Logic-Degradable Nanogels for Environmentally Triggered Chemotherapeutic Delivery

BioEn 402
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Abstract

The delivery of cell and drug-based chemotherapeutics to tumors have presented major challenges in effective cancer treatment. Opportunities to improve current small molecule drug delivery systems exist by increasing overall delivery specificity and decreasing harmful off-target effects. Towards this, we have developed a chemical framework for creating user-programmable hydrogels that undergo programmed degradation in response to multiple environmental cues following Boolean logic. Exploiting this methodology, user-specified combinations of environmental inputs (e.g., tumor-presented enzymes, reducing conditions) yield material breakdown, accompanying therapeutic release. To translate these materials for chemotherapeutic delivery, we have established strategies to formulate these stimuli-sensitive materials into nanogels that can circulate in the bloodstream before acting on the desired target site. We have demonstrated techniques to formulate gels on the 50-250 nanometers size scale, one which should enable circulation in the blood and uptake within tumors based on the enhanced permeability and retention effect. Different ultrasonication and chemical conditions allow us to tune nanogel size and dispersity. Through proof-of-concept degradation studies, we have characterized the nanogel platform. This system is scalable, translational, and can be created timely. In the future, these materials can effectively hone and selectively deploy small molecule chemotherapeutics to tumors in patients, as well as open doors to other delivery platforms.

Introduction

Cancer's impact on global health and economics in the world has been tremendous. In 2018, in the US alone, more than 1.7 million cases of cancer were diagnosed with more than half a million deaths [1]. The numbers translate to about an incidence of 440 per 100,000 people and a precedence of 160 per 100,000 people. In fact, the National Institute of Cancer predicts that 40% of the US population will be diagnosed with cancer during their lifetime. In the world, there are approximately 17 million new cancer cases each year with 9.6 million deaths [2]. Economically, in 2017, cancer alone accounted for $150 billions of national expenditures. This number is estimated to continue to grow as prevalence, incidence and more expensive advanced treatments continue to grow. Financially, the lost productivity caused by cancer diagnosis in 2005 has been accumulated to be well over $120 billion, with lung cancer accounting for more than $35 billion of the total [3].

Cancer is defined as uncontrolled proliferation of cells into malignant tumors that can often spread and travel to many areas of the body [4]. There are many factors that contribute to the development of cancer. Abnormal inactivation of X chromosomes is thought as the precursor in single cells that lead to the eventual development of tumors. At the cellular level, genetic mutation and specific selection for cells with increased capacity for proliferation and invasion are strong contributors to cancer growth. Tumor progresses in multiple stages. First, a single cell with genetic defect begins to proliferate uncontrollably in a particular organ. Cells with more proliferation potential are selected for and triggers more cancerous cell growth. This feedback underlies the molecular mechanism of cancer development. In addition to inherited genetics, there are multitude of other factors that increase the odds of cancer. Radiation, viruses and chemicals are some of the major contributors in epigenetics, where the genome is changed due to the environment. Many lifestyle choices such as diet, exercise and smoking can introduce chemicals into body that increase the possibility of genetic mutations. There are also hormones and neurotransmitters that are more prevalent in certain gender and age groups that play a factor. For example, excessive estrogen exposure increases the likelihood of women to develop endometrial cancer. Ultimately, cancer is a complex disease with many variations in different organs. Each type of cancer holds its own specific properties and they can vary from patient to patient as well. Therefore, it is tremendously important for research to continue to be done in the field to develop better treatment for all.

As cancer is such a prevalent problem worldwide, there have been a lot of treatments developed to combat the disease. Surgery, radiation therapy, cryoablation and radio frequency ablation involves direct physical removal of tumors via a variety of techniques. Immunotherapy is currently a very popular technique as it recruits the patients' own immune system to attack cancerous cells. They are more specific in target and is part of a more general concept called target therapy, where off target harmful side effects are minimized. Hormone therapy removes hormones that are critical in cancer formation and this is often done with drugs such as chemotherapeutics to target cancer cells. As cancer treatment varies significantly by type and stage, biomaterials serving as chemotherapy drug carriers are often designed to have more general applications to serve a wide range of drug molecules. To deliver these drugs to target sites, smart biomaterials are often used as carriers. Single-input responsive biomaterials are the basis of many drug delivery systems [5]. Often times, the magnitude of material degradation rely on the external or biological input amplitude delivered to the system. Some of the common inputs include redox conditions, biological enzyme presence, and external physical forces. In water-degradable systems, poly(lactic-co-glycolic acid) (PLGA) is one of the most common material used as it allows for user-defined stiffness, biocompatibility, and geometry [6-8]. In addition, its versatility allows for specific material degradation and cargo release rates for different applications [9]. Acidity and basicity are also common inputs as materials are able to take advantage of different pH's exhibited in different biological tissues and pathologies.

Tumor microenvironments have a pH of around 6.5-7.2, which is noticeably more acidic than healthy cellular environments [10]. Enzyme presence is another input that is widely used to degrade materials that include peptides, lipids, and other macromolecules. Specifically, matrix metalloproteinases (MMPs) are present in tumor tissues and often used to recognize and cleave specific peptide combinations [11]. Different redox conditions in the human body also present opportunities for varying material degradation. Glutathione (GSH) and glutathione disulfide are shown to have a significantly higher presence in tumors compared to healthy tissues [12]. Finally, external stresses and pressures can induce varying levels of biomaterial degradability. The vasculature exhibited in microtumor environments favor uptake of biomaterials in the nanoscale, known as the enhanced permeability and retention effect [13].

Dual-input responsive biomaterials are explored for many delivery applications. Different combinations and permutations of inputs can be programmed to trigger desired degradability. One example is a micelle system that can separate or coagulate with different polarities introduced to its components. Another application of this concept is a nanoparticle system that degrade if required reducing or light condition is present. In terms of materials, mesoporous silica nanoparticles are common platforms for dual-input responsive systems to its small size and compatibility as a drug carrier [14]. Hydrogels are also widely used in this application as it exhibits cytocompatibility and reversible degradation properties [15]. Many assembly methods are adopted to generate these smart materials. Covalently assembled materials have been shown to be more stable and more geometrically versatile compared to additive and subtractive physical methods. The ability to introduce peptides in these polymer networks is often utilized to introduce more input possibilities.

In comparison, greater-than-two-input responsive biomaterials present even more specificity and complexity in biomaterials engineering. Lower critical solution temperature (LCST) is commonly used in three-input responsive systems. Although not commonly investigated, four or five input systems are possible to be implemented. In one example, acidity, temperature, UV and visible light triggered micelle expansion and the materials were able to return to their original states after specific inputs. In summary, these systems provide platforms for exciting applications from drug delivery to tissue engineering.

Although existing technology show significant promise, there is still a lot of room for improvement when it comes to the delivery of cell and drug-based chemotherapeutics, and the specific deployment of these therapeutics to diseased sites present significant clinical barriers. Drug dosage often has to be increased and unintended toxicity to healthy cells often requires patients to replenish cells via bone marrow transplants. While current single-input drug delivery systems allow for localized enrichment, the biomarker triggers are rarely unique to these locations in the body. Treatment dosage, efficacy, and efficiency are often compromised as a result. The DeForest Research Group has established a modular chemical framework for creating hydrogel drug carriers that undergo programmed degradation in response to precise combinations of multiple environmental cues following Boolean logic. The innovative design allows the cytocompatible material to have unprecedented specificity in therapeutic release by possessing modular versatility and biocomputational functionalities. Although these systems present exciting opportunities in advancing efficacy of drug delivery, to date they have been formulated only as macroscopic hydrogels with limited physiological applicability. To translate these advanced biomaterials in biological settings, we have developed nanogel formulation strategies that equip these smart materials with the necessary size and geometry to travel in the bloodstream to the desired target site. In addition, due to the leaky vasculature of tumors, the enhanced permeability and retention effect further promotes accumulation of particles in the nanoscale. The designed nanogel system

has the desired multi-input degradability and specificity, as well as the necessary geometry and properties for biological applications.

Development of Design Specifications

To ensure the efficacy of the degradable nanogel platform, there are some specifications it must meet (Table 1). These specifications also take into consideration the constraints imposed by the materials, monetary resources and equipment available to the DeForest Laboratory during technology development. First, it is important that the nanogel platform are able to circulate in the blood stream and the smallest capillaries, with the maximum allowable diameter being a few micrometers. Ideally, the designed material should lie between 50-250nm to take advantage of the enhanced permeability and retention effect. Second, during material synthesis, the nanogels must be monodisperse as to ensure the heterogeneity and the uniformity of the materials in solvent. To characterize this, the standard measurement is known as the poly-dispersity index, where values of less than 0.3 are considered monodisperse. The final synthesized material in solution should be less than that value. Third, there is a need to tune the size of the nanogels to cater to drug molecule sizes and different biological applications. Whether through chemical tuning or differentiating sonication conditions, materials from a range of 50nm to 1um should be generated.

Parameter	Value
Size	<2um
Poly-Dispersity Index	<0.3
Size Tunability	50-1000nm
Degradability	Different depending on input
HeLa Cell Survival After Treatment	<20% dsDNA content

Table 1. Design specifications of final logic-degradable nanogel platform, not including intermediate steps for quality control.

To create nanogels that undergo disease-triggered degradation, logic-based responsive cross-linkers are used during nanogel synthesis. Synthetic peptides are incorporated as material cross-linkers as they are intrinsically biocompatible and can be chemically modified to include non-canonical functionality, connectivity and degradability. One example is a peptide sequence consisting of GPQG↓IWGQ, which is cleaved in the presence of matrix metalloproteinases, enzymes commonly overexpressed in tumor microenvironments. Another input are disulfide bonds that break under reducing conditions in disease sites, which will be incorporated to give nanogels high degrading specificity and versatility. These nanogels will be characterized by *in vitro* treatment of relevant input combinations. The exact values from characterization of degradability of nanogels are to be determined, whether from rheological or fluorescent based assays.

The final test of the platform involves analyzing nanogels' behavior in biological systems. To demonstrate the ability to deliver therapeutics in response to physiological factors present in tumor environments, I plan to attach a doxorubicin chemotherapeutic into nanogels cleavable by enzymes AND reducing conditions to start out. Other input combinations will be accessed over time. This nanogel functionalization is chosen such that the chemotherapeutic released after full material degradation will cause apoptotic death of cervical cancer-derived HeLa cells. Following treatment of nanogels by associated input combinations, the viability of these cancer cells will demonstrate the material's capability to specifically degrade and release drug cargo. To quantify this, the double-stranded DNA (dsDNA) content will be accessed as a measure for cell survival. Cell nuclear antibody stains will also be done to support such claims.

There are many standards and norms to satisfy when engineering a new technology. This will be accomplished all throughout the design process, especially towards commercialization or publication. Terminology and test standards will need to be well defined when accessing the viability of the design. Process and product standards will be needed to ensure the efficacy of the product as well as the safety of the patients and providers. Specifically, common chemotherapy side effects need to be avoided in my design for it to deem successful. Interface and data standards will be put in place to allow intuitive understanding of the product and convenience of analysis. If commercialized and globalized, company, industry and international standards will be followed for the technology to be distributed.

The US Food and Drug Administration (FDA) defines medical technology related to my work as the following: "an apparatus intended for use in the diagnosis of disease or other conditions, or in the cure, mitigation, treatment, or prevention of disease, in man or other animals" [16]. Specifically, nanoparticle-based drug delivery platforms are considered combination products with extensive regulations from molecular evaluation of the drug to approval of the manufacturing process [17]. The assessment of the drug and carrier includes guidelines such as safety, toxicity, biocompatibility, chemistry and metabolism. My proposed technology would be compared to current targeted chemotherapies where off-target side effects are significantly evaluated. The timeline and application fees of the approval process vary from technology to technology. To efficiently and successfully go through the FDA regulatory processes, my technology will need to be rigorously tested for safety and efficacy in *in vitro* and *in vivo* models. Specifically, mouse models and non-human primates are common in chemotherapeutic pre-clinical trials while human subjects will be preferred in clinical trials. These evaluations will be monitored closely after the technology is implemented in the clinics as well. If issues arise, the FDA may remove the product from the market. In which case, the technology will be redesigned and undergo another FDA approval process.

There are additional public health, safety, and welfare, global, cultural, social, environmental, and economic factors to be considered. During the engineering design process and later into clinical implementation, public health and human safety/welfare holds the upmost importance. Strict engineering standards and FDA regulations highlighted above will be met and scrutinized rigorously. In terms of environmental impact, the DeForest Laboratory strictly follows regulations for biosafety level 2 laboratories. Chemicals and other laboratory wastes are disposed correctly with minimal environmental impact. The lab is audited by University of Washington's Environmental, Health, and Safety department. For socioeconomic and cultural factors around the globe, the goal of this technology development is to have a far reach and impact as many patients around the world positively. Although chemotherapeutics is expensive and not widely available in low-resource settings, we hope that the contribution to the advancement in the field, especially from a non-profit academic research setting, drives the cost down and provides treatment to more patients in the world over time. Finally, cultural considerations are important when pursuing any scientific advancement. Although the designed technology is intended for a limited scope where chemotherapeutics is accepted, we hope that the decrease in chemotherapy side effects due to our technology will enable larger cultural acceptance for such treatments. Furthermore, injection of nanogels is less invasive than other surgical-based cancer treatments, which presents more feasibility in diverse cultures and potential for acceptance.

There are many social and ethical considerations with the research project itself as well as the successful translation to clinical use. Animal models will be crucial in determining the safety and efficacy of the technology. Specifically, mouse models are often used in cancer treatment studies

due to their reproducibility and similarities with the human genome [18]. Although they are not perfect models to translate to human use, they set the foundation in evaluating and identifying potential challenges. Ethically, I will use the minimum number of animals that will achieve a statistically significant result to justify the cost in animal life. This will be determined during experimentation. All of my experiments will be approved by the Institutional Animal Care and Use Committee prior to execution to understand and ensure the ethics of the study. If results are promising, non-human primates and human clinical trials will be the next step to advance the technology. Specifically, the human subjects division of the institutional review board will need to approve the study to protect the rights of the subjects without losing the scientific rigor. All data generated will be transparent to avoid controversy throughout the whole process. If the technology becomes available for human use, it will contribute to health equity and ideally low cost to increase access. Politics can definitely play a role in the end-user price depending on the translational pathway chosen, varying between academic and industry use. All of these aspects of commercialization will have to be considered before clinical use.

Materials and Methods

Synthesis and characterization of cross-linkers. The details of crosslinker and nanogel backbone synthesis were optimized by Dr. Barry Badeau, a previous graduate student in the research group. For details of all cross-linker syntheses and characterization, see Supplementary Methods of reference 19.

Characterization of water-in-solvent emulsion. Using cyclohexane, water, and a nonionic detergent, ultrasonication conditions for micelle preparation were optimized. The ranges of variables tested were sonication power (10-40% power), run time (0-20min), and concentration of Span 80 surfactant and water relative to cyclohexane (0-5 weight%). The sonicator used was a Branson 450 Digital Sonifire. The samples were characterized with Dynamic Light Scattering (Malvern ZetaSizer Nanoseries HT) for size and poly dispersity.

Synthesis of non-degradable nanogels. A 20mL cyclohexane solvent containing 1% PBS, 2.5 wt% Span 80, 2mM four-arm poly(ethyleneglycol) tetrabicyclononyne and 4mM two-arm poly(ethyleneglycol) diazide were agitated under ultrasonication every other second at 40% power for 20 minutes. The sample was allowed to rest for 1 hour at room temperature. The sample then underwent rotary evaporation exchange with PBS to remove unwanted organic solvent for 20 minutes. It is then put through a 220 nm cell filter (Millipore Express PES membrane) to remove larger particles. The nanogels were lyophilized for storage and resuspended in PBS when needed. DLS measurements were taken throughout the process for size and poly dispersity confirmation.

Synthesis of logic-degradable nanogels. Synthesis of logic-degradable nanogels follows closely with the synthesis of non-degradable nanogels as described above. The only difference is the cross linker utilized. Instead of using 4mM two-arm poly(ethyleneglycol) diazide as the sole crosslinker, some of the poly(ethyleneglycol) diazide crosslinkers are replaced with desired degradable linkers. The ratio of non-degradable to degradable linker is 3:2, with the total culminating to 4mM in total emulsion volume. The degradable linkers examined were Photo OR Reductive degradable crosslinker (N3-*o*NB-RGGRC(N3-C-OH)- NH2 with cysteines linked *via* disulfide bond) and Enzymatic YES degradable crosslinker (N3- RGPQGIWGQGRK(N3)-NH2). Details of these syntheses can be found in Supplementary Methods of Reference 19.

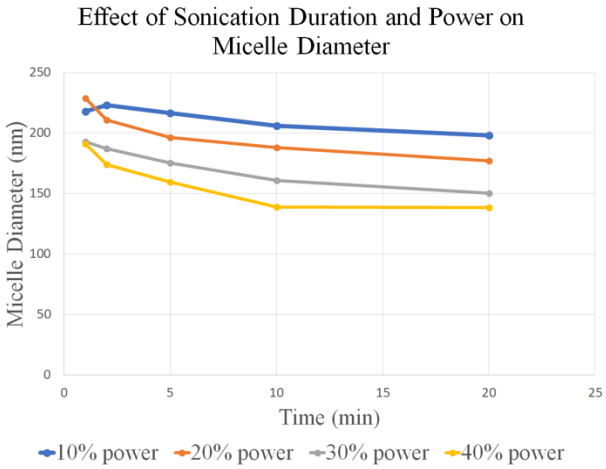
Recombinant expression and purification of matrix metalloproteinase-8 (MMP-8). The details of MMP-8 enzyme expression can be found in Supplementary Methods of Reference 19.

Degradation and characterization of logic-degradable nanogels. 1mL nanogel samples in plastic cuvettes were degraded and characterized. For light degredation experiments, samples were treated with 365nm wavelength UV light at 10mW/cm^2 ranging from 0 to 90 minutes. The reductive degradable samples were treated with tris(2-carboxyethyl) phosphine hydrochloride (TCEP·HCl, 200 nmol) then incubated overnight at 37 °C. The enzyme degradable samples were first resuspended in MMP-8 Buffer (see supplementary methods of reference 19). Then, they were treated with MMP-8 (5 μl, 0.2 mg ml−1 in MMP buffer) and incubated overnight at 37 °C. It is of note that the degredation protocols for enzymatic and reductive degradable nanogels are still actively being optimized.

Statistical analysis. All statistical calculations and data plotting from DLS measurements and other assays were done using Microsoft Excel. Size and poly dispersity were the primary metrics used. All experiments have appropriate sample sizes and control groups as to compare data meaningfully.

Results

Micelle synthesis and optimization. To understand the properties and contributing factors that govern nanoparticle synthesis, I first studied micelle formation in water-in-oil emulsion systems. As hydrogels are water-based polymer networks, in order to form nanogels, it is essential to create stable pockets in the aqueous phase for individual components to crosslink. In addition, the sensitive nature of ultrasonication equipment available further motivates micelle optimization studies before generating nanogels. Micelles are generated due to the hydrophobic effect as small pockets of water in the nanoscale can be created in hydrophobic solvents under intense sonication. With both hydrophobic and hydrophilic properties, surfactants are often utilized to stabilize these aqueous pockets by preventing individual micelles from aggregating. Due to the variety of methods and ingredients commonly used in nanoparticle synthesis, I first identified surfactants and solvents that are most chemically optimal for my system. Using cyclohexane, DI water and Span 80 surfactant, I then tested ultrasonication variables including sonication duration, power, and concentrations of each component relative to one other. The experimental results are presented in Figure 1. Over the course of 20 minutes of sonication agitation, micelles that experienced higher sonication up to 40% resulted in smaller emulsions, around 120nm. The power constraint on the sonication equipment did not allow for testing of higher sonication amplitudes. As sonication power and time increased, the micelle sizes decreased. However, the micelle sizes plateaued around 15 min, with addition sonication causing no further decrease in micelle size. In addition, a surfactant weight% of 2.5 with respect to total emulsion sample generated the smallest micelles. It is of note that small emulsions were not possible when no surfactants were added to the sample.



Surfactant Weight %	Micelle Diameter (nm)
0	3255
1	158.7
2.5	149.2
4	157.3
5	164.3

Figure 1. Optimization of sonication conditions using cyclohexane, DI water and Span 80 surfactant. Sonication power and duration were first varied to scale micelle diameter. The effect of Span 80 surfactant weight percentage on micelle diameter was evaluated afterwards. Micelle diameters were characterized with dynamic light scattering.

Non-degradable nanogels analysis. Different iterations were tested and optimized to arrive at the final design of non-degradable nanogels described in the methods above. Briefly, I first attempted a two-pot approach where the nanogel backbones and crosslinkers were emulsified separately before combined together to gel. The geometry of the nanogels from this approach were satisfactory but not as small or time efficient as the one-pot approach. Throughout the process, I assessed nanogel size and dispersity via DLS, observing nanogel stability over time (Figure 2). The nanogels were stable for at least a week with the desired geometry and mono-dispersity. This allowed me to design the purification process without having destabilization concerns with regards to time.

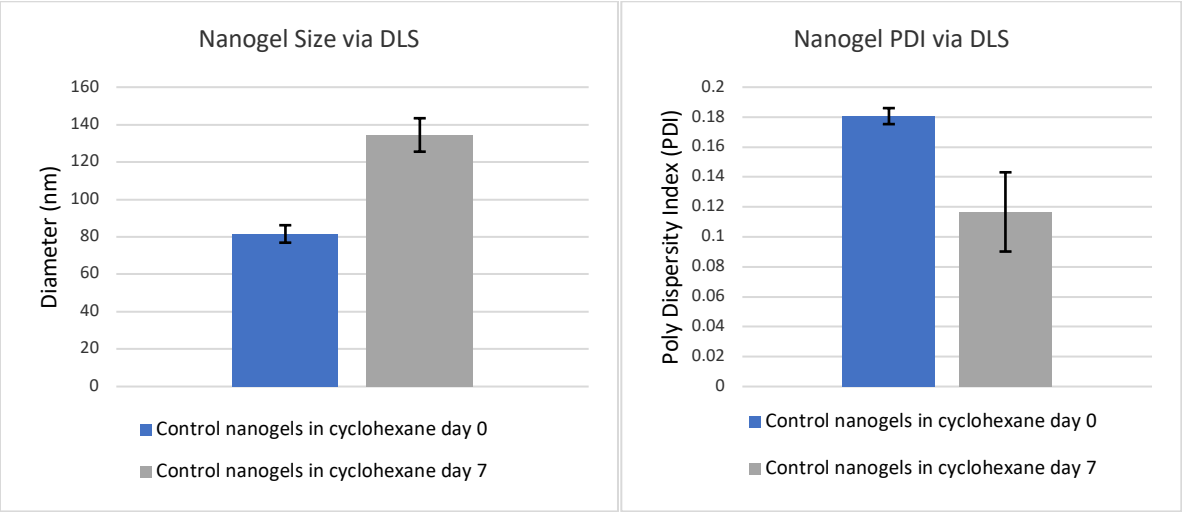


Figure 2. Non-degradable nanogel size and dispersity measured after synthesis and at a week timepoints.

With nanogel synthesis optimized, I then moved on to purifying the nanogels, putting them in the desired final solvents for different applications. Many iterations were also attempted here. A combination of rotary evaporation, centrifugation, dialysis exchange, and physical mesh filtering

were optimized in different orders to reach the final protocol described in the methods above. Throughout the optimization process, nanogel size, dispersity and stability over time were assessed to inform the next design iteration. The majority of the challenges laid in stabilizing the nanogels once the surfactant is removed. The results of the final design were in the acceptable range given in the design specifications, with final nanogel diameter less than 140nm and a dispersity index lower than 0.3 (Figure 3).

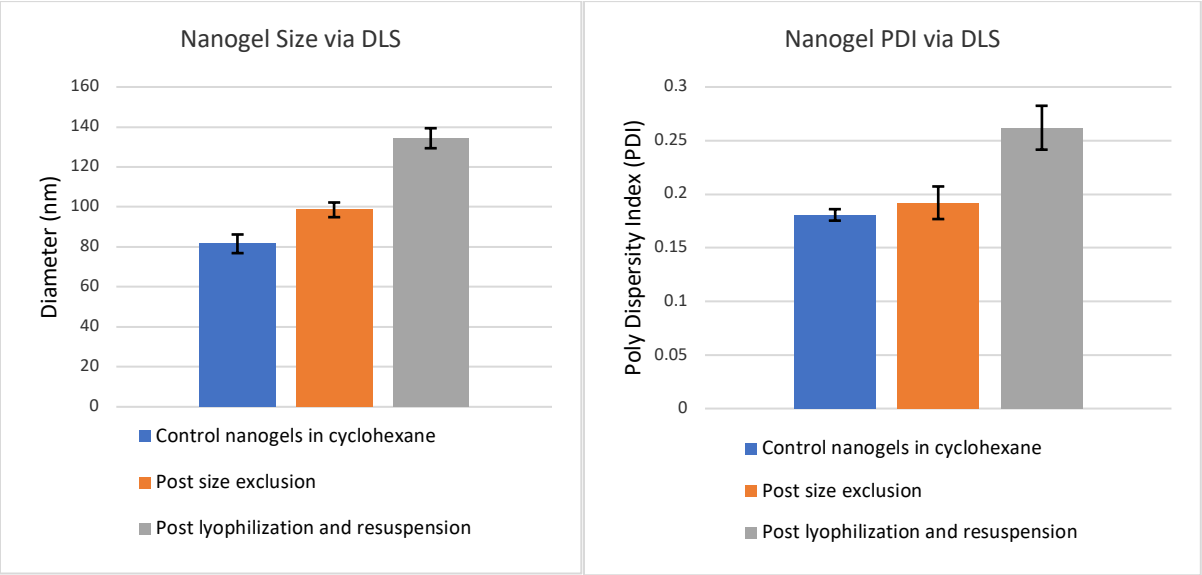


Figure 3. Non-degradable nanogel size and dispersity measured at different stages of the purification process.

Logic-degradable nanogels analysis. After optimizing general nanogel synthesis and purification protocols, I then incorporated logic-degradable linkers into my studies. During experimentation, it is clear that the purification techniques optimized did not translate directly for logic-degradable nanogels. Therefore, more optimization was done to ensure size, dispersity and stability after purification. Next, I experimented with photo or reductive nanogels. Photo-degredation studies were performed and nanogels exposed to 365nm wavelength UV light at 10mW/cm^2 degraded after 90 minutes (Figure 4). As the linkers become more and more degraded over time, nanogels become less and less stable, demonstrated by the increase in size. There were no measurements possible at 90 minutes due to complete degredation and the DLS was not able to measure non emulsified molecules. With the nanogels also degradable by reductive conditions, I also exposed the nanogels to TCEP-HCl. The protocol for chemical exposure for this condition is still being optimized.

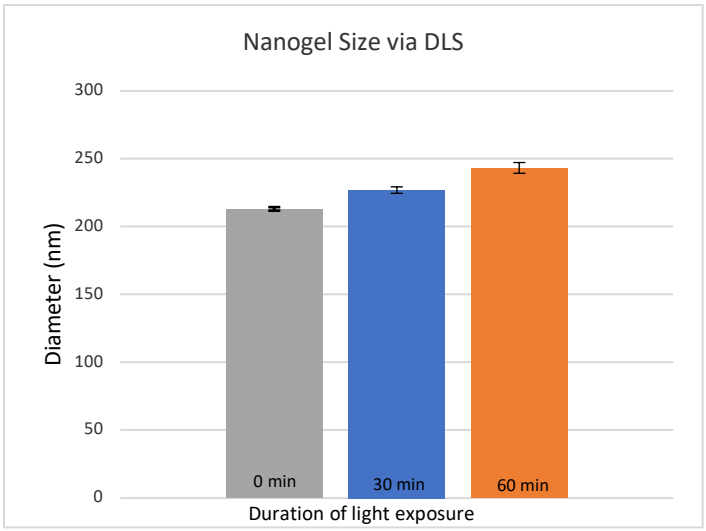


Figure 4. Photo or reductive degradable nanogel size measured at different stages of the degredation process. Nanogels were exposed to 365nm wavelength UV light at 10mW/cm^2. At 90 minutes, DLS measurements were not possible with no emulsion remaining.

Enzymatic degradable nanogels are also currently under investigation. While optimizing the purification protocol for MMP-8 degradable nanogels, I realized that not all of the linkers have to be degradable for the gels to degrade. It was experimentally determined with macro hydrogels that a stoichiometric ratio of 3:2 nondegradable to degradable nanogels were sufficient in material degradation given appropriate stimuli. The results of this study allowed for material conservation and higher purification yield, given that the purification protocols were optimized under non-degradable conditions. The degredation of enzymatic-degradable nanogels and other complex linkers are still areas of investigation and optimization.

Discussion

Over the course of the project, chemical components and conditions most suitable for nanogel formation were identified. Given the materials and equipment available, nanogel synthesis and purification protocols were turned. The desired nanogel geometry, dispersity and tunability first set out in the project were achieved. Finally, initial proof of concept experiments were performed on simple and more complex degradable nanogel systems. The studies bring the smart biomaterials in the DeForest Research Group closer to physiological relevance and introduces nanomaterial to the lab toolkit. Many nuances of nanogel formation can aid other group members in relevant drug delivery projects of their own.

While this foundational work brings exciting possibilities for targeted drug delivery, future work needs to be completed before the deployment of these materials into patients. Complex nanogels need to be developed further and optimized for geometry and degradability. Once they are able to be reliable produced, we can then apply these materials to biological systems to assess their performance in cancer relevant settings. For example, to demonstrate the ability to deliver therapeutics in response to physiological factors present in tumor environments, doxorubicin chemotherapeutic attached nanogels can be deployed in a cancer-cell line assay. Following treatment of nanogels by associated input combinations, the viability of these cancer cells will demonstrate the material's capability to specifically degrade and release drug cargo. The success of these in vitro experiments will bring exciting possibilities for this system, enabling the nanogel

platform to be studied in in vivo settings. As the studies move closer towards clinical applications, it is important to constantly recognize the global, economic, environmental, and societal impacts enabled by the project along the way, especially in cancer-relevant contexts. This would ensure the technology's positive impact to be maximized, relieving cancer patients of some of the many medical and socioeconomical burdens they face today.

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