to 5 days. Increasing micelle concentration in the hydrogel increased the amount of intact micelles that was released. Likewise, not decreasing the micelle
concentration in the hydrogel release also increased the amount of intact micelles released. Thus, we can conclude that polymer concentration both inside and outside the hydrogel determines the amount of intact micelles present in the hydrogel surroundings.

We’ve established that intact micelles can be released from hydrogels if they have been directly embedded in the mesh before polymerization. But what about micelles that have not been encapsulation and thus have not had the opportunity to interfere with native hydrogel mesh size? Using a Franz Cell system, a thin hydrogel layer was placed between a donor and acceptor chamber and then a fluorescently labeled micelle solution was poured into the donor chamber. Fluorescence can be measured over time in the acceptor chamber in order to calculate the diffusion coefficient or determine whether micelles are intact or not. Surprisingly, the measured diffusion coefficients for Pluronic® F-127 and Pluronic® F-127/L-101 micelles were found to be ten-fold higher than what was calculated using Stokes-Einstein Equation for particles of 10 and 150 nm in diameter. This leads to the idea that perhaps, the fluorescent dye (in this case, DiI) was diffusing out of the micelle which was trapped on one side of the hydrogel sheet and diffusing through the hydrogel. In such a situation, no intact micelle should be present in the acceptor cell. To answer this question, FRET-loaded Pluronic® F-127 or F-127/L-101 was passed through a sheet of 3.4 kDa, 10 kDa, or 20 kDa PEGDA hydrogel. Results showed no intact Pluronic® F-127/L-101 micelles were able to pass through any of the hydrogels for all molecular weight PEGDAs. However, a mixture of disassembled as well as intact Pluronic® F-127 micelles was able to pass through the mesh of the gel. This experiment showed that micelles larger than native mesh size cannot normally pass through the hydrogel intact (unless of course, micelles have been directly encapsulated in the hydrogel and have the ability to enlarge the mesh size). In such a situation however, the encapsulated drug or fluorescent dye is able to diffuse out
of the entrapped micelle and still leave the hydrogel. As already mentioned in a previous experiment, a more hydrophilic drug (such as DOX) can more easily leave the micelle/hydrogel system compared to a very hydrophobic drug (such as indomethacin). A possible future study can then be to load drugs of different hydrophobicities (DOX, indomethacin, curcumin) into Pluronic® F-127/L-101 micelles and pass the micelles through a hydrogel using the Franz Cell system. We would again expect to see a significant difference in rate of drug diffusion through the hydrogel based on drug hydrophobicity. Regardless of whether intact micelles are released or drug is first released from the micelle, our observations suggested that the presence of drug-loaded micelles will slow release of drug out of the hydrogel compared to drug in hydrogel only. This would be beneficial to extend drug release from the hydrogel for a variety of therapeutic purposes. An experiment which would be done to confirm such findings would be to put a hydrophobic drug-loaded micelle/hydrogel system into an organic solvent which would easily dissolve the micelle. In this case, the rate of hydrophobic drug release would be the same as hydrophilic drug release from only the hydrogel.

Lastly, released drug-loaded Pluronic® F-127 micelles from hydrogel were tested for therapeutic efficacy against tumor cell U87-MG. Ideally, this hydrogel/micelle drug delivery device would be implanted surgically next to or at a solid tumor mass or the cavity where tumor resection occurred (Figure 5.1). This is partly the reason behind selection of the human glioblastoma cell line U87-MG as it is capable of forming solid tumors in vivo.
Our results clearly showed that treatment with hydrogel releasate containing DOX loaded Pluronic® F-127 micelles was just as effective as DOX HCl and the IC50 was reached at the same drug concentration. Negative controls with no micelles or empty F-127 micelles showed little to no cytotoxicity. This clearly demonstrated that DOX-loaded Pluronic® F-127 released from hydrogels have cytotoxic activity towards tumor cells. However, there was no significant difference in activity when using different molecular weight PEGDA hydrogels. Previously, we had believed that a higher molecular weight PEGDA hydrogel would release micelles at a faster rate which then causes more cell death. This, however, was shown not to be the case. This might be due to the idea that an increased amount of DOX released by the 20 kDa PEGDA hydrogel compared to the 3.4 kDa hydrogel was not of such significance as to cause any considerable changes in cell death. DOX, as mentioned in chapter 1, mainly works by inhibiting
DNA and RNA synthesis thereby stopping cancer cell growth [6]. At high enough concentrations of 30-100 μM, IC50 for U87-MG cells can be reached within 24 hours. [7, 8] Perhaps, if we tried a more immediately cytotoxic drug towards glioblastoma cells, we might observe more of a difference when using higher molecular weight PEGDA hydrogels.

In this study, we have shown the ability of hydrogels to release intact micelles over time and that encapsulated micelles effect hydrogel mesh size. This idea and the method for determination can be utilized in future systems using different materials and different delivery platforms. Previous research using nanoparticles in hydrogel hybrid systems have tended to focus on therapeutic activity [9] or on the escape of the drug itself [10]. Now that we can indeed show that intact micelles can be released from the hydrogel, future studies might focus on optimizing the system by changing variables for type of drug release needed (whether it be burst or slow, extended release) and testing the system in-vivo. PEGDA is not considered to be a biodegradable polymer and our current system is a pre-polymerized implantable device in the tumor cavity [11, 12]. A possible study in the future would be to use a biodegradable polymer such as hyaluronic acid, gelatin, PLGA, etc, mixed it with lyophilized micelles and a photoinitiator in saline, inject the solution the solution directly at the tumor site, and use UV radiation to directly polymerize the gel in-vivo [13-15]. However, while this system might be more clinically feasible, rate of drug release and general kinetics would also be more difficult to measure and control [15]

Similarly and turning our attention to the micelle material in this system, Pluronics® and particular shorter more hydrophobic types have been known to resensitize multidrug resistant tumor cells to chemotherapeutics [16, 17]. Multidrug resistant cancer cells are shown to be extremely difficult to treat using typical chemotherapy. These cells overexpress efflux
transporters such as P-glycoproteins and multidrug resistant proteins (MRPs) which aid in pumping chemotherapy drugs out of the cell [18]. Similarly, MDR cells are able to maintain abnormally high pH levels across organelle membranes through the activity of ATPase pumps. This allows for the drug to be sequestered in acidic vesicles which can then be extruded from the cell [19]. Because MDR cells require higher levels of ATP in order to maintain efflux pumps and this H+ concentration gradient, they are much more susceptible to energy depletion compared to normal cells. Studies using Pluronic® L-61 and P-85 have shown that hydrophobic Pluronics® have the ability to intercalate into the cell plasma membrane and bind to P-glycoproteins as well as aid in ATP depletion (Figure 5.2) [20]. A recent study by Kabanov et al reported that Pluronics® can enter the mitochondria of MDR cells, block several respiratory complexes, deplete organelle membrane potential, and therefore stop ATP production [21]. As a result, Pluronics® are able to increase the effectiveness of DOX, paclitaxol, and other chemotherapeutics by 2-3 orders of magnitude [17, 22, 23].

**Figure 5.2. Diagram of Pluronic® activity on MDR cells.** This polymer can block P-glycoproteins and multidrug resistant proteins as well as deplete ATP production in the mitochondria
Future studies would focus on incorporating hydrophobic Pluronics® into mixed micelle systems (as we have already started to do with the Pluronic® F-127/L-101 micelles) and looking for possible drug synergy in MDR cell lines [24, 25]. Another alternative is simply to deliver small amounts of hydrophobic Pluronics® with the drug alone from the hydrogel. While researching the various drugs used in this study, we have also found evidence of synergy between the NSAID Indo and various chemotherapeutics [26, 27]. Numerous studies have shown indomethacin’s ability to block protein kinase receptors, blocking protein synthesis, and causing cellular apoptosis in human colorectal cancer cells [27, 28]. This could lead to the possibility where indomethacin is codelivered in the same mixed Pluronic® micelle as DOX which is predicted to greatly enhance cancer cell response towards DOX. Moving forward, there are multiple other directions where our system can be further optimized: In order to better control the biodistribution on the Pluronic® micelles towards tumor cells, we can conjugate targeting moieties to the micelle surface. For larger mixed Pluronic® micelles where cell uptake might be more difficult, a cell penetrating peptide might be added to micelle surface. We can also further adjust and vary the molecular weight of PEGDA, size and shape of hydrogels, size and type of micelles, type of drug to allow for faster or slower release kinetics. Furthermore, nanoparticles of a certain size (10-200 nm) are known to be better retained in the tumor due to the EPR effect [29, 30]. We can easily vary the size of our micelles by using different Pluronics® or a mixture of in order to better study this mechanism in-vivo. This would allow for selection of a micelle of some optimum size where EPR effects can be utilized and yet cell uptake is also at a maximum. Also, in order to better visualize and track the fate of micelles embedded deep within the hydrogel mesh, we might utilize confocal or SEM microscopy to image these particles over time. We can fluorescently label the polymer (in this case Pluronic®
with rhodamine for example) itself and look to see if both polymer and drug co-localizes both inside and outside of the hydrogel. This would further add to our knowledge of intact micelle release from the hydrogel. Similarly in the future to understand the fate of intact micelles once taken up by the cell or within the body (specifically the brain for the continuation of this project), FRET-loaded Pluronic® micelles can be imaged using confocal microscopy [31, 32]. It would be interesting to observe localization of these micelles in any specific organ or organelle and will help us better understand the biology of micelle activity.

The value of this dissertation lies in the fact that proving intact micelle release from hydrogel is definitely not specific to our set of biomaterials or disease model. The results presented highlight the mechanics of micelle release from hydrogel and so far show a promising future for use of this system in disease treatment. As noted above, there are still many challenges and future studies needed to fully optimize our system for use in patients. However, this is definitely a major step towards understanding the properties of nanoparticle in depot systems which could have vast effects in the future in the controlled release drug delivery field.
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